

Investigating Cell Lineage Specific Biosynthesis of Tenascin-C during Inflammation



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Abstract

The extracellular matrix (ECM) is a complex network of molecules secreted by cells, which is essential for providing structural support and facilitating cell processes including adhesion, migration and survival. Tenascin-C is an immunomodulatory ECM protein that exhibits limited expression in healthy tissues, but is transiently elevated at sites of tissue injury, and is persistently expressed in chronic inflammatory diseases and tumours. Alternative splicing of 9 of tenascin-C's fibronectin type III-like domains (FnIII- A1, A2, A3, A4, B, AD2, AD1, C and D) generates enormous diversity in form; yielding 511 possible isoforms. Post-transcriptional modification of tenascin-C has been studied in cancer and during development where disease and tissue specific isoforms exhibit distinct adhesive, migratory and proliferative effects. However, little is known of how tenascin-C is expressed or alternatively spliced during inflammation. This study characterises inflammation and disease specific tenascin-C isoforms made by immune cells and fibroblasts, and investigates their functional relevance.

Biosynthesis and alternative splicing of tenascin-C was examined using standard curve qPCR, ELISA, Western blot and confocal immunocytochemistry in resting and activated primary human immune cells, dermal fibroblasts, and in synovial fibroblasts isolated from healthy controls and from osteoarthritis (OA) and rheumatoid arthritis (RA) patients. Based on these data, three recombinant proteins comprising FnIII domains AD2-AD1, B-C-D and B-AD2-AD1-C-D were cloned, expressed and purified, and their impact on cell behaviour including adhesion, morphology and migration was assessed.

Basal tenascin-C expression was lower in myeloid and lymphoid cells than fibroblasts, and was induced in all following inflammatory stimulation. Tenascin-C expression was elevated in disease with RA and OA synovial fibroblasts containing higher levels than healthy

controls. Alternative splicing following cell activation was cell-type specific: all FnIII except AD2 and AD1 were upregulated in dendritic cells and macrophages, in T-cells all FnIII remained unchanged with FnIII A1 absent; and no change in splicing was observed in activated dermal fibroblasts. Normal and OA synovial fibroblasts exhibited similar tenascin-C splicing patterns, but FnIII B and D were specifically elevated in RA. Functional analysis revealed differences in the adhesion, morphology and migration of myeloid cells and dermal fibroblasts cultured on FnIII AD2-AD1, B-C-D, B-AD2-AD1-C-D and full length tenascin-C substrates; FnIII B-C-D promoted MDDC migration while B-AD2-AD1-C-D promoted fibroblast adhesion, compared to full length tenascin-C.

For the first time, this study reveals differences in tenascin-C biosynthesis and alternative splicing by immune cells and fibroblasts following activation with inflammatory stimuli; and starts to reveal how alternative splicing of tenascin-C may influence the behaviours of both stromal and immune cells types during inflammation and in inflammatory diseases.

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List of Abbreviations

APC – Antigen presenting cell

BCA – Bicinchoninic acid assay

BDNF - brain derived neurotrophic factor

BLAST - Basic local alignment search tool

BMP – bone morphogenetic protein

bp – Base pairs (nucleotide)

BSA – Bovine serum albumin

cDNA – complementary DNA

CL – Cell lysate

CNS- Central nervous system

CRC – Colorectal carcinoma

DAMP – Damage associated molecular pattern

DAPI - 4',6-diamidino-2-phenylindole

DCIS – Ductal carcinoma in-situ

dH₂O – Deionised water

DMEM – Dulbecco's modified Eagle's medium

EDA – Fibronectin extra domain A

EDB – Fibronectin extra domain B

EDTA - Ethylenediaminetetraacetic acid

EGF-L – Epidermal growth factor-like repeats

EGF-R – Epidermal growth factor receptor

ELISA – Enzyme-linked immunosorbent assay

Ex – Embryonic day x

FACS – Fluorescence associated cell sorting

FBG – fibrinogen like globe

FBS – Fetal bovine serum

FGF – fibroblast growth factor

FN – Fibronectin

FnIII – Fibronectin type III-like repeat

FOXO - Forkhead box transcription factor

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GM-CSF – Granulocyte macrophage-colony stimulating factor

HDF – Human dermal fibroblast

HGF – hepatocyte growth factor

IF - Immunofluorescence

IgG1 – Immunoglobulin G 1

IL – Interleukin

ISH – In situ hybridization

LPS - Lipopolysaccharide

mAb – Monoclonal antibody

M-CSF – Macrophage-colony stimulating factor

MDDC – Monocyte derived dendritic cells

MEFs – Mouse embryonic fibroblasts

MES - 2-(N-morpholino)ethanesulfonic acid

miRNA – Micro RNA

MMP – Matrix metalloproteinase

Mr – Molecular mass

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NIH-3T3 - mouse embryonic fibroblasts

NPC – No primary control

NSCLC – Non-small cell lung carcinoma

NSF – Normal synovial fibroblast

NT – neutrotrophin

OA – Osteoarthritis

OA-SF – Osteoarthritis synovial fibroblast

PAGE – Polyacrylamide gel electrophoresis

PAMP – Pathogen associated molecular pattern

PBMC – Peripheral blood mononuclear cell

PBS – Phosphate buffered saline

PBST – Phosphate buffered saline Tween-20

PCR – Polymerase chain reaction

PDGF – platelet-derived growth factor

PDGF-R β – Beta-type platelet-derived growth factor

PFA – Paraformaldehyde

PIGF - phosphatidylinositol-glycan biosynthesis class F protein

PLC – phospholipase C

PRR – Pattern recognition receptor

PS – Penicillin/streptomycin

PTPR β / ζ – Receptor-type tyrosine-protein phosphatase beta/zeta

Px – Postnatal day x

qPCR – quantitative PCR

RA – Rheumatoid arthritis

RA-SF – Rheumatoid arthritis synovial fibroblast

SCC – Squamous cell carcinoma

SDS – Sodium dodecyl sulfate

SEM – Standard error of mean

siRNA - Small interfering RNA

SN – Supernatant

STDEV – Standard deviation

TA – Tenascin assembly domain

TBS – Tris buffered saline

TGF β – Transforming growth factor beta

Th17 – T-helper 17 cells

TIMP – Tissue inhibitor of metalloproteinase

TLR4 – Toll-like receptor 4

TNC – Tenascin-C gene

TNC $-/-$ - Tenascin-C knockout

U-87 MG – Human neuronal glioblastoma cell line

UCC – Urothelial cell carcinoma

VEGF – vascular endothelial growth factor

WB – Western blot

wt – *Wild type*

Δ Ct – Delta Ct

$\Delta\Delta$ Ct – Delta delta Ct

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Thanks to all who made this project possible,

Sean Giblin

Declaration

I declare that I autonomously carried out the DPhil thesis entitled “Investigating cell lineage specific biosynthesis of tenascin-C during inflammation”. This dissertation is the result of my own work and all collaborations have been acknowledged at the appropriate locations within the text.

The following third-party assistance is listed below:-

Chapter 1 was adapted from my own review publication “Giblin SP, Midwood KS. Tenascin-C: Form versus function. *Cell Adhesion and Migration*. 2015; 9:48-82.”, and all of the included sections were authored by myself. Permission to include this publication as part of the thesis was obtained by my co-author Professor Kim Midwood, and a supporting letter is enclosed with the thesis. This publication is included at the end of this thesis for reference.

I did not receive any assistance in return for payment by consulting agencies or any other person. The project was completed at the Kennedy Institute of Rheumatology, University of Oxford.

This thesis has not been submitted elsewhere for examination

I affirm that the above statements are complete and true to the best of my knowledge.

Signature.....

“Sweet is the lore which Nature brings;

Our meddling intellect,

Mis-shapes the beauteous forms of things:-

We murder to dissect”

– William Wordsworth, The Tables Turned

Chapter 1 Introduction

1.1 The extracellular matrix

The extracellular matrix (ECM) is a complex network of secreted molecules including large structural fibrillar proteins, and a myriad of different glycoproteins and proteoglycans. The ECM is not merely an inert scaffold as it is part of the local microenvironment, or ‘niche’, and directly affects cell behaviours; for example it can anchor cells, provide migration tracks or barriers, signal external mechanical force, signal directly to cells in order to influence their phenotype, and it also concentrates soluble factors such as cytokines, growth factors and proteases where it can act as co-receptors for these factors.

1.1.1 *Tissue specificity of the ECM*

Different tissues have distinct ECM compositions with unique molecular components, which tailor the ECM for specific functions.

1.1.1.1 *The ECM of the basal lamina*

One such specialised tissue is the basal lamina, a major architectural and signalling component of epithelial tissues; which is composed of a simple layer of cells that adopt apical/basal polarity, with the basal side contacting the basement membrane, and the apical side facing the fluid filled lumen of the organ. In epithelial tissues, the basal lamina is a complex sheet-like meshwork of ECM molecules that is comprised of proteoglycans, collagen fibres and soluble multi-adhesive matrix proteins such as laminin, fibronectin and tenascin-C, which cross link with other ECM components and cell surface adhesion receptors such as integrins, to form hemidesmosomal junctions¹.

During the development of embryonic nervous systems, cells migrate along ECM tracts that contain basal lamina components. Additionally, at the blood-brain barrier two basal lamina form a tight barrier that prevents diffusion of molecules between the blood and cerebrospinal fluid². In this way, the basal lamina can compartmentalise cells, orchestrate tissue repair and guide migrating cells during development.

In epithelial tissues such as breast, pancreas, lung, salivary gland and the prostate, the ECM is mechanically compliant; partly due to the presence of elastin and type I and III collagens, which alongside fibronectin are incorporated into a relaxed meshwork which is embedded in a glycosaminoglycan (GAG)-rich proteoglycan hydrogel^{3, 4}. This makes these tissues resistant to a variety of tensile stresses.

1.1.1.2 The ECM of connective tissues

Connective tissue such as tendon and cartilage differ from most other solid tissues, in that most of their volume is made up of ECM instead of cells. In contrast to basal laminae, connective tissues can also be highly resistant of compressive stresses, due to the binding of the GAG-rich proteoglycans to fibrillar ECM proteins⁵. The incorporation of GAGs permits the matrix to form a hydrogel, which binds a large amount of water and generates *turgor pressure* in the extracellular space that is highly resistant to compression.

Connective tissue ECM also contains abundant levels of insoluble protein fibres, predominantly type I, II and III collagens. Additional protein fibres such as elastin are found at sites that can be deformed, such as skin, tendons and heart; while fibronectin forms fibrils in the matrix of most connective tissues⁶. Together, these findings show that the ECM is a finely tuned and dynamic environment, which is remodelled in tissue specific manners.

1.1.2 *Components of the ECM*

The ECM is predominantly comprised of two classes of macromolecules. The first are the fibrous proteins⁷, and the second are the proteoglycans⁸. Below is a brief overview of the predominant fibrous protein and proteoglycan components of the ECM.

1.1.2.1 *Laminin*

Laminin is the primary multi-adhesive ECM protein expressed in basal laminae; it is a large 820kDa heterotrimeric cross-shaped protein, with 15 different known isoforms. The α -chain of laminin contains a globular LG domain at the C-terminus, which mediates Ca^{2+} dependent binding to cell surface carbohydrates such as syndecans. Furthermore, laminin α -chain also contains binding sites for heparan sulfate proteoglycans, and cell surface integrin receptors such as $\alpha 6 \beta 4$, which promotes the integrity of the basal lamina by forming focal adhesions, and promoting endothelial hemidesmosomal contacts⁹.

1.1.2.2 *Fibronectin*

Fibronectin is a 440kDa hetero-dimeric, multi-adhesive matrix protein consisting of two C-terminally linked polypeptides; that has >20 isoforms due to the RNA alternative splicing of a single gene. Fibronectin is present in soluble plasma and cell associated tissue forms, and fulfils diverse functional roles due to its multiple ligand binding sites, which enable binding to extracellular molecules collagen, fibrin, heparan sulfate proteoglycans and tenascin-C, in addition to cell membrane receptors such as integrins and syndecans.

Fibronectin contains the Arg-Gly-Asp (RGD) motif, which is specifically recognised by $\alpha \beta$ integrins on the cell membrane, and leads to the stimulation of intracellular signalling pathways that drive focal adhesion formation, migration and differentiation of a range of fibroblast and immune cell types. These features allow fibronectin to bind both ECM

molecules, and cell surface receptors, to provide cell anchoring points and signalling cues to cells populating the ECM¹⁰.

1.1.2.3 Elastin

Elastin is a highly elastic ECM protein rich in hydrophobic amino acids glycine and proline, which form highly mobile regions crosslinked by lysine residues that resume their original shape after being deformed. Many tissues such as blood vessels, lung and skin require elastic properties to function properly. In tissues, elastin polymerises into large insoluble fibres; though these are susceptible to proteolytic degradation by elastase enzymes expressed by neutrophils during the immune response¹¹.

1.1.2.4 Collagen

Collagens are the largest class of fibrous ECM molecules, including 16 distinct types of collagen. Collagen types are tissue specific, for example type IV collagen binds to laminin and proteoglycans to form the basal lamina, to which epidermal cells attach; while dermal ECM contains 80% type I, and 8% type III collagens. Type I and III collagens contribute to the tensile strength of skin, as these fibrillar collagens self-assemble into micro fibrils in both head-to-tail and side-to-side lateral conformations¹².

1.1.2.5 Proteoglycans

Proteoglycans are heavily glycosylated proteins that fill the interstitial space in the ECM with a hydrogel-like medium through which hormones, growth factors, ions and nutrients can diffuse freely. Perlecan is a proteoglycan molecule that fulfils a filtering role in the ECM, and form gels with varying pore sizes and charge densities, which impact the diffusion of compounds through the ECM. Due to this feature, perlecan has a major role in glomerular filtration in the kidney glomerular basement membrane^{13, 14}. Furthermore, the heparin sulfate chains of proteoglycans can bind growth factors and promote receptor signalling^{15, 16}; and can

bind pro-inflammatory chemokine molecules in epithelial cells, prolonging the inflammatory response.

1.1.2.6 Glycosaminoglycans (GAGs)

Glycosaminoglycans are long unbranched polysaccharides consisting of repeating disaccharide units which are strongly negatively charged and hydrophilic, attracting large quantities of water into their structure. The hydrated GAGs occupy large volumes relative to their mass and form hydrogels at low concentrations; these gels have remarkable buffering, force-resistance, hydration and binding properties, making these molecules ideal components of the cartilage matrix in load bearing joints.

1.1.2.7 Extracellular proteases

The ECM turnover is highly dynamic, and its remodelling is impacted by a number of enzymes which maintain tissue homeostasis. The turnover of ECM components is controlled by fibroblast secretion of Ca^{2+} dependent, Zn containing endopeptidases called matrix metalloproteinases (MMPs), which degrade a wide range of extracellular matrix proteins and cell receptors¹⁷. This destructive measure is counterbalanced by the secretion of tissue inhibitors of metalloproteinases (TIMPs)¹⁸ and other enzymes such as lysyl oxidase and transglutaminases which stiffen the ECM by crosslinking ECM molecules¹⁹.

1.1.2.8 Growth factors

The ECM also serves as a reservoir for growth factors, which can bind to ECM components to increase their local concentration in the tissue, or to promote cell signalling by retaining the growth factors close to the cell. In this manner ECM bound growth factors differentially regulate cell growth, migration and differentiation^{20, 21}.

1.1.3 *ECM dysregulation in disease*

The ECM is essential for life and directs essential processes such as cell proliferation, migration, survival, and apoptosis. Changes in the ECM are associated with healing wounds, and a number of pathologies including, but not limited to, cancer²², atherosclerosis²³, rheumatoid arthritis (RA)²⁴, osteoarthritis (OA)²⁵, and fibrotic diseases²⁶; indicating the importance of a carefully regulated extracellular environment to maintaining healthy tissue homeostasis.

My thesis focuses on one particular matrix protein, tenascin-C; specifically, how alternative splicing of tenascin-C is key to controlling the activity of this matrix molecule.

1.2 **The multifunctional nature of tenascin-C**

Tenascin-C was independently and concurrently characterised in the 1980s by several research groups with interests in the fields of cancer, matrix biology and embryonic/neural development. Tenascin-C is highly expressed in the developing embryo in a strictly regulated spatio-temporal pattern, but most healthy adult tissues exhibit negligible tenascin-C levels. Here, expression is constrained to sites where high cell turnover, plasticity and tissue-remodelling are obligatory; such as stem cell niches and the central nervous system (CNS), in addition to regions which undergo significant tensile stress; such as tendons, ligaments and smooth muscle fibres. However, transient expression of tenascin-C is observed at sites of active tissue remodelling in the adult, such as during the healing of wounds, and persistent tenascin-C expression is detected in pathological states such as cancer or rheumatoid arthritis (RA) (reviewed in ^{27, 28, 29}).

Tenascin-C consists of 4 distinct domains, which can interact with pathogenic components, matrix constituents, soluble factors and cell surface proteins; conferring upon tenascin-C the ability to bind to more than 57 different molecules identified thus far²⁹. For example, the tenascin assembly (TA) domain forms inter-molecular hydrophobic interactions and disulfide bridges, the epidermal growth factor-like (EGF-L) repeats act as a low affinity ligand for the EGF-receptor (EGF-R), inducing mitogen-activated protein kinase (MAPK), and phospholipase-C (PLC)- γ signalling, the 17 fibronectin (FN) type III-like repeats (FnIII), of which 8 are constitutively expressed (FnIII 1-8) and 9 are alternatively spliced (FnIII-A1-D), interact with proteins ranging from $\alpha 7\beta 1$ integrin and syndecan-4 to Annexin II. The constitutive FnIII domains 4-5 promiscuously bind to growth factors including members of the platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and transforming growth factor- β (TGF β) families. The fibrinogen-like globe (FBG) domain binds to integrins, receptor-type tyrosine-protein phosphatase beta/zeta (PTPR β/ζ) and the pattern recognition receptor toll-like receptor 4 (TLR4). These binding partners enable tenascin-C to drive a range of processes from oligomerization to induction of mitogenic responses, cell migration, cell attachment, cell spreading, focal adhesion, cell survival, matrix assembly, neurite outgrowth and potentiation and protease and pro-inflammatory cytokine synthesis (reviewed in ²⁹). Further information regarding tenascin-C binding partners is summarised in Figure 1.1.

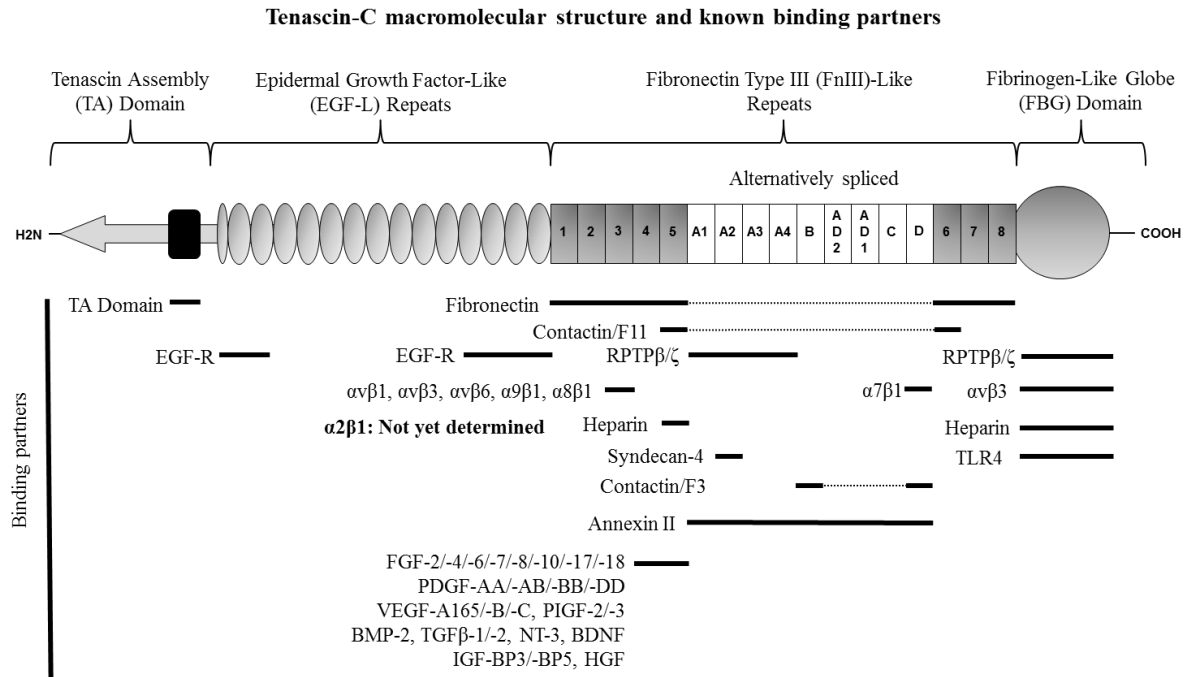


Figure 1.1. The domain structure and ligand binding sites of human tenascin-C. The human tenascin-C protein comprises 4 domains: a TA domain, 14.5 EGF-L repeats, up to 17 FnIII-like repeats (FnIII), and an FBG domain. Eight of the FnIII repeats are constitutively expressed (FnIII 1-8 (grey)), and 9 can be alternatively spliced (FnIII A1-D (white)). The TA domain binds other tenascin-C TA domains during oligomerisation³⁰, the EGF-receptor (EGF-R) bound to the EGF-L domains³¹. Fibronectin bound to the constitutively expressed FnIII but not the alternatively spliced FnIII³², contactin/F11 bound to the FnIII 5-6 domains when contiguous but not when interrupted by alternatively spliced FnIII domains A1-D³³; contactin/F3 bound to domains FnIII B,D when contiguous³⁴. Receptor protein-tyrosine phosphatase β/ζ (RPTPβ/ζ) bound to FnIII A1-A4³⁵ and FBG³⁶. The integrins αvβ1, αvβ3, αvβ6, α8β1 bound to the RGD sequence present in FnIII-3 (95). αvβ3 had an additional binding site in the FBG domain (34); while α9β1 bound to the AEIDGIEL sequence in FnIII-3 (24). The α2β1 binding site is unknown. α7β1 integrin bound to the VFDNFVLK sequence in FnIII-D (42). Heparin bound in FnIII-5³⁷, and syndecan-4 bound to a cryptic site within FnIII-A2^{38, 39}. Annexin II bound to the entire alternatively spliced FnIII cassette A1-D⁴⁰ while the growth factors fibroblast growth factor (FGF)-2/-4/-6/-7/-8/-10/-17/-18; platelet derived growth factor (PDGF)-AA/-AB/-BB/-DD; vascular endothelial growth factor (VEGF)-A165/-B/-C; phosphatidylinositol-glycan biosynthesis class F protein (PIGF)-2/-3; bone morphogenetic protein (BMP)-2; transforming growth factor beta (TGFβ)-1/-2; neurotrophin (NT)-3; brain derived neurotrophic factor (BDNF); insulin-like growth factor-binding protein (IGF-BP)3/-5; hepatocyte growth factor (HGF) promiscuously bound to FnIII 4-5⁴¹. Toll-like receptor 4 (TLR4) binds to three-epitopes within the FBG portion of tenascin-C^{42, 43}.

The diverse collection of functions for tenascin-C is reflected in the wide ranging consequences of genetic deletion of tenascin-C in mice. Whilst these mice are born viable and fertile with no obvious morphological defects in neural or organ architecture, there are noticable behavioural defects. Fukamauchi *et al.*, 1996 discovered that normal C57BL/6N mice and heterozygous littermates could swim for 3 minutes in a watertank without training, but that tenascin-C knockouts failed the test and must be rescued from drowning. Furthermore, normal mice usually move about their cages during the dark cycle, while tenascin-C knockouts move around their cages almost incessantly, independent of the dark-light cycle⁴⁴. In the wild, this hyperactive behaviour would expose the mice to predation more frequently, and may explain why tenascin-C is conserved throughout vertebrate evolution.

Additional abnormalities become apparent when the mice are stressed or subject to disease, with reported phenotypes including reduced fibronectin during dermal wound healing,⁴⁵ hyperactivity,⁴⁶ reduced kidney regeneration,⁴⁷ reduced haematopoiesis,⁴⁸ increased tumour monocyte population,⁴⁹ abnormal tumour organisation and angiogenesis,^{50, 51} and aberrant immune responses,^{43, 52, 53} among many others (reviewed in^{54, 55}). Genetic variation at the human tenascin-C gene locus is associated with 6-fold increase in risk of Achilles tendon injury,⁵⁶ non-syndromic hearing loss,⁵⁷ and increased risk of developing adult asthma⁵⁸.

Together these data point towards very diverse biological and pathological roles for tenascin-C that can be further modified by alternative splicing of the FnIII domains. In this chapter, I further discuss some of the mechanisms by which tenascin-C alternative splicing dictates the impact of tenascin-C expression on cell functions during embryonic development, and in cancer.

1.3 Tenascin-C gene

Tenascin-C is encoded by a large intron rich gene (*TNC*), which in humans spans 97.68kb of DNA on the antisense strand of chromosome 9, at locus 9q32-34/9q33.1^{59, 60} and of which only ~7.9% is protein coding (reviewed in ⁶¹). The first partial gene sequence derived for chicken tenascin-C identified several transcripts of various lengths prompting the speculation that the transcript is alternatively spliced.⁶² The first human tenascin-C exon sequence was published using complementary DNA (cDNA) clones isolated from U-373MG glioblastoma cells, identifying a clone with 8 consecutive FnIII like repeats, and another clone containing the same repeats but with a 1.9kb insert between FnIII 5 and 6; providing clear genetic evidence of alternative splicing within the tenascin-C transcript.⁶³

Alternative splicing allows a single gene to encode multiple proteins by the inclusion or exclusion of selected exons into the mature messenger RNA (mRNA), dramatically increasing the size and diversity of the proteome. Up to 95% of the ~21,000 protein coding genes in humans are alternatively spliced,⁶⁴ and 85% of these have a minor splice isoform with an expression frequency exceeding 15%. As a result this moderate number of genes is able to produce >290,000 non-redundant peptide combinations.⁶⁵ Following on from the first observations by Jones *et al*,⁶² and Gulcher *et al*,⁶³ that tenascin-C is subject to alternative splicing, many studies have expanded on this theme revealing that post transcriptional modification of tenascin-C has a profound effect on tenascin biology.

1.4 Tenascin-C exon structure

The first *TNC* human exon sequences published showed the gene to comprise 28 exons (accession number NM_002610). However, two further alternatively spliced FnIII domains (additional domains 1 and 2: FnIII AD1⁶⁶ and AD2⁶⁷ (accession numbers U88892.1 and EU295718.1 respectively) were later discovered. FnIII AD1 was identified within human U251 glioma cDNA clones as a single exon between FnIII repeats B and C. This study also showed that whilst AD1 was present in human glioblastoma, neuroblastoma and osteosarcoma tumour cells, it was absent in healthy human lung fibroblasts and umbilical vein endothelial cells (HUVECs) providing the first indication that tenascin-C splicing may play a role in tumorigenesis.⁶⁶ Human FnIII AD2 was discovered between FnIII B and AD1 in oral mucosa carcinoma samples and was named so on account of being in the same respective location as the avian FnIII AD2; situated between FnIII B and AD1, in addition to it sharing 70% amino acid and 55% nucleotide sequence homology with the avian FnIII AD2.^{67, 68} Thus human *TNC* contains 30 exons (Figure 1.2).

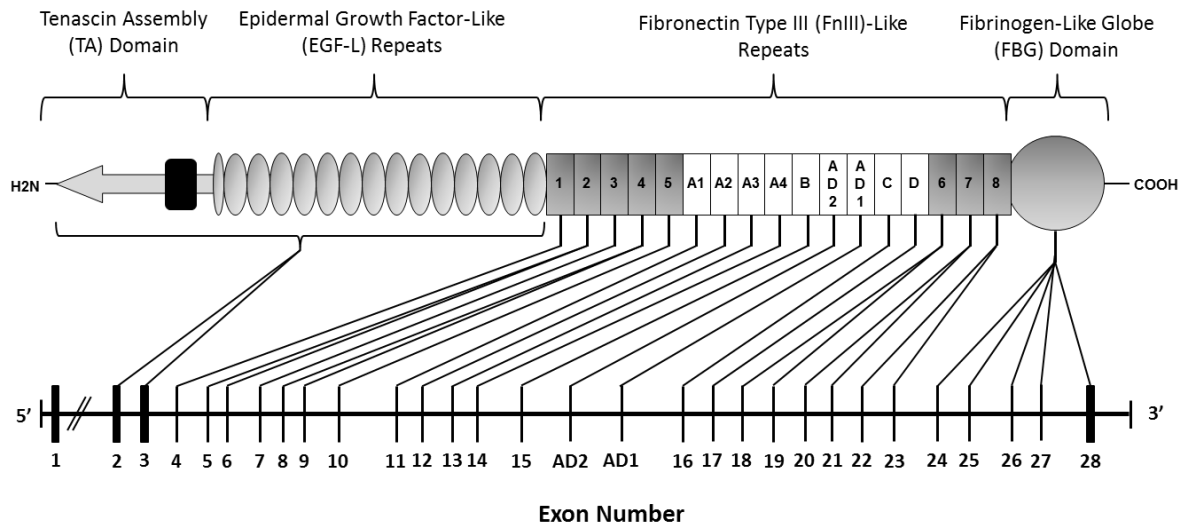


Figure 1.2. The exon structure of *TNC* with corresponding protein domains in tenascin-C. The *TNC* gene comprises 30 exons (1-28, plus AD2 and AD1). All exons are translated excluding the first. Exon 2 encodes the start sequence for translation of mRNA, and together exons 2 and 3 code for the signal peptide, the TA domain and all of the EGF-L repeats. The 8 constitutively expressed FnIII repeats are coded for between exons 4-10, and 18-23, and the 9 alternatively spliced FnIII from exons 11-17. Each alternatively spliced FnIII repeat is encoded by its own exon; in contrast, the constitutive FnIII repeats 1 and 3 are encoded by a single exon, but the remainder of the modules FnIII 2 and FnIII 4-8, are encoded by 2 exons each. Alternative splicing of FnIII domains within the tenascin-C pre-mRNA transcript means that the human *TNC* exon sequence varies in size from a maximum of 9154bp to a minimum of 6251bp. The FBG domain is coded by exons 24-28^{63, 66, 67, 69}.

Tenascin-C in other vertebrate species including chickens, rats, mice and pigs, exhibits similar domain organizations to the human protein. Each contains a TA domain, a series of EGF-L and FnIII like domains and an FBG domain. However, a number of differences exist between orthologs. For example, all vertebrate tenascin-C contains 13.5 EGF-L repeats, with the exception of mammals which possess 14.5 EGF-L repeats.⁷⁰ Furthermore, tenascin-C from humans, rats, mice and chickens each contain 8 constitutive FnIII repeats, with an additional cassette of FnIII which may be alternatively spliced between FnIII domains 5 and 6. However, the number of alternatively spliced FnIII repeats varies by species; with chickens and mice, rats, and humans having 6, 7 and 9 respectively^{67, 71, 72} (Figure 1.3).

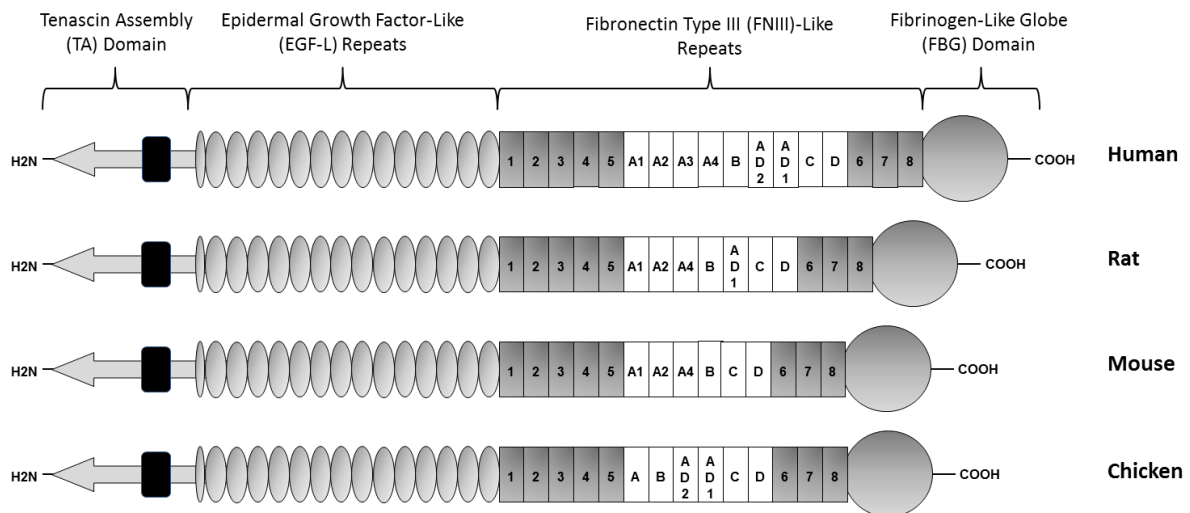


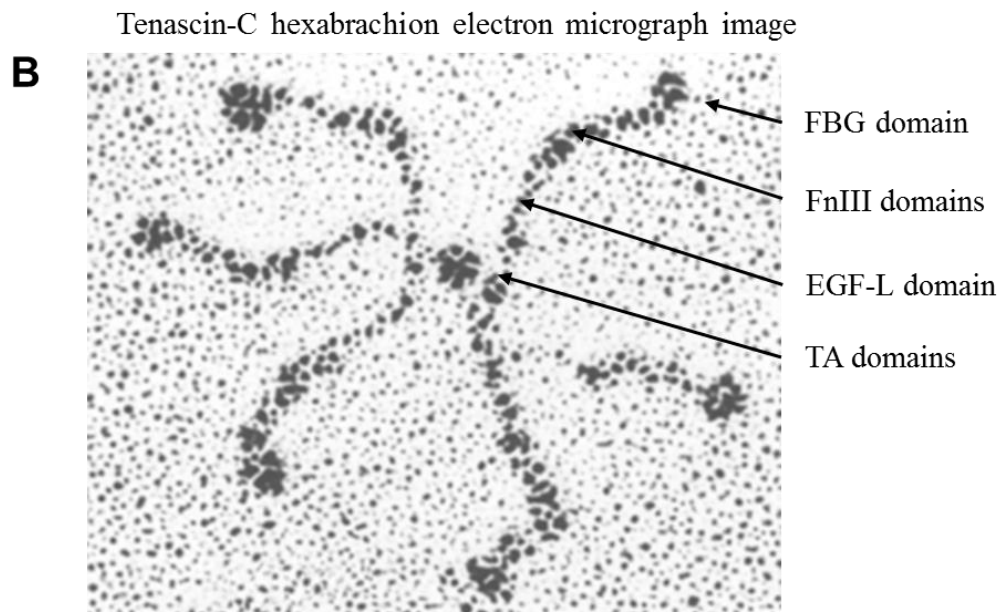
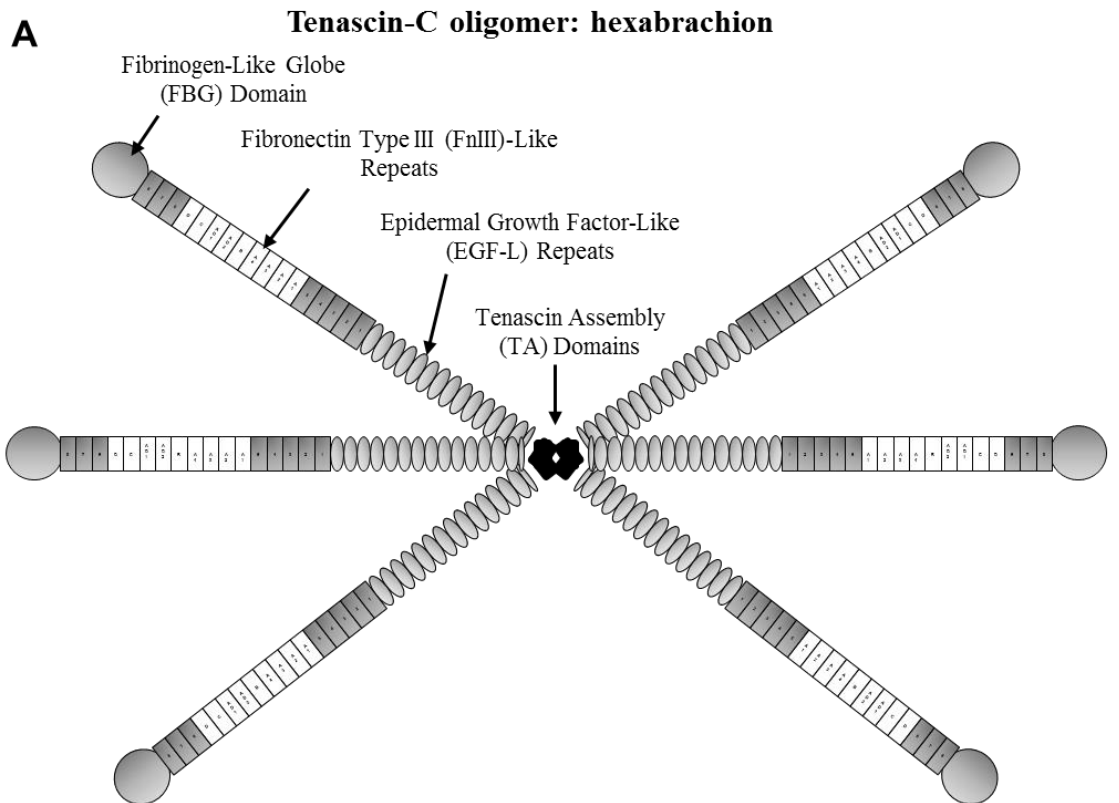
Figure 1.3. Schematic representation of human, rat, mouse and chicken tenascin-C. While each species contains 8 constitutively expressed FNIII domains, the number and content of alternatively spliced FNIII repeats varies. Human tenascin-C contains 9 alternatively spliced FNIII repeats, rat 7, and mouse and chicken 6 each. Alternatively spliced FNIII repeats are typically more homologous than constitutive repeats. For example, constitutive mouse FNIII repeats share on average 44% nucleotide sequence identity to each other, in contrast to alternatively spliced FNIII which share 52% identity. Of the mouse alternatively spliced FNIII, A2 and D share the lowest nucleotide sequence identity at 41%, while A1 and A4 share 80%. Analysis of human tenascin-C also noted 80% amino acid sequence homology between the first 4 alternatively spliced modules (A1, A2, A3 and A4), in contrast to the other alternatively spliced FNIII repeats; raising the possibility that these domains are the result of gene duplication of an ancestral FNIII module. The absence of any comparable homology between avian spliced FNIII repeats, allows for speculation that any such duplication occurred after the divergence of avian and mammalian lineages^{59, 73}.

Theoretically there are 511 possible human tenascin-C splice isoforms; this figure was calculated via the equation $\frac{9!}{(9-r)!(r)!}$ as follows, $\left(\left(\frac{9!}{(9-1)!(1)!} \right) + \left(\frac{9!}{(9-2)!(2)!} \right) + \left(\frac{9!}{(9-3)!(3)!} \right) + \left(\frac{9!}{(9-4)!(4)!} \right) + \left(\frac{9!}{(9-5)!(5)!} \right) + \left(\frac{9!}{(9-6)!(6)!} \right) + \left(\frac{9!}{(9-7)!(7)!} \right) + \left(\frac{9!}{(9-8)!(8)!} \right) + \left(\frac{9!}{(9-9)!(9)!} \right) \right)$ where 9 represents the total number of alternatively spliced FnIII, and r represents the total number of alternatively spliced FnIII included in a single variant. Despite this large theoretical variation, the number of tenascin-C splice isoforms recorded does not exceed 100. This is partly because to date no exhaustive analysis and identification of all tenascin-C splice variants expressed in human tissues has been performed. Aside from the currently incomplete picture of tenascin-C splice variants expressed in human tissues, the reasons accounting for the large discrepancy between the numbers of theoretical and recorded splice isoforms is not yet known, but may result from epigenetic regulation, miRNA interference or the presence of unknown alternative splicing regulatory factors that limit the total number of potential splice isoforms, by preferentially selecting for the inclusion of specific FnIII exons into the mRNA transcript. Furthermore, in a genome wide *in silico* analysis Wang *et al.*, 2008 described tissue specific regulation of alternative mRNA splicing that is characterised by skipped and mutually exclusive exons⁷⁴; which was supported by previous findings of tenascin-C splicing, where some alternatively spliced FnIII domains are preferentially expressed together. For example, human FnIII AD2 is observed to be included in the transcript only when linked to AD1, FnIII C only when alongside D and interestingly FnIII A4 and C have never been observed to be linked together in mice or humans^{67, 71, 73}. Therefore currently unknown splicing regulatory mechanisms may promote the skipping of exons, or promote expression of mutually exclusive FnIII repeats, which could account for the reduced number of tenascin-C splice variants which are observed.

1.5 Tenascin-C biosynthesis

Following translation, tenascin-C assembles intracellularly into a 1.1-2.0MDa six-armed hexabrachion structure comprised of six monomers.⁷⁵ This process is largely determined by N-terminal residues which are arranged into three cysteine-rich heptad repeats which form a coiled-coil region (amino acids 118-145 in human tenascin-C);^{30, 76} a feature common to other oligomeric matricellular proteins such as thrombospondin and cartilage oligomeric matrix protein-1⁷⁷.

While hexabrachions are often formed as homotypic molecules comprised of identical subunits, there are instances where heterotypic molecules can be formed. Kammerer et al.³⁰ demonstrated that two triplets of tenascin-C trimers; one missing both the FnIII and FBG domain, were able to heterodimerize forming a heterotypic hexabrachion. Furthermore, smaller oligomers comprising of 2-5 tenascin-C molecules have also been identified, although it is not known whether these smaller isoforms are deliberately assembled in this manner, or if they are merely remnants of hexabrachions degraded by proteases⁷⁸ (Figure 1.4.A,B).



Erickson and Inglesias., 1984

Figure 1.4. Tenascin-C structure and model of the tenascin-C hexabrachion. (A) Model of the tenascin-C hexabrachion oligomer, with the TA domain, EGF-L domains, FnIII domains and FBG domains labelled. (B) Electron micrograph image of the hexabrachion that was isolated from purified fractions of plasma fibronectin. Here the central globular TA domain has six-arms emanating from its centre, each containing a thin proximal EGF-L domain region, followed by the thicker FnIII domains, and a distal FBG globular domain. Modified from Erickson and Inglesias.,⁷⁸.

1.6 Tenascin-C splicing: embryogenesis and development

Alternatively spliced isoforms of tenascin-C have been shown to play integral roles in many different processes. In this section, I focus on describing how alternative splicing controls tenascin-C action during development.

Tenascin-C is abundantly expressed during embryogenesis in neuro-ectodermal tissues, and subsequently in several non-neural sites where high cell-turnover, tissue remodelling and epithelial-mesenchymal interactions occur (reviewed in ⁷⁹). Typically, the overall expression of tenascin-C decreases with increasing age, generally peaking during embryonic development and shortly after birth before decreasing into adulthood when expression is restricted to a few sites at relatively low levels.^{80, 81} Prior to the development of tools permitting the detection of specific alternatively spliced FnIII repeats, many studies reported the presence of high and low molecular mass (*Mr*) isoforms of tenascin-C, differentiated by their apparent *Mr* upon polyacrylamide gel electrophoresis (PAGE) under reducing conditions, at various stages of embryogenesis in chickens, mice and rats. Further analysis utilizing in situ hybridization (ISH) and immunostaining/western blotting (WB) with cDNA probes and monoclonal antibodies (mAbs) respectively, could distinguish between ‘long’ and ‘short’ tenascin-C forms; revealed that differently size splice variants are expressed in cell and tissue specific patterns that change over the course of development. These studies are described below and summarized in Table 1.1.

1.6.1 *Large versus small*

A good example of differential expression of tenascin-C splice variants during development was seen in an elegant series of studies in the developing chick, which revealed 4 main isoforms; Tn260, Tn230, Tn200 and Tn190, which have apparent *Mr* of 260, 230, 200 and 190kDa respectively.^{82, 83} Tn260 was rarely expressed in contrast to Tn230, Tn200 and Tn190, which were widely expressed during embryonic and early postnatal development.^{76, 84} High *Mr* variants (Tn230/200) were associated with regions of active tissue remodelling, cell migration and cell division; evidenced by their presence in epithelial substratum for migrating neurons, embryonic skin fibroblasts, whole brain, cerebellum, chord glia, Bergmann glia, endoderm-derived epithelium at developing lung bronchioles, growing wing bud tips, base of feather buds, major blood vessel endothelium, kidney, lung, osteoblasts and regions of osteogenesis; where the expression of tenascin-C is only required transiently.^{62, 80, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94} In these regions, large *Mr* tenascin-C is expressed by migrating glia and Bergmann glia in the developing chick spinal cord and cerebellum respectively; expression in the latter of which facilitates granule cell migration.⁹¹ In contrast, lower *Mr* isoforms (Tn190/200) are observed to be expressed more stably in dense connective tissue in areas such as gizzard tendons, intramuscular connective tissue, aortic mesenchyme, articular cartilage, inner layer of perichondrium and zones of active chondrocyte proliferation, where cell condensation and differentiation are more prevalent.^{62, 84, 86, 89, 90, 91, 92, 95, 96, 97}

Similar patterns of tenascin-C expression are observed in mice and rats, although significantly less is known about the presence of alternatively spliced tenascin-C during human foetal development. WB and real-time PCR techniques revealed that smaller *Mr* tenascin-C variants are the predominant isoforms expressed in embryonic day (E)14 mouse gut mesenchyme,⁹⁸ and in the developing thymus and skin from E17 to postnatal day (P)6 respectively.⁸¹ Furthermore, smaller isoforms are observed to be persistently expressed into

adulthood in thymus, colon, cerebellum, lymph nodes and splenic tissues.^{80, 81} In contrast, the larger *Mr* isoforms are the predominant variants expressed in embryonic mouse kidney and in developing rat lung.^{85, 99, 100} Small isoforms are also implicated in regulating development of rat lungs, where the expression of a small variant is shown to be preferentially induced by TGF β in explant cultures,¹⁰⁰ and low *Mr* isoforms have the effects of inhibiting lung branching morphology and aveolarization.⁹⁹

The splicing pattern within developing tissues was also observed to vary over time, as well as by location within the organism. For example, there is a shift in the relative abundance of splices away from large isoforms, in favour of smaller ones with increasing age in developing mouse kidney, cerebellum, skeletal muscle, stomach, bladder, duodenum, ileum, jejunum and colon.^{80, 85, 101, 102} In embryonic chicken brain and gizzard, the relative proportion of smaller variants also increases with developmental age,^{62, 88} as is also true in embryonic mouse intestine, gut mesenchyme, and chick cerebellum.^{85, 89, 98}

The smallest tenascin-C isoform, with no alternatively spliced FnIII included, is known to bind strongly to fibronectin and the glycosylphosphatidylinositol-anchored neural cell adhesion molecule contactin/F11^{33, 103, 104} and to promote cell attachment and the formation of focal adhesions. This is in contrast to larger tenascin-C isoforms, which prevent focal adhesion formation and drive cell migration.¹⁰⁵ These data imply that predominance of the larger isoform at sites of active tissue remodelling aid cell migration and dynamic tissue organization, whilst the smaller, pro-adhesive isoforms mediate stability of newly formed tissues towards the end of development and into adulthood. Indeed, in the prenatal chick brain, larger isoforms are abundantly expressed from E6 although the relative occurrence of smaller isoforms increases from E6 to E15;⁸⁸ and at postnatal day 3 only a single 7.2Kda message encoding a 220kDa peptide is detected.⁸⁹ Increased cell migration in the developing CNS was found to correlate with accumulation of long tenascin-C, but not short tenascin-C

isoforms;^{95, 102, 106, 107} indicating that long tenascin-C splices facilitate neurite motility in development.¹⁰⁸

These studies provided a wealth of information about tenascin-C splicing during development, but it is worth mentioning that two isoforms containing a different repertoire of alternatively spliced FnIII repeats can still exhibit the same molecular mass, as each alternatively spliced FnIII repeat is approximately the same size (89-92 amino acids), with a mass of ~10kDa each. Moreover, analysis of tenascin-C forms based purely on *Mr* does not allow distinction between tenascin-C that has been alternatively spliced or tenascin-C that has been proteolytically clipped. As it became apparent that each alternatively spliced FnIII repeat had unique functions, analysis of the precise FnIII repeats that make up each isoform became increasingly important.

Table 1.1. Association of ‘long/large’ and ‘short/small’ tenascin-C splice variants with stages of chicken, mouse and rat embryonic development.

Species	Size of splice variants detected (kb, kDa, if known)	Tissue or cell type	Features of study	Reference
Chicken	Small (150kDa, 170kDa, 190kDa, 200kDa) and large (220kDa)	Embryonic skin fibroblasts, breast muscle	E11 fibroblasts predominantly express Tn220 in ratio 4:1:1 with Tn200/190 respectively, and express 7x more tenascin-C than muscle cells. E11 myoblasts express Tn220, 200 and 190 in ratio 2:1:1 respectively	Chiquet and Fambrough. ⁸⁷
	Small and large	Embryonic brain, gizzard, wing and skin fibroblasts	E10 skin fibroblasts predominantly express Tn220, but also Tn200 isoform. Doublet at ~190kDa predominant in E11 brain, gizzard and wing. Bands also detected include 210, 220kDa and ~400kDa in brain	Erickson and Taylor. ¹⁰⁹
	Small (170kDa) and large (195kDa, 205kDa and 220kDa)	Sterna	Identified 6-armed oligomer from E17 sterna. Reducing-PAGE gave prominent major bands at 195/205kDa, and minor bands at 220/170kDa	Vaughan et al. ¹¹⁰
	Small (190/200kDa)	Embryonic chick retina	In E8 retina Tn-C 190/200kDa abundant along with ligand contactin/F11 in inner and outer plexiform layers. Identified possible binding site for HSPGs within FnIII-5	Vaughan et al. ¹¹⁰
	Small (180kDa, 160kDa) and large (200kDa, 220kDa, 250kDa).	Brain	Identified novel 250kDa chondroitin sulfate containing isoform. Larger isoforms are expressed extensively at E6 to E15, but prevalence of smaller isoforms increases over this time	Hoffman et al. ⁸⁸
	Small (220kDa, 200kDa/ 7.2kDa) and large (240kDa, 220kDa/ 8kb)	Embryonic gizzard, brain, liver	7.2kb and 8kb mRNA isoforms increased in expression in E9/E15 gizzard and brain respectively. Corresponding peptides of 220/240kDa and 200/220kDa were identified in gizzard and brain respectively	Jones et al. ⁶²
	Small (190kDa) and large (230kDa/ 200kDa)	Embryonic chick fibroblasts	Identified 3 cDNA clones generated from E11 skin fibroblasts with open reading frames of 1808, 1626 and 1535 amino acids, which correspond with <i>in vitro</i> translated tenascin identified at 200/180 and 170kDa	Spring et al. ⁷⁶
	Small (190kDa) and large (230kDa/ 200kDa)	Primary chick fibroblasts	Large isoform associated with gizzard smooth muscle layer and connective tissue below villi epithelium. Shorter isoform predominant in tendons and intramuscular connective tissue. Transfection with middle-T polyomavirus antigen induces preferential secretion of large but not small isoforms	Matsuoka et al. ¹¹¹
	Small (6.6kb, 6.4kb) and large (220kDa/ 7.2kb)	Cerebellum	Total tenascin-C increased E8 to E15, then decreased until barely detectable at P3. 7.2kb mRNA prominent in E6-15 cerebellum while 6.4kb decreases over this time. ISH probe for FnIII-B,C hybridized only to 7.2kb message in	Prieto et al. ⁸⁹

Species	Size of splice variants detected (kb, kDa, if known)	Tissue or cell type	Features of study	Reference
			CNS, and was absent in non-neural tissues (chondroblasts, tendons and lung mesenchyme)	
	Large	Embryonic skin fibroblasts	Proteolytic cleavage of the 230kDa variant isolated from E11 skin fibroblasts by pronase, and detected by mAb Tn68, produced C-terminal heparin binding peptide fragment specific only to cleavage of large isoform	Chiquet et al. ⁸³
	Small (190kDa) and large (200kDa and 220kDa)	Embryonic cornea	Close association between 220kDa isoform expression and embryonic corneal cell migration in E3-19	Kaplony et al. ⁹⁵
	Small (190kDa) and large (230kDa)	Embryonic lung bud/ bronchiole tube epithelium	Used ISH to detect mRNAs corresponding to Tn190 in tips of budding bronchioles but not older epithelia or dense mesenchyme. Tn230 probes had identical association to Tn190	Koch et al. ¹¹²
	Small (200kDa, 190kDa) and large (220kDa)	Neural crest	Identified Tn230, 200, 190 in E3 neural crest cell conditioned media. Identified neural crest as major expresser of tenascin-C in developing spinal cord until E18 after which all splices not expressed	Tucker and McKay. ⁹³
	Small (190kDa, 200kDa) and large (230kDa)	Periosteal cells, osteoblast enriched cultures and endochondral bone.	Used universal cDNA probe to detect tenascin-C in osteogenic and chondrogenic regions. Full length tenascin-C present only in osteogenic regions and expressed by osteoblasts. Periosteal cultures express 3 isoforms but enriched osteoblast cultures express Tn230 only	Mackie and Tucker. ⁹⁰
	Small (190kDa)	Embryonic chick brain	Identified Tn190 as ligand to contactin/F11. Contactin/F11 binds FnIII-5,6 via first 3 Ig-domains and binding efficacy is reduced by incorporation of alternatively spliced FnIII	Zisch et al. ¹⁰³
	Tn190, 200, 230	Embryonic knee cartilage	IHC revealed Tn190 present in E11 articular cartilage. Peripheral articular cartilage and fibrocartilage expresses Tn200, and Tn230 is expressed in perichondrium	Pacifici et al. ⁹⁶
	Small and large	Whole chick embryos	ISH revealed large and small tenascin-C abundantly expressed in embryo at E3-7. From E10-15 expression was spatially regulated; chord glia, Bergmann glia, endoderm-derived epithelium at growing tips of lung bronchioles, endothelium of major vessels, and osteogenic regions predominantly express large isoform. Small isoform associated with cartilage deposition and chondrocyte proliferation e.g. surrounding E10 notochord	Tucker. ⁹¹

Species	Size of splice variants detected (kb, kDa, if known)	Tissue or cell type	Features of study	Reference
	Small and large	Embryonic aorta and adjacent mesenchyme	Identified 3 isoforms containing 0, 1 or 3 alternatively spliced FnIII repeats, in haematopoietic progenitor/primordial germ cell migratory pathways of which the smallest was most prominent	Anstrom and Tucker. ⁹⁷
Mouse	Small (210kDa) and large (260kDa)	Gut mesenchyme	Small isoform predominant at E14, but after birth abundance of large isoform increases	Aufderheide and Ekblom. ⁹⁸
	Small (190kDa) and large (220kDa)	Embryonic intestine, brain	190kDa isoform more prevalent than 220kDa isoform in mouse ileum, but relative concentration is the same from E14 to adulthood. Adult brain expresses single 160kDa isoform. Developmental appearance of increasing concentration gradient of all tenascin-C from crypt to villus in ECM at epithelial-mesenchyme interface. Proposed to facilitate epithelial shedding in the villus	Probstmeier et al. ¹¹³
	Small (5.5kb) and Large (7kb)	Brain, submandibular gland, thymus, lung, heart, spleen, kidney, liver, pancreas, oesophagus, stomach, intestine, bladder, skin and skeletal muscle	Two isoforms observed between E17 and P6 in skeletal muscle, stomach, cerebellum, bladder, duodenum, jejunum, ileum, and colon. Large (7kb) isoform observed in lung, kidney and cerebrum. Small isoform observed in thymus and skin. Expression of all variants was decreased at P32, but small (5.5kb) message continued to be transcribed in thymus, colon and cerebellum	Saga et al. ⁸⁰
	Small (6kb) and large (8kb)	Kidney, intestine	Large variant predominant in neonatal mouse kidney but postnatally small variant increases in abundance. E13 intestines express small isoform, and by birth the larger isoform is predominant	Weller et al. ⁸⁵
	Small (190kDa, 200kDa/ 6kb) and Large (225kDa, 240kDa/ 8kb)	Cerebellum	Expression of larger isoforms down regulated faster than smaller isoforms from P0 to >P60	Bartsch et al. ¹⁰²
	Small (200kDa/ 6kb) and large (230kDa/ 8kb)	Thymus, spleen, lymph nodes, lung, skin, cerebellum	Small isoform abundantly expressed in adult thymus, weaker expression in cerebellum, skin and none in spleen, testes, skeletal muscle, liver, kidney and heart. WB revealed 200kDa tenascin in adult spleen and lymph nodes while thymus also contained 230kDa isoform	Ocklind et al. ⁸¹
	Small (200kDa) and large (250kDa)	mouse embryonic fibroblasts (NIH-3T3 cells)	TGFβ1 and FGF induce expression of small and large isoforms respectively in mouse embryonic fibroblast cell line	Tucker et al. ⁹²

Species	Size of splice variants detected (kb, kDa, if known)	Tissue or cell type	Features of study	Reference
Rat	Small (6.5kb) and large (7.2kb/280kDa)	Lung	7.5kb mRNA more abundant than 6.5kb in developing rat lung. Corresponds with prominent 280kDa isoform detected from E17. All tenascin-C expression increases at early postnatal age and decreases to levels found in adult by P21. Bacterially expressed FnIII-6,8 peptide inhibited lung branching morphogenesis though only slightly more than FnIII1-5 and A-D	Young et al. ⁹⁹
	Small (6.4kb/180kDa) and large (7.3kb/230kDa)	Lung explant culture	TGFβ preferentially induces expression of 180kDa isoform containing 1 alternatively spliced FnIII, over 230kDa isoform containing 5 in dose dependent manner. Strong expression of both occurs from P2 to P30, and decreases from P30 into adulthood, although 230kDa isoform more abundant from E19 onwards	Zhao and Young. ¹⁰⁰
	Small (180kDa) and large (230kDa)	Cultured lung epithelial, fibroblast and endothelial cells	The conditioned medium of lung fibroblasts and endothelium both expressed both 180 and 230kDa variants, whereas lung alveolar cells expressed very little total tenascin-C	Zhao and Young. ¹¹⁴
	Range from small (190kDa) to large (280kDa)	Cortex, thalamus and cerebellum	E16 and P7 cortex, thalamus, cerebellum. All tissues expressed isoforms ranging from 190-280kDa. Large variant is most prominent at E16 and P7 in all tissues. Expression of total tenascin-C increased most in P7 cortex and thalamus, but no major shift in the ratio of isoform expression was observed	Götz <i>et al.</i> , 1997 ¹¹⁵

1.6.2 *Analysis of specific alternatively spliced FnIII domains in development*

The pattern of expression of specific FnIII domains in developing tissues and the functional consequences of the expression of these different isoforms are described below and summarized in Table 1.2 and Table 1.3 respectively. In both the text and tables individual splice variants separated by (,) are included in the same transcript, while those separated by (/) are not.

Table 1.2. Associations between specific alternatively spliced FnIII repeats and developing tissues.

Individual splice variants separated by (,) are included in the same transcript, while those separated by (/) are not.

Cell/Tissue Type	Alternatively spliced FnIII repeats	Associations	Method of Identification	Reference
Chick embryo	A,B	FnIII-A,B containing tenascin-C synthesised by migrating glia and osteoblasts at sites of epithelial-mesenchymal interactions in feather buds, kidney, bronchiole tips and tendons	ISH, IHC, PCR	Tucker et al. ⁹¹
Embryonic mouse cerebellum	A1,A2,A4,B, D/ A1,A2,A4,D/ D/ no FnIII	Splices contained all FnIII or excluded C, B-C, A1-C or A1-D. Expression of isoforms containing 6, 5, 4, 1, 0 alternatively spliced FnIII decreased from E14 to adulthood, although expression of larger isoforms decreased faster than shorter isoforms	ISH, PCR, Northern Blot (NB), sequencing, Southern blotting (SB)	Dörries and Schachner. ¹⁰¹
Rat aortic smooth muscle cells (ASMCs)	Full length/ D/ none	Treatment with PDGF-BB subunit homodimer or Angiotensin II induced expression of mRNAs containing all, one or no variable FnIII repeats. Tenascin-C also inhibited cell adhesion of ASMCs to FN	Radiolabelling, WB, cell adhesion assays, PCR	LaFleur et al. ¹¹⁶
Embryonic chick spinal cord, tendons, base of feather buds, bronchiole tips, skin fibroblasts.	A/ B/ C/ A,B/ AD2/ AD1	First report of FnIII-AD1/ AD2/ C in chicken. ISH cDNA probes for FnIII-A/ B/ C hybridize in E7 bronchiole tips, ligamentum flavum, kidney mesenchyme, FnIII-A,B in E7 aorta endothelium, spinal-cord ependyma and E10 spinal cord, tendons and base of feather buds. FnIII-C absent in spinal cord. Identified FnIII-AD1 and AD2 in E11 skin fibroblasts by PCR	ISH, WB, IHC, PCR	Tucker et al. ⁶⁸
Chick embryonic lung bud tips, feather buds, bone	AD1/ AD2	AD2 observed in E10 bronchiole bud tips. AD1 expression more widespread and abundant in developing bone (where 85% tenascin-C contained AD1)	Quantitative WB, IHC, PCR and SB	Derr et al. ⁹⁴
Human Fetal Membranes	Predominant isoforms D/ A1,A2,A3,A4/ A1,A2,A4	Identified 8 mRNA isoforms associated with processes analogous to tissue remodelling and wound response prior to labour and delivery in normal membranes Speculated that inclusion of FnIII A3 provides substrate for MMP-2, 3, 7 digestion prior to membrane rupture	Sequencing, PCR	SB, Bell et al. ¹¹⁷

Cell/Tissue Type	Alternatively spliced FnIII repeats	Associations	Method of Identification	Reference
Mouse postnatal day 6 cerebellum	27 isoforms ranging from 1-6 FnIII repeats	Identified 27 tenascin-C isoforms (22 of which were novel) in P6 cerebellum. Cerebellum confirmed as major expresser of tenascin-C in P6 brain. Only splice containing FnIII-D found in adult brain	PCR, SB	Joester and Faissner. ⁷¹
Mouse embryonic whole tooth	D/ A1,D/ B,D/ B,C,D/ A1,A2,A4,B, D	E13 whole tooth expresses multiple alternatively spliced isoforms	ISH with cDNA probes, PCR, IHC	Sahlberg et al. ¹¹⁸
Mouse embryonic dental papilla mesenchyme	D/ B,D/ A1,A2,A4,B, D	E12 dental mesenchyme expresses FnIII-D following induction by FGF-4 and TGF β . TGF β induces expression of long mRNA containing 5 alternatively spliced FnIII. Propose mesenchyme becomes sensitive to epithelial induction of tenascin-C during E11	ISH with cDNA probes, PCR, IHC	Sahlberg et al. ¹¹⁸
Mouse NSCs	20 isoforms identified, novel A1,A4,B,D	Identified 20 isoforms in embryonic forebrain derived NSCs. Transcription factor Pax6 overexpression induced isoforms with 4, 5, and 6 alternatively spliced FnIII repeats, but downregulated smaller ones	PCR, gene overexpression	Von Holst et al. ¹¹⁹
Mouse embryonic NSCs	Full length	Identified N-and O-linked HNK-1 epitopes expressed in NSC rich regions is almost exclusively expressed on full length tenascin-C. Small interfering RNA (siRNA) knockdown showed HNK-1 on full length tenascin-C promotes NSC proliferation via modulating EGF-R expression	Immunocytochemistry (ICC), WB, siRNA knockdown, PCR, liquid chromatography MS/MS	Yagi et al. ¹²⁰
Rat Hippocampal Neurons	AD1, various others.	FnIII AD1 detected in VZ and area dentate of rat brain. Observable shift from short to long isoform expression in rat hippocampus from embryonic day 16, to postnatal day 5	ICC, PCR, ISH	Garwood et al. ⁷²

1.6.2.1 *Developing Mouse*

Tenascin-C is abundantly expressed in the mouse olfactory bulb, ventricular zone (VZ) and sub ventricular zone (SVZ) of the CNS during development and postnatally^{121, 122, 123} where it has been shown to orchestrate the development of niches permitting the development of neural stem cells (NSC) altering their response to FGF-2 and BMP-4.¹²⁴ Embryonic and postnatal day 6 mouse cerebellum express 4 and 27 different major mRNA isoforms respectively, of which the largest decrease in expression faster than the shorter variants during neural development.^{71, 101} Similarly, NSCs express 20 isoforms which are differentially regulated by Pax6 and Sam68 transcription factors.^{119, 125} In NSCs, expression of the largest isoform comprising FnIII-A1,A2,A4,B,C,D was demonstrated to contain *N*- and *O*-linked natural killer-1 (HNK-1) carbohydrate epitopes;¹²⁰ first described on tenascin-C by Kruse et al.¹²⁶ HNK-1 epitopes; otherwise known as CD57 are named after the HNK-1 mAb, and are found on the surface of a subset of T-lymphocytes and natural killer cells, but also on a number of cell adhesion molecules distributed throughout the nervous system including myelin-associated glycoprotein,¹²⁷ neural cell adhesion molecule,¹²⁸ and L1 cell adhesion molecule.¹²⁹ HNK-1 has functions in cell-cell and cell-substrate interactions, and promotes NSC proliferation via modulating the expression of the EGF-R.¹²⁰

Further functional studies demonstrated how tenascin-C splice variants are capable of modulating the response of neurons during CNS development. In E18 rat and P6 mouse neurons, FnIII-A1,A2,A4,B,D coated plates, supported initial attachment.¹³⁰ FnIII-B,D/ D,6 and 6 containing isoforms facilitate neurite outgrowth in P6 granule cell neurons and E18 rat hippocampal, P0 mouse dorsal root ganglia explants respectively, and even increase the proportion of neurite bearing cells in culture.^{130, 131} Mercado et al.¹³² demonstrated that the FnIII-D repeat is strongly inductive of neurite outgrowth on account of two short sequences within the VFDNFVLK amino acid sequence, which permit interactions with $\alpha 7$ and $\beta 1$

subunit containing integrins. The FnIII-A1,A2,A4 region in contrast is anti-adhesive and was demonstrated to promote neuronal migration and repulsion in rat E18 and P0 mouse root dorsal ganglia neurons,^{130, 131} and E16 rat cortical and thalamic axonal outgrowth.¹¹⁵

Non-neural sites exhibiting a diverse expression of splice variants include the developing tooth, which at E13 expresses D/ A1,D/ B,D/ B,C,D/ and A1,A2,A4,B,D.¹¹⁸ Nearby dental mesenchyme papilla expressed FnIII-D following induction by FGF-4 and TGF β , the latter of which also induced the expression of a long mRNA variant containing 5 alternatively spliced FnIII repeats. Interestingly, the response of dental mesenchyme papilla to growth factor induction always occurred in E12 but not always in E11, suggesting that the dental mesenchyme becomes responsive to the induction of tenascin-C at some point during E11.¹¹⁸ In the mammary gland, full length tenascin-C isoforms are highly prevalent during involution, and recombinant proteins containing FnIIIA1-D inhibit milk protein synthesis, suggestive of a role for tenascin-C in facilitating the cessation of lactation in mammals.¹³³

1.6.2.2 Developing Chicken

In-situ hybridization (ISH) experiments using cDNA probes complementary to FnIII AD2 or C revealed a strict spatial pattern of tenascin-C splicing in the developing chick. These probes did not hybridize to chondrogenic or osteogenic regions; while AD2 cDNA probes did hybridize at regions of epithelial-mesenchymal interactions including lung bronchioles and the base of feather buds.^{28, 48} In contrast probes for AD1 and A,B hybridize strongly in almost all regions where tenascin-C is detected in the E10 chick embryo, such as feather buds, lung bronchioles, tendons, ligamentum flavum, cartilage and bone; with exceptions including spinal cord glia and sternal perichondrium.^{28, 48} In developing bone, up to 85% of the tenascin-C transcripts detected via quantitative immunohistochemistry (IHC) included the AD1 repeat.⁹⁴ FnIII-C localises in a pattern which exclusively overlaps the expression pattern for FnIII-A,B, with the exception that FnIII-C cDNA probes never hybridize in the spinal

cord, or aorta endothelium, indicating a more tightly regulated pattern of expression in this location.⁶⁸

Functionally, tenascin-C was shown to promote adhesion and outgrowth of E3 spinal cord, E8 sensory, E8-11 sympathetic and E6 retinal ganglion cell neurons in chickens.^{134, 135, 136} In CNS neuron neurons this effect was mediated by FnIII-A, but in peripheral nervous system (PNS) neurons was mediated by FnIII-A and 3. Cell adhesion to FnIII-A and 3 was inhibited by anti- β 1-integrin antibodies, and arginyl-glycyl-aspartic (RGD) peptides respectively, illustrating that the adhesive effects of FnIII-A on neurons is β 1-integrin dependent.¹³⁷

In chicken embryos, the expression of isoforms containing FnIII-AD2/ AD1/ and C are dramatically up-regulated at sites of active tissue remodelling and fibronectin expression within the developing feather-bud and sternum. *In vitro* these variants have been shown to decrease cell attachment and organisation of actin microfilament bundles in myoblasts cultured on FN.¹³⁸ However, cells bound to a FnIII-AD2/AD1/C containing substrata developed stronger adhesions than those bound on tenascin-C containing no alternatively spliced FnIII, but did not form focal adhesions as is the case on FN. Instead they formed F-actin microfilament bundles at non-uniform adhesion points, giving the cells an irregular shape much like when adherent cells are cultured on thrombospondin-1.^{138, 139} The introduction of alternatively spliced FnIII modules may conversely disrupt adhesions between tenascin-C and other ECM molecules, as was demonstrated by FnIII A/B/C splices binding less strongly to fibronectin and the GPI-anchored immunoglobulin-superfamily neural cell adhesion molecule contactin/F11.¹⁰³ This is because in contactin/F11, the first 3-Ig domains preferentially bind to the uninterrupted FnIII-5,6 region;³³ as a result, contactin/F11-tenascin-C binding is attenuated by the inclusion of alternatively spliced FnIII in the tenascin-C molecule which interrupt this binding region.¹⁰³ Larger chick tenascin-C isoforms possess annexin II binding ability, and hence promote proliferation, cell migration and induce loss of

focal adhesions hence encouraging cells cultured on high Mr tenascin-C to become motile.^{40,}
¹⁴⁰ In addition, contactin/F11 binding to FnIII-5,6 was shown to be inhibited by heparin sulfate and dermatan sulfate.¹⁰⁴ All in all, the inclusion of adhesion modulating FnIII repeats such as AD2/AD1/C in the developing chick embryo create an environment conducive to tissue remodelling by permitting changes in motility, adhesion and cell shape.

1.6.2.3 *Developing Rat*

AD1 containing variants are found in the highly plastic SVZ and dentate gyrus of the developing rat brain, with an observable shift in expression from shorter to longer isoforms as development progresses from embryonic day 1 to postnatal day 5.⁷² An early mAb perturbation study revealed that FnIII-4,5/ A1,A2,A4/ D but not the EGF-L was required for axonal outgrowth from E16 cortical and thalamic explants¹¹⁵. *In vitro*, FnIII-C facilitates the orientation of rat cerebellar granular neurons, but has no effect on outgrowth.¹⁴¹ The variable rat FnIII B-D promotes contactin/F3 dependent neurite outgrowth when FnIII-C is excluded from the transcript; as its inclusion disrupts the contactin/F3 binding site formed between the adjacent FnIII-B and D.³⁴ FnIII-B,D containing tenascin-C is widely reported to promote neuron outgrowth in embryonic rat hippocampal neurons, mesencephalic neurons, cortical astrocytes and retinal neurons.^{34, 130, 142, 143, 144} This repeat has a conserved function in P6 mouse cerebellar neurons, where it does not alter the rate of cell proliferation, but does increase the proportion of neurite bearing cells in culture.¹³¹ Similarly to mouse neurites, FnIII-D also promotes rat neurite outgrowth in a manner dependent on Ca²⁺, PLC, protein kinase-C and contactin.¹⁴⁵ The outgrowth of E18 rat hippocampal neurons by FnIII-D was also inhibited by addition of anti- α 7/ β 1 integrin mAbs, caffeine, thapsigargin, inositol triphosphate receptor, ryanodine, 3,4,5-trimethylbenzoic acid 8-(diethylamino)octyl ester and proved dependent upon Ca²⁺ mobilisation from the endoplasmic reticulum.¹⁴⁵

Interestingly the abundance of FnIII-B,C in regions of high cell motility and turnover led some investigators to assess any potential functions of the repeat in developing organs outside of the CNS. However, despite a high abundance of tenascin-C in the E14 rat kidney mesenchyme, antibody perturbation of FnIII-B,C and A1,A2,A4 failed to exhibit any negative effect on the development of the kidney, suggesting that in an *in vivo* model at least, the presence of alternatively spliced FnIII in these tissues have no developmental function.¹⁴⁵ The model did not take into account the potential roles of variable FnIII repeats on *in vivo* specific processes in development, such as vascularization which would make good considerations for the future.

1.6.2.4 *Developing Human*

A wealth of tenascin-C splice variants was found within human fetal membranes which encapsulate the developing foetus and amniotic fluid. Bell et al.¹¹⁷ identified 8 different tenascin-C splice variants containing between 0 and 7 spliced FnIII repeats, which were speculated to promote spontaneous membrane rupture during labour by contributing to tissue remodelling processes in a manner analogous to a wound response. The predominant isoforms detected by PCR contained FnIII-D/ and A1-A4, either with or without FnIII-A3.¹¹⁷ The selective inclusion/exclusion of tenascin-C isoforms containing repeat FnIII A3, may provide functional significance with regard to mediating the susceptibility of tenascin-C to proteolytic degradation by matrix metalloproteinase (MMP) -2 and 3; both of which can cleave tenascin-C at a site located within the FnIII A3. MMP-7 also exhibits protease activity, and can digest tenascin-C between FnIII repeats A3-D;^{117, 146} perhaps facilitating fetal membrane rupture prior to birth. In this way, 'long' tenascin-C isoforms would be susceptible to MMP-2, 3 and 7 mediated fragmentations, whereas 'small' tenascin-C isoforms lacking A3 would be resistant. This process may generate a novel regulatory pathway where FnIII A3 containing isoform dependent processes can be regulated by MMP-2 and MMP-3¹¹⁷.

Table 1.3. Functional consequences of specific alternatively spliced FnIII domains during development.

Individual splice variants separated by (,) are included in the same transcript, while those separated by (/) are not.

Cell/Tissue Type	Alternatively spliced FnIII repeats inc.	Function	Method of Identification	Reference
Chick embryonic skin fibroblasts	A,B,C/ C/ none	FnIII-A,B,C/ C/ none containing fusion protein had no effect on promoting cell adhesion. FnIII-7,8 promoted adhesion as efficiently as full-length tenascin-C evidenced by perturbation with mAb Tn68	Generated fusion proteins, cell attachment assays, WB, electron microscopy, antibody perturbation experiments	Spring et al. ⁷⁶
Embryonic rat hippocampal and mesencephalon neurons	B,D	Promotes neurite outgrowth and cell adhesion to substratum. Effects inhibited by anti FnIII-B,D mAb J1/tn2	Antibody perturbation, cell substrate adhesion assay, ICC, rotary shadowing and electron microscopy	Lochter et al. ¹⁴²
Bovine aortic endothelial cells	A3/ D	Antibody perturbation revealed FnIII-A3/ D mediate loss of focal adhesions. Confirmed by addition of recombinant protein containing FnIII-A1,A2,A3,A4,B,C,D	Antibody perturbation experiment, focal adhesion assays	Murphy-Ullrich et al. ¹⁴⁷
Early postnatal mouse cerebellar cortex	A1,A2,A4/ B,D	FnIII-A1,A2,A4 promotes P6 granule cell neuron migration, but not outgrowth. B,C has no effect on migration but promotes outgrowth and increases the proportion of neurite bearing cells	Antibody perturbation experiment, cell migration assays, neurite outgrowth assays	Husmann et al. ¹³¹
Rat Lung (Fetal/ Postnatal)	A-D	Inhibits lung branching morphogenesis and aveolarization	Produced spliced domain anti-serums, IHC	Young et al. ⁹⁹
Adult mouse mammary gland	A1,A2,A4,B,C, D	FnIII 1-3/ A1-D/ all (1-8) inhibit β -casein expression and milk production during involution of mammary gland	Generated recombinant FnIII fragments, ICC, NB, WB	Jones et al. ¹³³
Chicken PNS and CNS neuron cultures	A	FnIII-A promotes PNS and CNS neuron adhesion, increasing proportion of cells with extending neurites. Adhesion effects inhibited by anti- β 1 integrin antibodies	Generated FnIII-A fusion protein, cell adhesion assays, antibody perturbation experiments	Phillips et al. ¹³⁷

Cell/Tissue Type	Alternatively spliced FnIII repeats inc.	Function	Method of Identification	Reference
Embryonic and Postnatal Mouse and Rat CNS Neurons	A1,A2,A4,B,D/ A1,A2,A3/ B,D/ D,6	FnIII-A1,A2,A4,B,D supported initial attachment in E18 rat and P6 mouse neurons. FnIII-A1,A2,A4 was repulsive to neurons, while B,D/ D,6/ and 6 promoted neurite outgrowth in E18 rat hippocampal and P0 mouse dorsal root ganglia explants	Cell binding assays, repulsion assays, neurite outgrowth assays, WB	Götz et al. ¹³⁰
Chicken Embryos	AD2/AD1/C	Decreased cell attachment and actin microfilament bundle organisation on cells adherent to FN. Increased adhesion on AD2/AD1/C containing substrata without focal adhesion	Cell adhesion assays, immunofluorescence (IF), PCR, SB, IHC, in-situ hybridization	Fischer et al. ¹³⁸
Rat cortical and thalamic explants	4,5/ A1,A2,A4/ D	Tenascin-C IHC and western blot staining identified isoforms ranging from 190-280kDa in E16-P7 cortical tissue. mAb perturbation with J1/Tn1, J1/Tn2/ J1/Tn4 inhibited axon outgrowth by binding FnIII-A1,A2,A4/ D and 4,5 respectively. J1/Tn3 to EGF-L had no effect	IHC, ISH, mAb perturbation, WB	Götz et al. ¹¹⁵
Rat embryonic cerebral cortical and hypothalamic neuronal cells	D/ A1,A4/ A4	Surface bound long and short isoforms promote E17 neurite process extension. Soluble long and short variants have no effect, or inhibit outgrowth respectively. FnIII-A1,A4/ D/ 6 are permissive and 6-8 are inhibitory. Different sites are masked/exposed when surface bound	Neurite outgrowth assays, antibody perturbation, generating recombinant FnIII proteins, ICC, WB	Meiners and Geller. ¹⁰⁸
Embryonic rat kidney	A1,A2,A4/ B,D	Tenascin-C expressed in kidneys from E14 past birth, strongest expression in cortical regions at newest growth. No alternatively spliced FnIII are implicated in kidney development <i>in vitro</i>	Antibody perturbation experiments (used 11 antibodies, 6 of which were novel)	Talts et al. ¹⁴⁸
Rat embryonic cerebral cortical	Full/ A1,A2,A3,A4/	Bound human FnIII-A-D promotes neurite outgrowth.	mAb perturbation, neurite outgrowth	Meiners et al. ¹⁴³

Cell/Tissue Type	Alternatively spliced FnIII repeats inc.	Function	Method of Identification	Reference
neurons, rat cerebral cortical astrocytes, baby hamster kidney cells (BHKs)	B,C,D/ D	mAb perturbation revealed FnIII-D as outgrowth permissive region on FnIII A-D bound to astrocytes, and A1-4/ D as permissive on A-D bound BHKs. Bacterially expressed FnIII A1-A4 and B,C,D promoted astrocyte outgrowth on BHK cells	assays, binding assay, WB	
Rat embryonic cerebellar granule neurons	Full/ C/ D	Discovered FnIII-C mRNA in early postnatal rat cerebellum. FnIII-D permits neurite extension, C regulates orientation and growth	PCR, neurite guidance assays, mAb perturbation, IF, WB	Meiners et al. ¹⁴¹
Rat Embryonic Hippocampal Neurons	B,D/ D	FnIII-B,D/ D promote neurite process extension and outgrowth. The B,D effect was contactin/F3 dependent	Expressed hybrid-fusion proteins, Neurite outgrowth assays, PCR	Rigato et al. ³⁴
Early postnatal rat and mouse cerebellar granule neuronal cultures	D	Unique amino acid sequence VFDNFVLK within FnIII-D promotes neurite outgrowth in $\alpha 7/ \beta 1$ integrin subunit dependent manner	Antibody perturbation, synthesized recombinant wild type and mutant FnIII-D, neurite outgrowth assay, ICC, affinity chromatography, WB	Mercado et al. ¹³²
Embryonic rat hippocampal neurons	D	FnIII-D mediated E18 hippocampal neurite outgrowth in Ca^{2+} , PLC, contactin and $\beta 1$ integrin dependent manner	Neurite outgrowth assay, antibody perturbation, inhibitor experiments, pull down assay, WB, video microscopy	Michele and Faissner. ¹⁴⁵
Embryonic rat retinal explant	B,D/ D,6/ A1,A2/ A1,D	FnIII-B,D fusion protein promoted strongest fibre outgrowth in E18 retinal explants, followed by A1,D. FnIII-A1,A2 is inhibitory. The FnIII-D responsible for outgrowth with effects modulated by neighbouring FnIII	Generated alternatively spliced FnIII fusion proteins to human Ig-Fc fragment, antibody perturbation, axon/ neurite outgrowth assay	Siddiqui et al. ¹⁴⁴

1.7 Tenascin-C splicing: cancer

Tenascin-C is abundantly expressed in the stroma of many solid tumours (reviewed in ^{27, 149, 29}). In addition to elevated tenascin-C in tumours, the splicing pattern often differs compared to healthy tissues. Studies examining ‘long’ and ‘short’ tenascin-C isoforms are summarized in Table 1.4 whilst Table 1.5 details specific alternatively spliced FnIII detected in tumours and Table 1.6 highlights the functional consequences of particular FnIII repeats on tumour cell biology.

1.7.1 *Tissue levels of large tenascin-C variants*

What little tenascin-C is present in healthy or benign tissues generally consists of the small variant containing no alternatively spliced FnIII repeats (*Mr* 180-190kDa). In contrast, a range of larger isoforms with *Mr* 210, 220, 230, 250, 260, 280, 320 and 330kDa, which would be predicted to contain one or more alternatively spliced FnIII repeats, have been described to be deposited in the ECM of a variety of human tumours including breast, colon, bladder, ovaries, prostate, pancreas, kidney, liver, uterus, brain, mouth, lung, skin, cartilage, connective tissues and peripheral nervous system (Table 1.4).

For example, total tenascin-C expression was observed to dramatically increase in invasive and intraductal breast carcinomas relative to normal breast tissues, with two major isoforms of 180kDa and 250kDa detectable via WB.¹⁵⁰ Subsequent analysis revealed that 85% of tenascin-C in normal breast tissue is a small 180kDa isoform, but in malignancy the larger 330kDa variant becomes predominant, with the small isoform always accounting for less than 40% of the total tenascin-C.¹⁵¹ Similarly, elevated expression of large tenascin-C isoforms relative to smaller ones in lung cancer, was reported by Oyama et al.¹⁵² who found elevated mRNA isoforms encoding a large tenascin-C variant with 7 alternatively spliced FnIII repeats in 6 of 10 adenocarcinomas, and 3 of 3 squamous cell carcinomas (SCCs) tested.

However there are exceptions to this rule; the expression of tenascin-C in cancer is highly polymorphic, depending on the type of cell involved, and the stage of disease. This is evidenced by examples of cancers where the smallest tenascin-C isoform is predominantly expressed, such as in some renal, colon and breast carcinomas.^{150, 153, 154}

1.7.2 *Association of large isoforms with disease progression*

A number of studies have suggested that large tenascin-C isoforms may correlate with prognosis, invasion and cancer progression in the bladder, brain, colon, lung, breast, B-cell non-Hodgkin's lymphoma, and adipocytic tumours. Furthermore, the presence of these large isoforms in some cases can also indicate disease recurrence and chemotherapy resistance (Table 1.4).

In the case of non-small cell lung carcinoma (NSCLC), an 18-fold increase in the expression of large tenascin-C variants was predictive of disease recurrence.¹⁵⁵ Similarly, large 8kb mRNA variants correlated with high stromal cellularity in breast cancer, and 330kDa protein variants with extra domain-B containing fibronectin (FN-EDB) positive colon cancer; linking large tenascin-C isoform expression with a tumour permissive stroma and the presence of invasive and angiogenic tumour marker protein FN-EDB, the expression of which is otherwise absent in healthy tissues.^{151, 156} Likewise, in normal and benign breast cancer, the 190kDa isoform was predominantly expressed, with a shift to expression of large 330kDa variant in invasive disease. Furthermore in 3 of 16 cases of invasive disease, a number of intermediate splice variants were identified which suggested that while stromal cells are capable of synthesizing tenascin-C of two major isoforms, 190 and 330kDa, epithelial cells can express tenascin-C isoforms of intermediate mass.¹⁵¹

Again though, whilst elevated tenascin-C expression in tumours is often associated with poor prognosis and patient outcome, there are many examples of tumours where there is no such

correlation. There is no correlation between tenascin-C expression and prognosis or disease stage in stomach adenocarcinoma;¹⁵⁷ metastasis, invasion or survival in gastric carcinoma;^{158,}¹⁵⁹ survival in oral and pharyngeal SCC;¹⁶⁰ survival, clinical stage or metastasis in pancreatic carcinoma.¹⁶¹ There are also cases where tenascin-C expression is absent in poor prognosis tumours, such as in cases of cervical carcinoma.¹⁶² Other studies found no associations between tenascin-C expression pattern and a number of clinical features such as nodal metastasis, tumour necrosis or blood vessel invasion in 32 invasive breast carcinomas.¹⁶³ Interestingly, one study found that in colorectal carcinoma (CRC) low expression of tenascin-C is associated with lymph node metastasis, perhaps indicating that in some cancers tenascin-C is capable of enforcing some anti-metastasis mechanisms¹⁶⁴ (Table 1.4).

Table 1.4. Association of ‘long/large’ and ‘short/small’ tenascin-C splice variants with cancer

Species	Alternatively Spliced FnIII Repeats (or size of splice variant if known)	Cell or Tissue Type	Features of Study	Reference
Human	Small (210kDa) and large (230kDa)	U-251MG Glioma	First identification of tenascin-C then called glioma-mesenchymal extracellular matrix antigen (GMEM). Identified major 230kDa isoform and minor 210kDa isoform. Did not specify whether difference in size of isoform is due to proteolysis or molecular heterogeneity	Bourdon et al. ¹⁶⁵
	Large (220, 230, 280kDa) Large (320kDa)	U-251 MG Glioma	Purified hexabrachions from conditioned media. Identified hexabrachions of different sizes, created by incorporation of different sized tenascin-C isoforms. In glioma, 220, 230 and 280kDa tenascin-C isoforms were in roughly equal abundance	Aukhil et al. ¹⁶⁶
	Small (180kDa) and large (250kDa)	Ductal and lobular breast carcinomas	Assessed distribution using mAbs in breast tissue from fetal, adult resting, lactating, ageing parenchyma, fibrocystic, fibroadenomas, cystosarcoma phylloides and ductal and locular carcinomas. Total expression increased during fetal growth, gestation, hyperplasia, dysplasia, benign tumours, and much increased in infiltrating and intraductal breast carcinomas	Howeedy et al. ¹⁵⁰
	Small (0 AS-FnIII), large (7 AS FnIII – 1.9kb insertion between FnIII 5- 6	Lung cancer tissues	6 of 10 and 3 of 3 adenocarcinomas and SCC respectively, exhibited elevated expression of large isoforms relative to small ones. Variant containing 7 FnIII comprised 27%, 24%, 54% and 42% of the total tenascin-C in adenocarcinoma, SCC, large cell carcinoma and small cell carcinoma respectively	Oyama et al. ¹⁵²
	Small (~6kb/190kDa) and large (~8kb/330kDa)	Normal, hyperplastic and neoplastic breast tissue	Invasive carcinoma expressed 6 and 8kb tenascin-C mRNA isoforms. Small variant accounts for 85% tenascin-C in healthy tissues, and <40% total in malignant ones. Intermediate sized variants detected in 3 of 16 invasive carcinomas. In fibroadenomas, 8kb isoform associated with high stromal cellularity, findings supported by intermediate protein bands also detected from these donors	Borsi et al. ¹⁵¹
	Small (190kDa) and large (280kDa and 330kDa)	Fibrosarcoma (HT-1080), Rhabdomyosarcoma (RD) and SV40 transformed fibroblast cell lines (WI-38-VA and AG-280),	In WI-38-VA and SK-MEL-28 cell lines, only 330kDa isoform visible in conditioned media. RD cells expressed low amounts of 330kDa isoform in conditioned media and faintly in cell extract. HT-1080 and AG-280 expressed no detectable tenascin-C. Normal fibroblasts expressed 190 and	Carnemolla et al. ¹⁶⁷

Species	Alternatively Spliced FnIII Repeats (or size of splice variant if known)	Cell or Tissue Type	Features of Study	Reference
		Melanoma (SK-MEL-28)	280kDa isoforms in cell extracts and conditioned media	
	Small (190kDa) and large (280kDa)	Transfected BHKs with long and short tenascin-C cDNAs using λ gt11 vector. Tenascin-C purified from SK-MEL-28 cells	Characterized binding locations of 11 mAbs to regions of tenascin-C, and found that mAbs BC-2, α -A2, α -A3, α -B and α -D bind to alternatively spliced FnIII repeats A1 and A4, A2, A3, B and D respectively	Balza et al. ¹⁶⁸
	Small and large	Prostatic hyperplasia and carcinoma	WB and PCR analysis identified small and large isoforms of tenascin-C protein	Ibrahim et al. ¹⁶⁹
	Small (190kDa) and large (250kDa)	Invasive CRC	Tenascin-C absent in normal tissues. Variants purified from invasive CRC were 190 and 250kDa in size, identical to sizes those found in human fetal fibroblasts	Sakai et al. ¹⁵⁴
	Large (330kDa)	CRC and colorectal adenomas	Large tenascin-C splice variant (330kDa) was detected in 7 of 15 carcinomas. Presence correlated with expression of fibronectin (FN) extra domain-B (ED-B)	Hauptmann et al. ¹⁵⁶
	Small (190kDa)	Renal cell carcinoma (RCC) and Oncocytoma	Small 190kDa isoform is predominantly expressed in RCC. Large isoform is almost absent	Lohi et al. ¹⁵³
	Small and large	Malignantly transformed fibroblasts	Normal human fibroblasts predominantly express smaller or larger tenascin-C isoforms when cultured in more acidic or basic culture medium respectively (pH 6.8-7.2) Malignantly transformed cells are resistant to external pH regulation of splicing and predominantly express large variant because a more basic cytosolic pH is maintained	Borsi et al. ¹⁷⁰
	Small (284bp PCR product), and large (490, 556, 750, 1651 and 1924bp) A4/ B/ C containing isoforms	Malignant and benign ovarian tumours	The smallest splice variant mRNA fragment (284bp) found in all tumours tested. FnIII-B expression was widespread in all except smallest variant. Larger variants also expressed A4 and C. 9/12 malignant compared to 1/6 benign tumours exhibited increase in intensity of larger ~490 and 556bp products relative to smallest 284bp	Wilson et al. ¹⁷¹
	Small and large	Endometrial adenocarcinoma	Total tenascin-C expression induced by 20ng/ml TGF β . Many individual splice isoforms identified, the most abundant contained none, 1 or 7 alternatively spliced FnIII repeats	Vollmer et al. ¹⁷²
	Small (190kDa/ 5.5kb) and large (220, 250kDa/ 7.5kb)	Lung cancer	All 30 lung cancers tested expressed 190kDa isoform (5.5kb mRNA), and also in 28 cases a larger 250kDa band (7.5kb mRNA); sometimes accompanied by a 220kDa band. Normal lung only expressed 5.5kb mRNA transcript, but 190 and 250kDa	Kusagawa et al. ¹⁷³

Species	Alternatively Spliced FnIII Repeats (or size of splice variant if known)	Cell or Tissue Type	Features of Study	Reference
			tenascin-C could be very weakly detected by WB	
	Small (5.8kb) and large (7.5kb)	Skin (dermal keratinocytes and fibroblasts) – non cancerous	In keratinocytes treatment with IFN γ and TNF α slightly increased mRNA expression of large and small isoforms respectively, while interleukin (IL)-4 increased both small and large isoforms equally	Latijnhouwers et al. ¹⁷⁴
	Small (220kDa) and large (320kDa)	Chondrosarcoma Clinical Specimens, and Cell Line JJ012	High small:large isoform ratio found in normal human articular chondrocytes. Low small:large isoform ratio found in chondrosarcomas and correlates with low survival. Determined via Semi-quantitative PCR (qPCR), IHC, survival analysis	Ghert et al. ¹⁷⁵
	Large isoforms (unspecified)	Neurofibromatosis, plexiform neurofibromas and malignant peripheral nerve sheath tumours (MPNSTs)	Relative expression levels of total tenascin-C in neurofibromatosis, plexiform neurofibromas and MPNSTs were 1: 2.98: 4.95, with larger spliced variants accounting for 27.6%, 54.1% and 60.3% respectively. Determined by PCR	Lévy et al. ¹⁷⁶
	Small (210kDa) and large (260kDa)	Amdc-s cells (NIH-3T3 transfected with human AdoMetDC), Ras-E4 (transfected with c-Ha-ras ^{Val12} oncogene) and Odc-n (NIH-3T3 cells transformed by overexpression of human ODC)	S-adenosylmethionine decarboxylase overexpressed in NIH-3T3 cells produced aggressive transformed cells. Amdc-s express 9.2-fold more tenascin-C than non-transformed cells, with the 260kDa isoform abundantly expressed in Amdc-s, and the only isoform expressed in Ras-E4 cells; while Odc-n cells additionally expressed 210kDa isoform	Paasinen-Sohns et al. ¹⁷⁷
Rat	Small (180kDa) and large (220kDa, very weak 280kDa)	Hepatic and sarcoma derived cell lines	Two hepatic and one sarcoma-derived cell lines shown to express major isoform 220kDa isoform. Cell lines explanted into nude mice, epithelial sarcoma induced tenascin-C expression in stromal mouse tissue but no-longer expressed tenascin-C themselves. After transplantation the stromal hepatic-derived cell lines still prominently expressed tenascin-C	Sakai et al. ¹⁷⁸

1.7.3 *Specific alternatively spliced FnIII repeats and their association with tumours*

Technological advances in the field now permit the identification of specific spliced tenascin-C variants in cancer, and many studies have used these tools to identify subsets of tenascin-C isoforms, or single alternatively spliced FnIII repeats in tumours. Whilst a systematic analysis of every splice variant in specific tumours is for the most part still lacking, these studies illustrate the distribution of specific FnIII repeats in malignancy, and shown that in some cases distinct FnIII repeats are associated with increased tumour grade, invasion, migration and metastases (Table 1.5).

For example, many IHC studies have utilised mAbs specific to regions FnIII-A1/A4, such as BC-2; showing that FnIII A1/A4 containing variants are not detected in normal cells, but are found at the tumour invasion front with apparent associations with migrating tumour cells. In oral SCC FnIII-A1, A4 containing variants are localised to the tumour stroma at the interface near invading cells, with greater expression correlating with higher disease stage.¹⁷⁹ Similarly, FnIII-A1,A4 and A3,A4,B are absent in normal prostate, yet strongly localised to neoplastic glands and the tumour invasion front in prostatic adenocarcinoma by IHC and ISH respectively.¹⁸⁰ In addition, FnIII-C containing tenascin-C isoforms were identified in brain cancer tissues via IHC using the mAb TN-11,¹⁸¹ and subsequently using a catalogue ISH probes and PCR techniques. This study found strong associations of FnIII-C with high grade astrocytoma and glioblastoma, cerebral cavernomas and lung cancers where the distribution of FnIII-C was consistently associated with tumour stroma, tumour blood vessels and proliferating cells in these tissues.^{181, 182, 183} Likewise, FnIII-B containing tenascin-C isoforms co-localise to laminin-5/ γ 2 in the basement membrane region of oral SCC, where increased expression positively correlates with malignancy grade.¹⁸⁴ Another study revealed by IHC and PCR that invasive urothelial cell carcinoma (UCC) exhibits restricted expression of FnIII-A1/ B or D which is closely associated with tumour blood vessels, invasive tumour

stroma and damaged muscle. Moreover, the same study identified an association between AD1 and a compact invasion pattern¹⁸⁵.

Similarly, the preferential expression of tenascin-C isoforms containing FnIII repeats B/ D/ AD1/ AD2/ B,D and B,AD1,D is well characterised in breast cancers including ductal carcinoma in-situ (DCIS) and is known to occur at the tumour invasion front, and in cancers with elevated risk of metastasis.^{94, 186, 187, 188, 189} These isoforms were originally thought to be tumour specific; although a more recent study identified FnIII-AD1 and AD2 expression in the myoepithelium of larger normal ducts in the breast, while also detecting their presence in 34.9% and 23.1% of invasive breast carcinomas respectively.¹⁸⁸ There is also a clear correlation between expression of AD1 and AD2 containing tenascin-C isoforms and invasive oestrogen receptor (ER) negative breast cancer in younger women (≤ 40 years of age), however no such correlation was observed between FnIII-AD1 and AD2 prevalence with human epidermal growth factor receptor 2 (HER2) expression. Since one of the primary chemotherapeutics for the treatment of breast cancer is the ER antagonist tamoxifen, the prevalence of FnIII AD1 and AD2 may be suitable as predictor for tamoxifen-resistant breast cancer, or breast cancer which is not yet tamoxifen resistant; yet may become so. In this way, surveillance of the tenascin-C isoform expression pattern could positively impact patient treatment.¹⁸⁸

Other IHC studies illustrated how the pattern of tenascin-C splicing changes dynamically during disease progression. For example, anti-FnIII-D mAbs stained strongly in early stage I/II CRC, but less so in later stage III/IV tumours and liver metastases. However, FnIII-A1,A2,A4 containing isoforms were much more abundant in metastases than early stage tumours demonstrating a shift in expression from FnIII-D to A1,A2,A4 during the development of CRC.¹⁹⁰

These observations may prove useful in cancer patient diagnosis or stratification. For example, Driemel *et al.*, demonstrated the diagnostic potential of combining haematoxylin and eosin (HE) staining with BC-2 IHC, in atypical oral brush biopsies, which would allow for the additional detection of FnIII-A1/ A4 containing isoforms. This combination protocol increased the sensitivity and specificity of conventional HE staining from 96 to 99% and 78 to 95% respectively¹⁹¹.

Table 1.5. Associations of specific tenascin-C splice variants, or individual alternatively spliced FnIII repeats in cancer. Individual splice variants separated by (,) are included in the same transcript, while those separated by (/) are not.

Species	Alternatively Spliced FnIII Repeats (or size of splice variant if known)	Cell or Tissue Type	Features of Study	Reference
Human	None/ D/ A4,B,D/ A1,A2,A3,A4/ A1,A2,A3,A4,B,D/ A1,A2,A3,A4,B, AD1/ A1,A2,A3,A4,B,C,D	U-251MG and U87-MG glioblastoma cell lines, MG-63 human osteosarcoma cell line, SK-N-SH neuroblastoma cell line, IMR-90 human lung fibroblast cell line, and HUVECs	Identified the novel FnIII-AD1 repeat located between FnIII-B and C, via PCR and sequencing. Identified other large splice variants also by PCR	Sriramara o and Bourdon et al. ⁶⁶
	A1,A2,A3,A4,B,C,D	U-251MG Glioblastoma cell line	Utilised radio-ligand binding assay to identify cell surface annexin II as a high affinity receptor for the whole alternatively spliced FnIII A-D region of tenascin-C (at the time this excluded FnIII AD2 and AD1)	Chung and Erickson. ⁴⁰
	AD1	Hs578T breast ductal carcinoma, SK-MEL-24 melanoma, WERI retinoblastoma and BCC basal cell carcinoma, A431 epidermoid carcinoma	Identified the presence of AD1 in human cell lines Hs578T, SK-MEL-24, WERI, BCC but not in A431 epidermoid carcinoma cells	Derr et al. ⁹⁴
	Small (190kDa) and large (200kDa, 230kDa) Japanese Quail fibrosarcoma cell line (QT6) contained AD1,D/ A,AD1,C/ A,AD1,D/ AD1,C,D/ A,AD1,C,D/ A,B,AD1,D/ A,B,AD2,AD1,D	QT6, SL-29 chick embryo fibroblast	SL-29 express 190, 200 and 230kDa isoforms encoding 0, 1 and 3 alternatively spliced FnIII in equal ratios. QT6 fibrosarcoma expressed predominantly 230kDa isoform containing 3 spliced FnIII but has minor bands containing 0, 1, 2 and 4 alternatively spliced FnIII. In QT6 cells splices containing more than 3 alternatively spliced FnIII are rare in protein form	Derr et al. ⁹⁴
	AD2	Malignant oral mucosae	Discovered human FnIII-AD2 repeat. Possesses 70% amino acid and 55% amino acid sequence similarity with chicken-AD2. Identified in 2/10 oral cancers, but was absent in 40/40	Mighell et al. ⁶⁷

Species	Alternatively Spliced FnIII Repeats (or size of splice variant if known)	Cell or Tissue Type	Features of Study	Reference
			normal, reactive, pre-malignant and other oral mucosae specimens	
	Large A1,A2,A3,A4,B,AD1,C, D	Melanoma SK-MEL-28	Large isoform is predominant isoform expressed by SK-MEL-28 although others are expressed in lesser abundance. Identified via sequencing, SB, PCR	Bell et al. ¹¹⁷
	C	Astrocytoma and glioblastoma	FnIII-C associated with high grade (III) astrocytoma and glioblastoma tumours, blood vessels and proliferating cells. Absent in healthy tissues and barely detected in meningioma, low grade astrocytoma, breast, lung and gastric carcinomas. Used TN-11 (mAb for FnIII-C)	Carnemolla et al. ¹⁸¹
	D/ A1,A2,A4 containing large isoforms.	CRC, ulcerative colitis and liver metastases	IHC with mAbs K8 (small isoform), 19H12, 201A, J1/tn1 (A1,A2,A4 region) and J1/tn2 (FnIII-D region). J1/tn2 stained strongly in stage I/II CRC but less so in stage III/IV tumours and liver metastases. The 19H12 staining was much stronger in metastases than early stage tumours	Dueck et al. ¹⁹⁰
	Small and large (A1,A4 containing variants)	Oral SCC	Large tenascin-C variant stained strongly in tumour stroma, and expressed in single positive layer of cells at the tumour-stroma interface near invading cells, illustrated by ISH and IHC with mAb BC-2. Higher expression of large variant associated with increasing tumour stage	Hindermann et al. ¹⁷⁹
	D, B/D	Breast Cancer cells MDA-MB 231, MDA-MB 468, MCF-7, T47D	Associated with metastasis and elevated invasion risk. Via PCR, SB, sequencing. These variants are synthesised by stromal fibroblasts in malignant tissue, and by periductal fibroblasts, and myoepithelial cells in DCIS	Adams et al. ¹⁸⁶
	C containing large isoforms	Cerebral cavernomas	Total tenascin-C localised to vascular walls and in interspace between blood cavities in cavernomas. FnIII-C localised to sub-endothelium of blood vessels in cavernomas and white matter surrounding the lesion sites. No FnIII-C was found in normal brain tissue. FnIII-C associated to tumour blood vessels in brain cancer	Viale et al. ¹⁸²
	B containing large isoforms	Breast cancer (intraductal cancers)	Recombinant full length FnIII region of tenascin-C promoted in vitro migration and mitotic activity, effects perturbed by adding mAb 4C8MS (against FnIII-B). Large tenascin-C variants localised	Tsunoda et al. ¹⁸⁷

Species	Alternatively Spliced FnIII Repeats (or size of splice variant if known)	Cell or Tissue Type	Features of Study	Reference
			to invasion front of intraductal and ductal cancers. Positive correlation between large tenascin-C isoform expression and proliferation rate	
	A1,A4/ A3, A4, B	Prostatic Adenocarcinoma	IHC revealed normal prostate tissue devoid of FnIII-A1,A4 containing tenascin-C, but staining is observed in tumour stroma with strong deposition around neoplastic glands. ISH revealed FnIII-A3,A4,B abundant in tumour cytosol and is associated with tumour invasion front and loss of cell adhesion in all adenocarcinomas tested	Katenkamp et al. ¹⁸⁰
	A1, B, D, AD1	UCC	A1, B, D Restricted to invasive tumours, tumour blood vessels and destructed muscle. AD1 associated with compact invasion pattern. Studied via Semi-quantitative IHC and PCR correlated to tumour stage	Berndt et al. ¹⁸⁵
	A1/ D	U87 Glioblastoma	Generated recombinant human mAbs to FnIII A1 and D (F16 and P12 respectively). F16 selectively localised at tumour site in U87 glioblastoma, and was rapidly cleared from other organs. F16 identified for potential antibody-based pharmaceutical development	Brack et al. ¹⁹²
	Small (322bp fragment) and large (2243bp fragment) A1,A2,A3,A4,B,C,D)	Cutaneous SCC and actinic keratosis (AK)	Large isoform found in 5%, 63% and 88% of normal, AK and SCC samples respectively. In SCC, tenascin-C associated with basal cells at invasion front and papillary/reticular dermis	Dang et al. ¹⁹³
	A1 and A4 containing large isoforms	Immortalised human corneal epithelial cells (HCEs)	Co-deposited laminin-332 and large tenascin-C variant in plaque beneath adhering cells and was Golgi dependent. HCE adhesion to laminin and large variant tenascin-C was dependent on $\alpha\beta 1$ integrin	Katz et al. ¹⁹⁴
	C	Lung cancer	Generated mAb G11 specific to FnIII-C and via IHC observed expression in majority of lung cancers, in vascular and stromal pattern. G11 demonstrated preferential localisation to tumour site in rats grafted with U87 gliomas, so may have application in delivery of imaging or therapeutics to glioma or lung tumours	Silacci et al. ¹⁸³
	None and A1,A2,A3,A4,B,AD2,A D1,C,D	Thyroid carcinoma. TT medullary carcinoma, ARO/ FRO anaplastic carcinoma, WRO follicular carcinoma, BHP 2/ BHP 5/ BHP 7/ BHP 10/ BHP 14/ BHP 17/ BHP 18,	PCR illustrated large transcript fragment containing full alternatively spliced cassette (variable splice region 1 - VS1) was more prevalent than the small variant in all cancers tested, except for medullary carcinoma cell line TT	Tseloni-Balafouta et al. ¹⁹⁵

Species	Alternatively Spliced FnIII Repeats (or size of splice variant if known)	Cell or Tissue Type	Features of Study	Reference
		BHP 19 and SW579 papillary carcinoma		
	A1 and A4 containing large isoforms	Atypical oral brush epithelium	Specificity and sensitivity of conventional HE staining for atypical oral brush biopsies increased from 96-99% and 78-95% respectively when combined with BC-2 immunostaining for large FnIII-A1/A4 containing tenascin-C isoforms	Driemel et al. ¹⁹¹
	B containing large variants	Oral SCC	IF with BC-3 mAb (for FnIII-B). No large tenascin-C variant identified in normal oral basement membrane. In dysplastic and neoplastic oral mucosa long tenascin-C variant containing FnIII-B was co-localised to laminin-5/gamma-2 in the basement membrane region. Extent of reorganisation of large variant in basement membrane correlated with malignancy grade	Franz et al. ¹⁸⁴
	C	CRC	Serum levels of large-tenascin-C variants containing FnIII-C quantified by enzyme-linked immunosorbent assay (ELISA). Primary CRC patients 5260±3243.3pg/ml, recurrent CRC patients 4106±2261.1pg/ml and healthy donors 2364±749.6pg/ml. Sensitivity for detecting CRC via serum levels was 56.6%, exceeding conventional tumour markers CEA at 40.1% and CA19-9 at 23.6%	Takeda et al. ¹⁹⁶
	D (~250kDa)	NSCLC	18-fold increase in large isoform expression observed in recurrent NSCLC compared to non-recurrent NSCLC, determined via quantitative PCR and WB	Parekh et al. ¹⁵⁵
	C	Hepatic recurrence of CRC	High serum levels of large tenascin-C splice variants in two patients; as detected via ELISA sensitive to FnIII-C domain, were associated with hepatic recurrence of CRC	Takeda et al. ¹⁹⁷
	B, C	UCC	Measured urine concentration of FnIII-B, C in 104 UBC patients, 11 patients with cystitis and 15 healthy donors. Increased urinary FnIII-B concentration correlates with tumour progression in UBC. Proteolytic fragmentation of tenascin-C was also observed in urine from invasive tumour patients	Richter et al. ¹⁹⁸
	AD1, AD2	Breast cancer DCIS, ductal carcinomas, lobular carcinomas, fibroadenoma	AD1 and AD2 present in 34.9% and 23.1% of invasive breast carcinomas respectively. AD1 and AD2 not tumour specific but expression is increased in carcinomas from younger women. AD1 localised to tumour cells and	Guttery et al. ¹⁸⁸

Species	Alternatively Spliced FnIII Repeats (or size of splice variant if known)	Cell or Tissue Type	Features of Study	Reference
			myoepithelium of normal breast ducts. AD1 associated with ER negative and grade III tumours	
	A1	Melanoma	24 primary and 29 metastatic melanoma lesions stained with F8, L19 and F16 mAbs for extra domain-A containing FN (EDA-FN), EDB-FN and FnIII-A1 respectively. F16 strongly stained basal lamina and deeper layers of tissue compared to others. F16-IL2 could therefore be useful as therapeutic for malignant melanoma	Frey et al. ¹⁹⁹
	B	UBC	Determined urine levels of FnIII-B/ C containing tenascin-C in 35 patients via ELISA. FnIII-B could predict cases without tumour recurrence, or with tumour existence. Could also predict whether UBC was muscle/or non-muscle invasive	Gecks et al. ²⁰⁰
	A1/ C	Clear cell RCC (ccRCC), papillary (pRCC), chromophobe-primary RCC (chRCC)	Detected FnIII A1/C in RCC samples. FnIII A1 in addition to FN ED-A/B was associated with vascular structures. By contrast FnIII-C was absent in ccRCC, strongly expressed in 80% of pRCC and was widely expressed in chRCC	Galler et al. ²⁰¹
Mouse, human	Large isoforms	NIH-3T3, human melanoma cells	Overexpression of SRSF6 transcription factor in mice induces hyperplasia of sensitized skin, and in melanoma is associated with increases in expression of full length tenascin-C isoforms found in invasive carcinomas.; Knockdown of SRSF6 in NIH-3T3 down-regulated large tenascin-C variant. SRSF6 associates with exons 10-14 (encoding FnIII-5,A1,A2,A3,A4)	Jensen et al. ²⁰²

1.7.4 *Consequences of spliced FnIII domain expression in cancer*

Tenascin-C possesses the ability to modulate cell behaviour in normal, tumour and tumour-associated cells in a manner which is cell-type specific, and which may either directly or indirectly affects cancer initiation and progression. Functional studies have identified roles for large tenascin-C variants in promoting a tumour supportive tissue microenvironment, with early studies identifying roles in the down-regulation of focal adhesion activity,¹⁴⁷ promotion of cell migration,⁹⁵ up-regulation of ECM degrading enzymes²⁰³ and prevention of binding to FN;^{32, 84, 105} processes which would favour the development of malignancy by reducing adhesion and facilitating cell migration (Table 1.6).

Large tenascin-C isoforms may promote the destruction of the local tissue environment during tumorigenesis and metastasis by inducing the expression of ECM degrading enzymes such as MMP-1 in chondrosarcoma²⁰³ and MMP-13 in breast cancer. Although here MMP-13 expression is accompanied by increased levels of TIMP-3, and was not tenascin-C isoform specific.²⁰⁴ The degradation of the ECM and the driving of an epithelial-mesenchymal transition is crucial in the process of tissue invasion as observed in metastasis. In chondrosarcoma, addition of the large 320kDa tenascin-C isoform conferred a 3-fold increase in collagenase and invasion activity via modulation of MMP-1 expression.²⁰³

Functional studies in human cells have also served to elucidate the role of specific tenascin-C isoforms in malignancy, and these have been best characterised in breast cancer (reviewed in¹⁸⁹). AD1 containing isoforms promoted cell growth and invasion above control levels in breast cancer cells.¹⁸⁸ These data are supported by previous findings of FnIII-AD1 expression in breast ductal carcinoma, melanoma, retinoblastoma, basal cell carcinoma cell lines and in highly invasive Japanese quail fibrosarcoma cells (QT6 cell line).⁹⁴ QT6 predominantly express a major 230kDa isoform containing 3 alternatively spliced FnIII, although minor

isoforms containing 0, 1, 2 and 4 spliced FnIII are detected in low abundance. FnIII-AD1 is abundantly expressed in QT6 cells, and correlates with these cells exhibiting invasive capabilities as demonstrated in a Boyden chamber assay where they passed through a fibronectin coated filter.⁹⁴ Other studies have also identified AD1 containing tenascin-C isoforms at sites of high cell motility.²⁰⁵ Together, these findings may suggest that AD1 containing tenascin-C isoforms modulate cell-FN interactions and contribute to a tissue microenvironment that facilitates changes in cell morphology, proliferation and migration in cancer.^{94, 188, 205}

Large tenascin-C isoforms have been directly implicated in the induction of proliferation, migration and control of cell spreading *in vitro*. MMP-2 mediated proteolytic cleavage of large tenascin-C variants reveals a cryptic syndecan-4 binding site within the FnIII-A2 domain. Here, a 22mer fusion peptide containing an adhesive YTITIRG sequence bound to the heparin sulfated side chains of the β 1 integrin co-receptor syndecan-4, hence inducing cell adhesion and spreading on FN³⁹. This study found that mutation of the cytosolic region of syndecan-4 had no effect on promoting the inhibition of β 1 integrin action, suggesting the extracellular portion of syndecan-4 binds FnIII-A2 and hence promotes β 1 integrin clustering and activation, rather than mediating this effect via downstream targets of syndecan-4. In mouse colon cancer cell lines, invasion and metastasis was promoted by full length tenascin-C and FnIII A2 via MMP-2 induction²⁰⁶. Elevated levels of both activated MMP-2 and degraded tenascin-C were identified in NSCLC, with proteolytically degraded tenascin-C serving as a good indicator of stage-1 NSCLC recurrence.²⁰⁷ It is therefore possible to speculate a role for MMP-2 in degrading tenascin-C in lung cancer, and that FnIII-A2 cryptic site interaction with syndecan-4 in lung tissues play a role in mediating early stage recurrence of the disease.

A follow up study identified that sustained activation of $\alpha 5\beta 1$ by the FnIII-A2 cryptic site caused non-transformed mouse embryonic fibroblasts (NIH-3T3) to become resistant to serum deprivation-induced anoiksis by activating the pro-survival Akt/Bcl-2 pathway, and enhanced PDGF-dependent proliferation of NIH-3T3 cells via promoting the association between PDGF receptor β (PDGF-R β) and the activated molecular complex that comprises of $\alpha 5\beta 1$ and syndecan-4.²⁰⁸ This extended the activation of the PDGF-R β and its downstream Ras/MAPK pathways. Furthermore, the study suggested that tenascin-C may de-regulate cell growth processes by triggering the continual activation of $\alpha 5\beta 1$ by the FnIII-A2 cryptic site; this mechanism was implied by the observation that confluent monolayers of fibroblasts continued to proliferate in an $\alpha 5\beta 1$ dependent manner, and had apparently overridden their innate cell-density mediated cell cycle arrest mechanisms.²⁰⁸ This demonstrates a highly significant role for MMP-2 cleaved tenascin-C in the promotion of cancer.

Chung and Erickson,⁴⁰ first showed that the large tenascin-C isoform containing the full alternatively spliced FnIII cassette is a high affinity binding partner of the cell surface located calcium-dependent phospholipid-binding protein annexin II, binding of which reduces focal adhesions, enhances mitogenesis and promotes cell migration.^{140, 147} Similarly, addition of full length tenascin-C to pancreatic cancer cells ameliorated the cytotoxic response to chemotherapeutic agent gemcitabine, in a manner which was dependent upon binding between FnIII A1-D and cell surface annexin II.²⁰⁹ Addition of recombinant FnIII A1-D increased the intracellular phosphorylation status of protein kinase B signal cascade constituents including PI3k, Akt, IKK α and NF-kB.²⁰⁹ Although in this study the effects of FnIII A1-D were not assessed in the context of promoting cell survival, proliferation or growth, such phosphoproteins are central mediators of these processes; for example, phospho-Akt is a major promoter of cell survival and effects this via phosphorylating and

hence inactivating a number of pro-apoptotic target proteins including Bad, Bax, Bim and Forkhead transcription factors (FOXOs).²¹⁰

The anti-FnIII-B mAb 4C8MS attenuated *in vitro* migration and mitotic activity in intraductal breast cancers.¹⁸⁷ Functional overexpression of FnIII-D/ B,D and B,AD1,D in human breast cancer cell lines or FnIII B and B,D in fibroblasts significantly increased proliferative and invasive capabilities of breast cancer cells compared to vector controls *in vitro*; in addition to inducing a 2- and 4-fold increase in expression of MMP-13 and TIMP-3 respectively.^{188, 204} Interestingly, during embryonic development, FnIII B and D promote neurite outgrowth in a manner dependent upon interactions with the F3/contactin and $\alpha 7\beta 1$ integrin,^{34, 132} permitting speculation that conserved mechanisms promoting cell migration in embryogenesis are being re-activated in tumorigenesis.

A further antibody perturbation study determined that specific splice variants of tenascin-C containing FnIII-D reduced immune surveillance in solid tumours, evidenced by the large tenascin-C variant inhibiting CD3-FN mediated lymphocyte proliferation and interferon- γ (IFN γ) production in tumour invading lymphocytes.¹⁵⁵ The presence of FnIII-D would therefore be expected to be accompanied by increased tumour recurrence rates on account of an impaired immune clearance mechanism. Interestingly, Parekh et al.¹⁵⁵ found that large 250kDa isoform expression is increased 18-fold in recurrent NSCLC compared to non-recurrent NSCLC; which if presuming FnIII-D expression was increased also, would be consistent with this hypothesis (Table 1.6).

Table 1.6. Functional consequences of tenascin-C alternative splicing in cancer. Individual splice variants separated by (,) are included in the same transcript, while those separated by (/) are not.

Species	Alternatively Spliced FnIII Repeats (or size of splice variant if known)	Cell or Tissue Type	Features of Study	Reference
Human	none and A1,A2,A3,A4,B,C,D	U-251MG cell line, and BHKs transfected with human tenascin-C	Small isoform tenascin-C binds purified and mixed FN substrate via FnIII-3 and a region within FnIII-6,7,8. Solid phase binding assay demonstrated stronger binding of small rather than full length tenascin-C to FN. FnIII-A-D fusion protein showed minimal displacement of bound FN-Tenascin-C complexes compared to FnIII-3/3-5/1-5	Chung et al. ³²
Human	Small and large (220 and 320kDa isoforms respectively)	Chondrosarcoma Cell Line JJ012	Small isoform of tenascin-C binds FN and promotes adhesion when bound to plastic. Large isoform does not bind FN and fails to promote cell attachment. Determined via ELISA, cell attachment assays, antibody blocking experiments	Ghert et al. ¹⁰⁵
Human	D (~250kDa)	Non-Small Cell Lung Carcinoma	Inhibits CD3-dependent lymphocyte proliferation and INF γ secretion in tumour-invading lymphocytes. Observed via Lymphocyte proliferation assay, ELISPOT assay and antibody perturbation. 18-fold increase in large isoform expression	Parekh et al. ¹⁵⁵
Human	A2	SV40-transformed human embryonic lung fibroblasts (WI-38-VA-13 cell line)	FnIII-A2 domain contains syndecan-4 binding cryptic site exposed by MMP-2 digestion. Stimulates β 1 integrin mediated cell adhesion to FN. Suggests the extracellular portion of syndecan-4 binds FnIII-A2 and subsequently promotes β 1 integrin clustering and activation	Saito et al. ³⁹
Human	D/ B,D	Breast cancer (MCF-7, T47D, MDA-MD-231, MDA-MB-468, GI101) and fibroblasts	Overexpression of full length tenascin-C increased mean invasion index (MII) in MDA-MD-231 and T47D cells, but overexpression of FnIII- D and B,D by tumour cells enhanced proliferation and invasion significantly. Fibroblasts overexpressing full length, or FnIII-B/ B,D also promoted invasion of tumour cells. Tenascin-C upregulated expression of MMP-13 and TIMP-3	Hancox et al. ²⁰⁴
Human	A1,A2,A3,A4,B,AD2,A D1,C,D	Pancreatic cancer	Recombinant FnIII-A-D bound cell surface annexin II, and suppressed gemcitabine mediated cytotoxicity in pancreatic cancer cells in dose dependent manner. This interaction increased intracellular phosphorylation status of PI3K, Akt, IKK α/β and NF-kB. NF-kB inhibition by siRNA restored gemcitabine cytotoxicity	Gong et al. ²⁰⁹
Human	B,AD1,D	Breast cancer cell lines (MCF-7, T-47	Overexpression of FnIII B,AD1,D enhanced tumour cell invasion and	Guttery et al. ¹⁸⁸

Species	Alternatively Spliced FnIII Repeats (or size of splice variant if known)	Cell or Tissue Type	Features of Study	Reference
		D, ZR-75-1, MDA-MB-231 and GI-101)	growth relative to baseline levels	
Human	Small (220kDa) and large (320kDa)	Chondrosarcoma	Exogenous addition of 320kDa isoform stimulates twice the levels of MMP-1 expression observed when adding 220kDa isoform. 30-fold activation of MMP-1 promoter by 320kDa isoform, compared to 220kDa isoform. Furthermore, collagenase and invasion activity of chondrosarcoma increased 3-fold in 320kDa variant treated cells	Galoian et al. ²⁰³
Mouse	A2	Colon cancer cell lines, Colon26-M3.1, PMF-Ko14	Cancer cell invasion and pulmonary metastasis increased by adding FnIII A2, and was suppressed by adding MMP inhibitor, and by FnIII-A2 antibody perturbation.	Suzuki et al. ²⁰⁶

1.8 Therapeutic exploitation of tenascin-C splice variants

The strong association between the expression of alternatively spliced FnIII repeats of tenascin-C with tumours, and in many cases very aggressive tumours, combined with their reduced expression in normal healthy tissues has raised interest in pharmacological targeting of these modules (reviewed in ²⁹).

Most studies to date have focused on using antibodies to alternatively spliced FnIII domains to deliver cytotoxic or anti-inflammatory factors direct to the tumour, but given the active role these domains play in driving tumour cell biology, there also exists the potential for developing antagonists of these domains for use in cancer treatment. Antibodies specific for FnIII-A1 (F16) and FnIII-C/D (81C6) have been best characterized, although others are in development. ¹³¹I-labelled 81C6 has been trialled as a therapeutic in malignant glioma where it targets residual cancer cells following brain surgery²¹¹. ¹³¹I-81C6 was shown to increase median survival of patients with Grade III anaplastic astrocytoma from 77.2 to 162.2 months, and increased median survival of WHO Grade IV glioblastoma multiforme patients from 18.9 to 25.3 months²¹². Despite findings showing ¹³¹I-81C6 increased median survival of

glioblastoma multiforme patients, correlations were observed between irreversible neurotoxicity and decreased survival. Nevertheless, ¹³¹I-labelled 81C6 delivers highly targeted radio-isotope therapy and is now undergoing phase III trials with the trade name Neuradiab^{213, 214}.

Three other anti-tenascin-C antibody conjugates are currently under development, all of which use the FnIII A1 targeting antibody F16.¹⁹² F16 is conjugated with interleukin (IL) -2, ¹³¹I, and ¹²⁴I for the therapy of lung and breast cancer, Hodgkin's lymphoma and various tumours respectively²¹⁴. F16-IL-2 is reportedly a highly efficacious and safe immunotherapeutic when administered in combination with the chemotherapeutic temozolomide to BALB/c nude mice with sub-cutaneous and intracranial human glioblastoma tumours, F16-IL-2 induced a complete remission in these animals, and promoted leukocyte infiltration of the tumours. Following complete remission, the mice remained tumour free for 160 days.²¹⁵ The toxicity of F16-IL-2 was assessed in combination with doxorubicin, for future studies of its efficacy on treatment of patients with solid tumours and metastatic breast cancer²¹⁶. Other more recent studies using F16, found that when conjugated to anthracycline PNU159682, the linker peptide was stable in serum, but was cleaved by the sub-endothelial ECM enzymes, released by dying cells²¹⁷. Most recently, the F16-monomethyl auristatin E, cured BALB/c nude mice transplanted with A431, U-87 and MDA-MB-231 tumours²¹⁸.

1.9 Summary of tenascin-C splicing in development and cancer

The functional role of tenascin-C can be modified by alternative splicing in a number of ways. Firstly, by including an alternatively spliced FnIII domain that introduces a new binding site. Secondly, by omitting an alternatively spliced FnIII domain to remove a binding site; and thirdly, by omitting alternatively spliced FnIII domains, new binding sites can be formed when non-contiguous FnIII domains are expressed together. To illustrate this, a figure containing examples of how splicing can impact tenascin-C function via these 3 modes of a function during development and cancer is shown below (Figure 1.5).

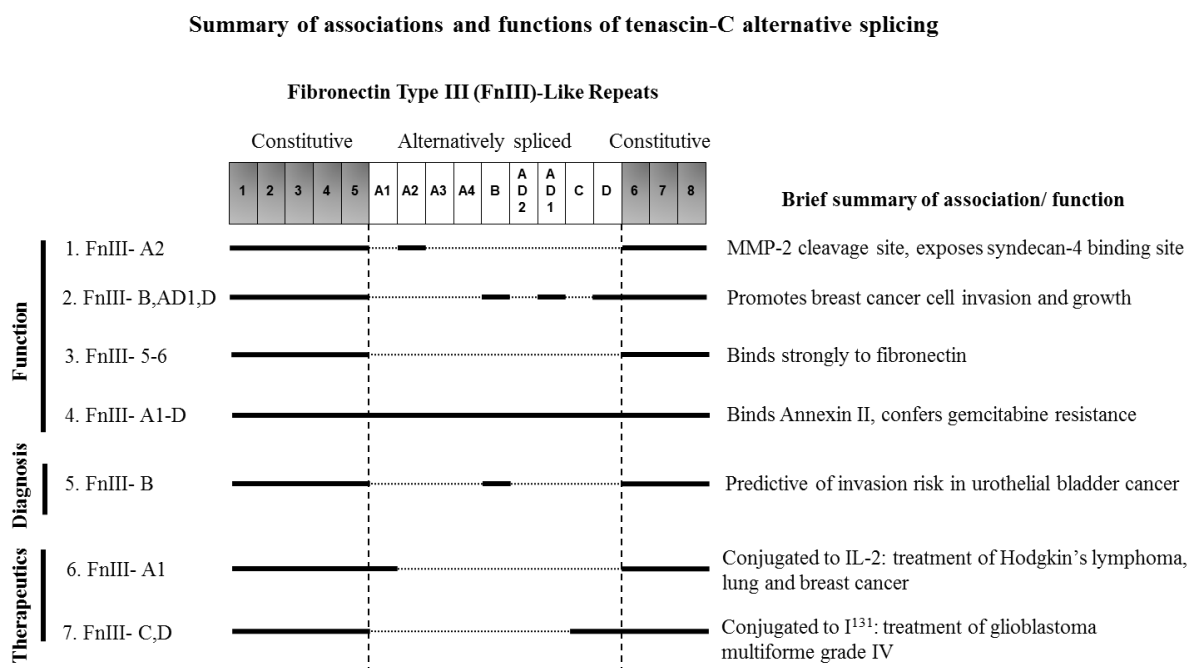


Figure 1.5. A recap of the associations and functions of alternatively spliced FnIII domains. 1. FnIII-A2 contained a site that was cleaved by MMP-2, and promoted β 1 integrin dependent cell adhesion³⁹, 2. FnIII-B,AD1,D enhanced breast cancer invasion and growth¹⁸⁹. 3. Small but not large tenascin-C isoforms bound strongly to fibronectin³². 4. FnIII-A1-D containing tenascin-C isoforms bound annexin II, and conferred gemcitabine resistance in pancreatic cancer cells²⁰⁹. 5. FnIII-B served as a predictive biomarker for invasive urothelial bladder cancer¹⁹⁸. 6. An FnIII-A1-IL-2 conjugate is currently undergoing clinical trials for the treatment of Hodgkin's lymphoma, lung and breast cancer²¹⁴. 7. An FnIII-C,D – I¹³¹ conjugate antibody is undergoing clinical trials for the treatment of late stage glioblastoma²¹¹.

1.10 Alternative splicing beyond development and cancer

Alternative splicing of tenascin-C is not restricted to developing tissues and tumours, but is more likely a universal feature of tenascin-C expression during tissue remodelling. Indeed 22 different tenascin-C splice isoforms were identified in pseudophakic and aphakic bullous keratopathy corneas, with the predominant isoforms containing one, two, both or neither of the FnIII A1/D modules^{219, 220}. These data suggest that a detailed and systematic examination of tenascin-C splicing will reveal important information about the role of this molecule in different locations.

1.11 The role of tenascin-C in inflammation

In this section I will provide a brief overview of the cell types which comprise the innate and adaptive immune systems, and discuss the currently understood roles for tenascin-C in modulating inflammatory processes.

1.11.1 *Cells of the innate immune system*

The innate immune system provides immediate defence against infection by recognising pathogens; but unlike the adaptive immune system, it cannot provide long term immunity to specific antigens. A brief overview of the cells present in the innate immune system is included below.

1.11.1.1 *Monocytes, macrophages and dendritic cells*

Monocytes are a myeloid component of the vertebrate immune system that comprise of 3-8% of the total circulating white blood cells. Monocytes differentiate from monoblast precursors in the bone marrow, and migrate to the blood where they circulate for 1-3 days, before extravasating into splenic and other tissues throughout the body²²¹. Monocytes terminally

differentiate in situ into tissue resident macrophages and dendritic cells; which are present in high abundance in the inner linings of the nose, lungs, stomach and intestines. Once activated, the macrophages and dendritic cells migrate to the lymph nodes where they activate T- and B- cells of the adaptive immune system.

Altogether, monocytes, macrophages and dendritic cells serve three main purposes in the immune system; Firstly, to perform phagocytosis by engulfing damaged host cells, invading organisms, or cell debris remnants, before killing and digesting the particles with lysosome enzymes. Secondly, to present digested antigens on major histocompatibility complex (MHC) peptides to cells of the adaptive immune system, to trigger polarisation of T- and B- lymphocytes, this can then mount a targeted immune response against the antigen, and generate long-term humoral immunity. Thirdly, they express pro-inflammatory cytokines and chemokines to recruit other immune cells to the site of infection or tissue damage; and after a delay, they can express anti-inflammatory cytokines so the inflammatory stimulus can be cleared, and inflammation can be resolved.

1.11.1.2 Neutrophils

Neutrophils comprise of 40-70% of the white blood cells in most mammals. Neutrophils are short-lived phagocytic cells that mature from stem cells in the bone marrow, and are considered an early responder in response to inflammation; as they rapidly migrate along chemotactic gradients of inflammatory cytokines to the site of inflammation. The anti-microbial effects of neutrophils are mediated by phagocytosis, degranulation of cytoplasmic granules storing immunomodulatory molecules²²², and the formation of neutrophil extracellular traps²²³.

1.11.1.3 Basophils, eosinophils and mast cells

Basophils, eosinophils and mast cells are myeloid granulocytes, and are key regulators of allergic immune responses that develop from stem cells in the bone marrow, before migrating to the circulating blood, after which they terminally differentiate and no longer multiply.

Basophils are the least common of the granulocytes, and account for 0.5-1% of circulating white blood cells; and are named so due to their susceptibility to staining with basic dyes²²⁴.

Basophils exacerbate acute and chronic allergy symptoms by producing the vasodilator histamine, and the anticoagulant heparin, which promotes blood flow to inflamed tissues, and exacerbates acute and chronic allergic diseases such as asthma, anaphylaxis, dermatitis and hay fever²²⁵.

Eosinophils comprise of 1-3% of total circulating white blood cells and contain large acidophilic cytoplasmic granules, which can be stained with acidic coal tar dyes. Eosinophils reside in tissues such as the thymus, lower gastrointestinal tract, spleen and lymph nodes, but not the skin, lung or oesophageal tract²²⁶; where their aberrant presence is associated with disease, such as eosinophilic asthma exacerbations²²⁷. Their cytoplasmic granules contain inflammatory mediators and enzymes such as eosinophil peroxidase, ribonuclease (RNase), deoxyribonuclease (DNase), lipase and plasminogen, which are toxic to host tissues, multicellular parasitic tissues and viral particles.

Mast cells are tissue resident cells, and are found in tissues surrounding nerves and blood vessels, and in boundary regions separating the internal milieu from the external world, such as the skin, lung mucosa, digestive tract as well as oral and nasal mucosa²²⁸. Mast cells like other granulocytes contain granules rich in histamine and heparin, and mediate the early immune response by sensing tissue injury or infection, via signalling from PRRs that detect DAMPs and PAMPs. Mast cells degranulate in response to PRR signalling, or via allergen

crosslinking with IgE receptors, and while this is widely known to exacerbate allergic reactions and anaphylaxis, mast cells are also involved in wound healing and maintaining immune tolerance²²⁹.

1.11.1.4 Natural killer cells

Natural killer cells are cytotoxic lymphocytes that are a component of the innate immune system, that differentiate from the same lymphoid progenitor cells that form B- and T-lymphocytes. NK cells are analogous to cytotoxic T-lymphocytes in the adaptive immune system, and provide rapid responses to virally infected cells, and tumour cells due to their ability to detect stressed cells in the absence of antibodies and MHC I/II molecules, which is particularly important as damaged or harmful cells lacking expression of MHC I cannot be detected or targeted by T-cells²³⁰.

1.11.2 Cells of the adaptive immune system

The adaptive immune system is comprised of highly specialized, systemic cells that eliminate pathogens in an antigen targeted manner. The adaptive immune system generates immunological memory after first being exposed to a pathogen, so that subsequent exposures to the same pathogen elicit enhanced and more efficient immune responses. Like the innate immune system, the adaptive immune system contains cell mediated immune processes, but also utilises humoral antibody mediated immunity.

1.11.2.1 T-cells

T-cells are lymphocytes which mature in the thymus from thymocytes, which play a role in cell mediated adaptive immunity²³¹. T-cells are distinguished from other lymphocytes by the presence of the T-cell receptor (CD3). During inflammation, T-cells become activated by co-stimulation of CD3 and a costimulatory molecule such as CD28, alongside interactions of co-receptors CD8 or CD4, by MHC I/II peptides and other co-stimulatory molecules present on

antigen presenting cells; typically macrophages and dendritic cells. There are many subsets of T-cells with highly specialised functions such as regulatory T-cells, memory T-cells, $\gamma\delta$ T-cells and natural killer T-cells, though below only brief descriptions of T-helper cells and cytotoxic T-cells are included.

1.11.2.2 T-helper cells (CD4+)

T-helper cells (Th cells) express the CD4 glycoproteins on their cell membrane, and are activated by antigens presented on MHCII molecules. They contribute to inflammatory processes by assisting the activation of B-cells into plasma and memory B-cells, by activating cytotoxic T-cells, and by stimulating macrophages²³². Once T-helper cells are activated they express inflammatory cytokines such as IL-2 which induce autocrine signals for the cells to proliferate rapidly; they then differentiate into Th1, Th2 and Th17 subsets depending on the cytokines present in the microenvironment. Th1 production is promoted by IFN γ , but is inhibited by IL-10 and IL-4, Th2 differentiation is promoted by IL-4 but is inhibited by IFN γ , while Th17 differentiation is promoted by IL-23 and IL-6, and is characterised by cells expressing the pro-inflammatory cytokine IL-17.

1.11.2.3 Cytotoxic T-cells (CD8+)

Cytotoxic T-cells are T-lymphocytes that express the CD8 glycoprotein on the cell membrane and can recognise foreign or self-antigens presented on the MHCI complex by non-professional antigen presenting cells. After activation by co-stimulation of CD3 and CD28 by MHCI, the cytotoxic T-cells clonally expand by releasing IL-2. In this way, cytotoxic T-cells can recognise and subsequently destroy tumour cells, virus infected cells or damaged cells, by delivering cytotoxic perforins, granzymes and granulysin to the targeted cell; which initiates the caspase cascade of serine proteases, leading to programmed cell death, apoptosis. Cytotoxic T-cells can be inactivated into an anergic state by exposure to IL-10, secreted by regulatory T-cells.

1.11.2.4 B-cells

B-cells are a lymphocyte subgroup that differentiates from haematopoietic stem cell progenitors in the bone marrow. B-cells express the B-cell receptor on the cell membrane, which when engaged by antigen, initiates the humoral immune response. These cells mediate humoral immunity by secreting antibodies, presenting antigens to other cells of the immune system, and by secreting cytokines²³³. During development B-cells undergo strict selection; firstly, positive selection is mediated by antigen independent signalling, which requires constant signalling to continue B-cell development. Secondly B-cells undergo negative selection through the binding of self-antigen with the BCR. There are a wide variety of B-cells in the immune system that fulfil diverse roles; such examples include plasma cells which secrete antibodies, memory B-cells which recognise previously detected antigens to initiate rapid humoral immune responses and regulatory B-cells which suppress pathogenic lymphocyte proliferation by secreting IL-10, IL-35 and TGF β ²³⁴.

1.11.3 *Sensing danger signals by pattern recognition receptors*

The immune system is challenged by dangers from highly diverse origins that mainly fall into one of two categories. Firstly, the immune system's primary role is to protect the body from infection, which it does by detecting the presence of foreign non-self organisms. This detection is largely sensed by innate immune cells via membrane bound and intracellular pattern recognition receptors (PRRs), which detect highly conserved, pathogen derived molecular motifs called pathogen associated molecular patterns (PAMPs). The recognition of PAMPs by PRRs triggers the rapid release of pro-inflammatory cytokines and chemokines, which recruit phagocytes and antigen presenting cells to phagocytose and destroy the invader to prevent colonization of the tissues by the pathogen; and also to ingest and present pathogen antigens to the adaptive immune system, to trigger long lasting immunity to prevent repeated invasion²³⁵.

Threats from microbes are not the only danger signal detected by the immune system, as innate immune responses can also be triggered by antigens of non-microbial or endogenous origin. Such antigens are detected as a result of tissue damage and cell stresses, and may be released following cell death, or as components of a damaged ECM. These endogenous activators of innate immunity are called damage associated molecular patterns (DAMPs), and these also trigger inflammatory activation of innate immune cells via PRRs. DAMPs serve to stimulate inflammatory signalling pathways that initiate the removal of damaged cell or ECM remnants, so that tissue homeostasis may be restored^{236, 237, 238}. To date, a range of endogenous molecules has been shown to trigger innate immune activation, including amyloid-beta²³⁹, cholesterol²⁴⁰, tumour specific antigens²⁴¹ and tenascin-C⁴³.

1.11.4 *Tenascin-C expression during inflammation*

Tenascin-C expression is rapidly and transiently induced at sites of inflammation, including during sterile injury^{242, 243} and infection^{244, 245, 246}; where levels of tenascin-C protein and mRNA transiently spike during inflammation, but are completely absent by the time tissue repair has been resolved²⁴⁷. In contrast, persistent tenascin-C expression is associated with chronic pathological states, including cancer and atherosclerosis, in addition to fibrotic and autoimmune disorders such as RA²⁴⁸. Tenascin-C expression correlates with excessive matrix deposition in a number of fibrotic diseases, such as chronic liver disease^{249, 250}, but also pulmonary fibrosis; a complication associated with RA²⁵¹. Despite this widely reported pattern of expression, the ability of tenascin-C to modulate inflammatory processes was only reported relatively recently, and this lack of current understanding of the role of tenascin-C and its alternatively spliced isoforms during inflammation forms the basis for the investigations described later in this thesis.

1.11.5 *Pro-inflammatory roles of tenascin-C*

To date, tenascin-C has been found to promote its inflammatory effects in cell based assays via interactions with 3 main receptor types; TLR4, $\alpha 9\beta 1$ integrin and $\alpha v\beta 3$ integrin. The impact of tenascin-C signalling via these receptor types is discussed in various immune cell types below, and is further discussed in the review paper Marzeda and Midwood,²⁵².

1.11.5.1 *Tenascin-C interactions with TLR4*

TLR4 is one of 13 TLRs identified in humans, and is a member of the toll-like receptor superfamily of PRRs that detects a variety of PAMPs to stimulate innate immunity in response to microbial challenge. TLR4 is a cell surface transmembrane receptor expressed on leukocytes and stromal cells that promotes innate immune responses to bacterial infection by sensing of the bacterial virulence factor and PAMP lipopolysaccharide (LPS); but it also detects other endogenous DAMPs, including tenascin-C, fibrinogen, biglycan, S100 proteins and opiate drugs among others²⁵³.

Our group previously showed that in macrophages, tenascin-C's FBG domain serves as an endogenous activator of TLR4, but that the other constitutively expressed domains of the molecule did not⁴³. Additionally, our group revealed that tenascin-C's FBG domain binds to TLR4 via three epitopes within a lysine rich cationic ridge on FBG; in a manner that was not dependent on the LPS binding cofactor MD2, revealing fundamental differences in the manner by which the DAMP FBG and PAMP LPS initiate TLR4 signalling⁴².

The most widely reported impact of TLR4-tenascin-C interactions is the induction of the pro-inflammatory cytokines and chemokines. Tenascin-C binding to TLR4 induces the expression of $\text{TNF}\alpha$, IL-6 and IL-8; which to date has been observed in macrophages^{42, 43, 254, 255}, dendritic cells²⁵⁶, fibroblasts^{43, 257} and chondrocytes²⁵⁸.

Tenascin-C mediated induction of TLR4, by FBG, is an important driver of PRR signalling in antigen presenting cells. Through interactions with TLR4, tenascin-C drives inflammasome priming, which results in elevated expression of specific cytokines including IL-1 β and IL-18, and induces pyroptosis; a form of programmed cell death²⁵⁹. Inflammasomes require 2 stimuli to be instigated, firstly a primer promotes pattern recognition receptor dependent cytokine production, and this is then followed by inflammasome assembly, caspase-1 mediated maturation and subsequently the release of IL-1 β and IL-18²⁶⁰.

These pro-inflammatory roles for tenascin-C, suggest that sites of elevated tenascin-C expression may also exhibit higher tissue levels of pro-inflammatory cytokines and chemokines that would promote immune cell differentiation, activation and recruitment. Studies have shown that tenascin-C dependent cytokine release is vital for generating an environment permissive for T-cell differentiation and polarisation. Dendritic cells isolated from tenascin-C deficient mice exhibited greatly diminished IL-6 and IL-23 cytokine release following LPS stimulation, and a reduced capacity to drive Th17, but not Th1, Th2 or Treg cell polarisation²⁶¹. Th17 cells are the dominant cellular component in many inflammatory autoimmune diseases, and are a key driver of pathology in RA²⁶². Furthermore, it was shown that stimulation of DCs with exogenous tenascin-C restored their ability to drive Th17 cell generation; which was abrogated by IL-6 and TLR4 blocking antibodies, revealing that IL-6 secretion triggered by TLR4 activation was an important factor in driving Th17 cell polarisation²⁵⁶. Furthermore, tenascin-C also stimulated chondrocyte expression of prostaglandin E2²⁵⁸, another inflammatory mediator involved in Th17 polarisation^{263, 264}.

Tenascin-C was also shown to modify neutrophil responses *in vitro* via TLR4 signalling. Neutrophils derived from murine bone marrow upregulated MMP-9 expression in a TLR4 dependent manner²⁶⁵. MMPs are important for facilitating leukocyte transmigration across vascular barriers; and elevated MMP-9 expression was shown to mediate leukocyte

transmigration in livers following ischaemic reperfusion injury^{266, 267}. Previously tenascin-C has been shown to upregulate MMP-9 synthesis in fibroblasts²⁶⁸, macrophages²⁶⁹ and tumours²⁷⁰. The TLR4 dependency of MMP-9 expression by neutrophils was also confirmed by showing lower MMP-9 levels in TLR4 knockout mice after experimental stroke²⁷¹. Furthermore IL-6 regulated MMP-9 activity in neutrophils²⁶⁶, and IL-6 levels are significantly reduced in tenascin-C knockout mouse livers following ischaemic reperfusion injury²⁶⁵.

Other cell types are also reported to be behaviourally modified by tenascin-C's interactions with TLR4. In a human hepatoma cell line, TLR4 activation by tenascin-C promoted cell migration, and induced epithelial to mesenchymal transition²⁵⁴. Tenascin-C also induces TLR4 dependent expression of TNF α and IL-6 in synovial fibroblasts, but not IL-8, as was previously reported in macrophages⁴³. Furthermore, TLR4 activation by tenascin-C in human foreskin fibroblasts induces type I collagen synthesis²⁷². Altogether, these studies identify a role for tenascin-C/TLR4 signalling in driving both immune cell mediated inflammation, and stromal cell immune defence responses; and reveal that tenascin-C contributes to acute and chronic inflammatory environments resulting from both non-sterile infection and sterile tissue damage injury via modulation of TLR4 signalling.

1.11.5.2 Differences between LPS and tenascin-C dependent TLR4 signalling

In addition to the pro inflammatory effects of FBG – TLR4 signalling, tenascin-C mRNA and protein is expressed in response to TLR4 stimulation with LPS. Previous work by our group showed that tenascin-C is expressed not only by fibroblasts, but also by myeloid and lymphoid cell populations isolated from synovial membranes of patients with rheumatoid arthritis; and in dendritic cells stimulated with the pathogenic TLR4 agonist LPS²⁷³. While LPS and tenascin-C both share the ability to induce cytokine expression via binding TLR4, both ligands exhibit distinct effects on cell signalling and behaviour. Despite Tenascin-C and

LPS signalling being NF- κ B dependent, LPS dependent activation of macrophages induced an aggressive phenotype that expressed high levels of pro-inflammatory cytokines and ECM degrading proteases; whereas tenascin-C stimulation produced macrophages with a more reparative phenotype, with greater ECM protein expression and phosphorylation²⁵⁵. Furthermore, tenascin-C mediated TLR4 dependent macrophage differentiation to foam cells in atherosclerosis²⁷⁴.

TLR4 dependent activation of bone marrow derived macrophages with LPS resulted in NF- κ B and PI-3K dependent upregulation of tenascin-C mRNA, which was shown to control the activity of the regulatory micro RNA miR-155. When miR-155 was not matured in the absence of tenascin-C signalling, the expression of LPS dependent pro-inflammatory cytokines such as TNF α was inhibited²⁷⁵. Furthermore, tenascin-C in human breast milk was shown to interfere with HIV transmission. Tenascin-C bound to the HIV-1 envelope protein, which neutralised the infectious properties of the virus in peripheral blood mononuclear cells and reporter cell lines²⁷⁶. Together these data show that tenascin-C is expressed in response to TLR4 activation where it regulates pro-inflammatory cytokine expression on the miRNA level, and it induces a macrophage phenotype that is distinct to that caused by LPS dependent activation of TLR4.

1.11.5.3 Tenascin-C interactions with $\alpha 9\beta 1$ and $\alpha v\beta 3$ integrins

Tenascin-C also modulates inflammatory processes by interacting with other cell surface receptors. Integrins are heterodimeric transmembrane receptors that facilitate communication between the ECM and the cell; and activate signal transduction pathways that affect cytoskeletal reorganisation, cell motility and regulation of the cell cycle. Each integrin comprises of one alpha (α) and one beta (β) subunit, of which there are 24 and 9 identified in mammals respectively.

Integrins $\alpha 9\beta 1$ and $\alpha v\beta 3$ have previously been reported to bind other ECM components such as osteopontin, periostin, fibronectin and tenascin-C. Tenascin-C binding to both $\alpha 9\beta 1$ and $\alpha v\beta 3$ induces the synthesis of pro-inflammatory cytokines; with the epitope for $\alpha 9\beta 1$ binding to tenascin-C has been localised to the constitutively expressed FnIII-3 domain²⁷⁷, while $\alpha v\beta 3$ can be bound by tenascin-C via two sites, in the FnIII-3²⁷⁸ and FBG domain²⁷⁹.

In vivo studies in mouse synovial fibroblasts and macrophages, revealed that tenascin-C dependent $\alpha 9$ signalling induced the expression of pro-inflammatory cytokines IL-1 α , IL-6, CCL2, CCL4, CXCL5²⁷⁷; while $\alpha 9$ signalling acted synergistically alongside TLR4 signalling, to promote Th17 cell differentiation by inducing IL-6 and IL-23 expression in dendritic cells²⁸⁰.

The role of $\alpha v\beta 3$ signalling in promoting tenascin-C dependent cytokine expression was previously assessed in peritoneal macrophages, where it resulted in the NF-kB dependent expression of IL-1 β , IL-6 and TNF α ²⁸¹. Together these findings show that the constitutive domains of tenascin-C can synergistically induce expression of pro-inflammatory cytokines in myeloid cells, lymphocytes and fibroblasts, by inducing signalling from different receptor families.

1.11.5.4 Tenascin-C signalling via non cell membrane receptor routes

Tenascin-C has also been shown to regulate cell signalling processes in the absence of binding with cell surface receptors. In human glioblastoma cells, tenascin-C downregulated Wnt inhibitor DKK-1 and increased Wnt signalling by stabilising β -catenin²⁸². Furthermore, tenascin-C interacts with Wnt3a, and human adenocarcinoma cells plated in the presence of Wnt3a demonstrated enhanced Wnt signalling. However, when the cells were incubated with soluble tenascin-C, Wnt signalling was reduced²⁸³. Therefore perhaps cell associated tenascin-C sequesters Wnt ligands and retains them close to the cell membrane to enable the activation of signalling; while perhaps soluble tenascin-C diffuses from the point of expression, and binds free Wnt ligands to reduce Wnt signalling. As Wnt and Nf- κ B pathways exhibit significant cross-talk, Wnt signalling by tenascin-C may exert positive and negative modulation of inflammatory responses²⁸⁴.

1.11.6 Tenascin-C and the resolution of inflammation

Transient expression of tenascin-C is detected during acute inflammation, but is not detected following the resolution of tissue repair. However, during chronic inflammation persistent tenascin-C expression is detected, and it is possible that here, due to tissue damage, cellular stress and pro-inflammatory cytokine synthesis, that tenascin-C may signal in an autocrine manner via cellular receptors to exacerbate the chronic inflammatory process^{61, 285, 286}. Therefore it is reasonable to consider whether tenascin-C exhibits anti-inflammatory functions and may serve to resolve inflammation during, or whether its absence in acute inflammation creates a less inflammatory environment. Firstly, tenascin-C binds and sequesters a range of growth factors including TGF β a restorative growth factor, which reduces inflammation. Tenascin-C therefore may serve to increase cellular concentrations of the growth factor^{41, 287}. Furthermore, tenascin-C can also exert immunosuppressive functions

on T-lymphocytes. Tenascin-C inhibited T-cell activation by natural antigens²⁸⁸, anti-CD3 and fibronectin²⁸⁹, anti-CD28, ICAM-1 and laminin²⁹⁰. It also significantly inhibited mitogen-induced and anti-CD3 dependent proliferation, and IFN γ production in lung tumour lymphocytes¹⁵⁵. While in myeloid cells, it was reported that tenascin-C inhibited monocyte chemotaxis *in vitro*²⁹¹. Altogether, this evidence suggests tenascin-C can exert pro- and anti-inflammatory effects in a sophisticated manner, though further study is required to elucidate these subtly balanced and complex mechanisms.

1.11.7 *Tenascin-C in adaptive immunity*

Tenascin-C has also been shown to directly stimulate lymphocytes in the adaptive immune system, in a manner that was independent of antigen presenting cells.

Tenascin-C has been shown to directly impact the function of lymphocytes in the adaptive immune system. Previously tenascin-C protein was detected at regions of high CD4⁺ T-cell density in the lymph nodes^{81, 292}, and activated dendritic cells²⁷³; and was found to be required for adequate Th17²⁶¹ and Th2⁵³ immune responses *in vivo*. Tenascin-C modifies T-cell behaviour directly by supporting adhesion and rolling²⁹³, and by inhibiting anti-CD3 induced T-cell proliferation and cytokine expression¹⁵⁵. Furthermore, tenascin-C stimulates Th2 and B-cell activation^{53, 294}, promotes lymphocyte migration^{52, 293, 295}, and restricts T-cell actin polymerization and migration²⁹⁶. Tenascin-C induced expression of IL-13, IFN γ , IL-5 and immunoglobulin E in mouse splenocytes when cultured on fibronectin⁵³, and induced Ca²⁺ influx in jurkat T-cells²⁹⁷. Therefore, it is feasible that inappropriate expression of tenascin-C by resting immune cells may dysregulate their function, leading to the initiation of inappropriate immune cell behaviours.

1.11.8 *Tenascin-C alternative splicing and inflammation*

Alternatively spliced FnIII domains of tenascin-C also exert immunomodulatory effects, offering another mechanism by which tenascin-C's activity may be regulated during inflammation. In humans, a Leu1677Ile single-nucleotide polymorphism in the FnIII-D domain of tenascin-C is associated with a high risk of asthma; partly via causing FnIII-D domain instability that affects its elasticity, and promotes airway stiffness²⁹⁸; suggesting that alternatively spliced FnIII domains facilitate normal immune cell migration by maintaining permissive tissue structures. Furthermore, alternatively spliced FnIII domains can inhibit T-lymphocyte polarization. A study using recombinant purified FnIII domains, found that FnIII-A1,A2 domains of tenascin-C, prevented anti-CD3/fibronectin mediated peripheral blood T-cell activation; by preventing internalisation of the T-cell receptor complex²⁹⁹.

1.11.9 *Inflammatory effects of tenascin-C in vivo*

Tenascin-C has been shown to exert immunomodulatory properties in vivo. For example, during acute inflammation induced by zymosan in mouse joints, inflammation resolved faster in tenascin-C knockout mice than in wild type mice; furthermore, the wild type mice were protected from synovitis, cartilage and bone destruction in a model of erosive arthritis induced by methylated BSA⁴³. In this model, levels of pro-inflammatory cytokines such as TNF, IL-6 and IL-17 were reduced in the joints of *TNC* ^{-/-} mice²⁶¹. Additionally, in a model of mouse acute encephalomyelitis, tenascin-C knockout mice exhibited reduced levels of IL-17 expressed by Th17 cells, and a reduction in the number of IFN γ expressing Th1 cells³⁰⁰. *TNC* ^{-/-} mice are also less susceptible to LPS induced sepsis³⁰¹, and are protected from experimentally induced asthma due to an impaired production of Th2 associated cytokines IL-3 and IL-13⁵³. Furthermore, tenascin-C also affects myeloid cells in a cell type specific manner; as the absence of tenascin-C reduced macrophage migration but not neutrophil

migration in wounded mouse corneas³⁰². These mouse studies support the previous in vitro findings, that tenascin-C impacts T-cell polarisation and myeloid cell function in a cell-type specific manner, by impacting Th17 cells in RA³⁰³, Th2 in asthma³⁰⁴, Th17 and Th1 in encephalomyelitis³⁰⁵, and macrophage migration in corneal wound healing³⁰².

1.11.10 *The clinical applications of tenascin-C in autoimmune diseases*

Due to the role of tenascin-C in driving inflammatory processes, tenascin-C has been investigated as a potential diagnostic biomarker for several inflammatory conditions. In arthritis, tenascin-C levels are elevated in the RA synovial fluid, but did not correlate with C-reactive protein (CRP) levels³⁰⁶. In addition to elevated synovial levels in RA, circulating serum levels of tenascin-C were elevated in RA patients, confirming that tenascin-C could be detected distally from the joint³⁰⁷. Furthermore, antibodies targeting citrullinated arginine epitopes within the tenascin-C FBG domain, could be detected in RA patients years before the onset of symptoms, and may assist early RA diagnosis³⁰⁸. Tenascin-C levels in patient serum correlated with levels of CRP in a number of diverse conditions including malaria, cancer and sepsis³⁰⁹; and circulating levels of tenascin-C correlated with disease severity in ulcerative colitis, Crohn's disease and systemic lupus erythromatosus, where they were a better predictor of flares than conventional diagnostic markers. Altogether, these data highlight tenascin-C as a useful diagnostic agent for the prediction and detection of autoimmune diseases, and further development of such tools could positively impact patient treatment.

1.11.11 **Summary**

The elevated level of tenascin-C expression in response to acute and chronic inflammation suggests that tenascin-C serves a role that is shared in global inflammatory responses. However, despite these studies, relatively little is known of the spatio-temporal manner in which tenascin-C is expressed during inflammation or in inflammatory diseases; as no systematic characterisation of tenascin-C expression or alternative splicing has been performed. Further examples of associations between tenascin-C expression and inflammation are reviewed by Udalova *et al.*,⁶¹ and Marzeda and Midwood.,²⁵², and are discussed at relevant locations throughout this thesis.

1.12 Aims and hypothesis

I hypothesised that fibroblasts and immune cells express inflammation and disease specific tenascin-C isoforms, which directly impact cell behaviour and facilitate cell processes during inflammation.

In order to test this hypothesis, a number of key aims and objectives were assessed as follows:

- 1) To quantify the levels of tenascin-C mRNA and protein in resting and activated primary human fibroblasts and immune cells; and in normal and diseased fibroblasts from RA and OA patients. This was to be done using standard curve quantitative PCR (qPCR), ELISA and confocal immunofluorescence microscopy to assess the distribution of tenascin-C in cell cultures.
- 2) To use Western blotting to assess the sizes of inflammation and disease specific tenascin-C isoforms expressed by fibroblasts and immune cells, and quantify the abundance of each alternatively spliced FnIII in these cell types at the mRNA level; using standard curve qPCR methods optimised as part of this thesis.
- 3) To clone, express and purify functionally active recombinant FnIII proteins in *E.coli*; which correspond to spliced domains regulated in fibroblasts and immune cells during inflammation and disease. Then, to assess the impact of these proteins on cytokine expression by ELISA, cell adhesion by crystal violet cell adhesion assays, and on cell migration using Transwell migration assays.

The overall aim of the study was to answer the following questions:

- a) How abundant is tenascin-C expression in resting fibroblasts and immune cells?
- b) How does tenascin-C expression change following cell activation?
- c) Is tenascin-C expression elevated in diseased fibroblasts?
- d) Are there inflammation or disease specific tenascin-C isoforms that are expressed?
- e) Do inflammation or disease specific tenascin-C isoforms impact fibroblast and immune cell behaviours?

Chapter 2 Materials and Methods

2.1 Buffers and recipes

Unless otherwise specified, all reagents used in this study were purchased from ThermoFisher Scientific. For the reagent list see Table 2.1.

Table 2.1. Lists the names and reagents required to prepare buffers and solutions described in this study.

Buffers and solutions	Reagents Required
25mM MES buffer (MES)	0.48g 2-(N-morpholino)ethanesulfonic acid (MES), 100ml deionised water (dH ₂ O), titrate to pH6.0 using 1M NaOH
4% paraformaldehyde solution (PFA)	36% (v/v) paraformaldehyde, dH ₂ O,
Coomassie blue destainer	30% methanol, 1% formic acid in dH ₂ O
Coomassie blue solution	0.1% coomassie blue, 50% methanol, 20% acetic acid solution, dH ₂ O
Crystal violet solution (0.1%)	MES buffer (1x), 0.1% (w/v) crystal violet powder
DNA Loading Buffer (10ml)	4g Sucrose, 25mg Bromophenol blue, dH ₂ O
EDTA (1Litre, 0.5M)	186.1g EDTA (SIGMA), NaOH dropwise to pH8, dH ₂ O
Flow cytometry (FACS) buffer	1x phosphate buffered saline (PBS) pH 7.2, , 0.5% (w/v) bovine serum albumin (BSA), 0.1% (v/v) PFA
Immunofluorescence and Western blot antibody dilution buffer	1xPBS, 1% BSA
Immunofluorescence blocking buffer	1xPBS, 1% BSA, 5% Goat serum
Immunofluorescence permeabilising buffer	1xPBS, 0.2% Triton X-100
Immunofluorescence wash buffer	1xPBS, 0.05% Tween-20, 0.1% BSA
Low-SDS RIPA buffer	50mM Tris-HCl, 0.5% Na-deoxycholate, 0.05% SDS, 150mM NaCl, 2mM EDTA, 0.1% Triton X-100
Lysogeny broth (1x)	10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl, to 1L dH ₂ O
MACS buffer	1xPBS pH 7.2, 0.5% (w/v) bovine serum albumin (BSA), 2mM EDTA
Membrane stripping buffer (1L)	5g Glycine, 2g SDS, 10ml Tween20, HCl to pH2.2, add dH ₂ O to 1L
PBS (1x)	10x PBS stock solution (SIGMA-ALDRICH)
PBST (1x)	PBS (1x), 0.05% Tween-20

Buffers and solutions	Reagents Required
Ponceau Red Stain	0.1% (w/v) Ponceau S, 5% (v/v) acetic acid in dH ₂ O
SDS loading buffer 5x (10ml)	250mM Tris-HCl pH6.8, 5% β-Mercaptoethanol, 0.02% Bromophenol blue, 30% glycerol, 10% SDS
Silver stain developer	6g sodium carbonate and 300ml dH ₂ O
Silver stain fix solution	75ml methanol, 7.5ml acetic acid and 67.5ml dH ₂ O
Silver stain sensitizing solution	30mg sodium thiosulfate-5-hydrate, and 150ml dH ₂ O
Silver stain solution	150mg silver nitrate, 120μl 36% paraformaldehyde, and 150ml dH ₂ O
Silver stain stop	7.5ml acetic acid and 142.5ml dH ₂ O
SOC media	2%w/v tryptone (SIGMA), 0.5%w/v yeast extract (SIGMA); 10mM NaCl; 2.5mM KCl; 10mM MgCl ₂ ; 10mM MgSO ₄ ; 20mM glucose
T ₁₀ E ₁ TE (10x)	10mM Tris-HCl pH8.0, 1mM EDTA, dH ₂ O
TAE buffer (1Litre, 50x)	242g Tris, 57.1ml Glacial acetic acid (SIGMA), 100ml 0.5M EDTA, dH ₂ O
Tris-buffered saline (TBS) (1x)	50mM Tris-Cl, 150mM NaCl, dH ₂ O
TN Buffer	50mM Tris pH 8.0, 150mM NaCl
TopVision agarose gel (1%-3%)	0.5-1.5g TopVision agarose, 50ml 1x TAE buffer, 5μl GelRed DNA stain (Biotium)
Tris-Glycine Buffer	0.06M Trizma base, 0.5M Glycine, 1% w/v SDS
Urea Buffer	8M urea (SIGMA), 20mM Tris pH 8.0
Western blot blocking buffer	1xPBS, 5% BSA

2.2 Isolation of primary human immune cells

Primary human monocyte and lymphocyte fractions were isolated from single donor blood residues, left over from plateletpheresis (North London Transfusion Centre) by ficoll gradient and counterflow centrifugation (as previously described in ^{273, 310, 311}). Blood was diluted in Hanks Balanced Salt Solution with phenol red (HBSS; Lonza), and layered on Lympholyte®-H cell separation media (CEDARLANE) and centrifuged at 2200rpm for 15 minutes with the deceleration brake off. The white cell layers were suspended in HBSS and centrifuged at 1800rpm for 10 minutes. The suspended white cell layers containing peripheral blood mononuclear cells (PBMC) were passed through a nylon cell strainer (VWR) prior to loading onto the elutriator (Beckman GEL).

The elutriator is an instrument which uses the principal of counterflow centrifugation, to selectively elute objects of a given size and density, based on the balance between centrifugal force, and the counter flow drag force. This causes smaller cells to elute at lower flow rates, and larger cells eluting at higher flow rates; permitting the isolation of monocyte and lymphocyte cell populations, from the platelet-depleted blood sample.

The elutriator was equilibrated in RPMI-1640 media (Lonza) with 1% (v/v) foetal bovine serum (FBS; Gibco), 1% (v/v) penicillin/streptomycin (PS; Lonza), and the centrifuge set to 2500rpm. Cells were loaded at 9ml/minute, and eluted at 16-23ml/minute flow rates. The purity of monocyte and lymphocyte fractions was estimated by forward scatter and side scatter, using the FACScan flow cytometry instrument, and fractions of <94% purity were discarded.

2.3 Cell culture, differentiation and purification

All cells were cultured in a humidified incubator at 37°C, 5% CO₂

2.3.1 *Monocytes*

The purity of human monocyte fractions obtained from elutriation was enriched by plating in canted neck 150cm² tissue culture flasks (Falcon), in serum free RPMI-1640 1% (v/v) PS at 1x10⁶cells/ml for 30 minutes. Non-adherent cells were washed away with PBS, and monocytes were then plated with RPMI-1640 5% (v/v) FBS and 1% (v/v) PS for stimulation or differentiation as described below.

2.3.2 *Macrophages and Monocyte derived dendritic cells (MDDCs)*

Human monocytes were plated in canted neck 150cm² tissue culture flasks (Falcon), in RPMI-1640 5% (v/v) FBS and 1% (v/v) PS, at 1x10⁶cells/ml in 20ml volume; and were differentiated into granulocyte-macrophage colony-stimulating factor (GM-CSF) macrophages by culturing for 5 days in RPMI-1640 5% (v/v) FBS and 1% (v/v) PS with 50ng/ml GM-CSF (Peprotech), or into macrophage colony-stimulating factor (M-CSF) macrophages by culturing for 5 days in RPMI-1640 5% (v/v) FBS and 1% (v/v) PS with 100ng/ml M-CSF (Peprotech). Monocytes were differentiated into MDDCs by culturing for 7 days in RPMI-1640 5% (v/v) FBS and 1% (v/v) PS with 50ng/ml GM-CSF and 10ng/ml interleukin-4 (IL-4) (Peprotech) respectively.

2.3.3 *CD3+, CD4+ and CD8+ T-cells*

Lymphocytes were collected from the elutriator after depletion of monocytes, and were purified into CD3+, CD4+ or CD8+ populations by MACS Cell Separation technology (Miltenyi Biotec), using the CD3+ Pan T-cell isolation kit (#130-096-535), CD4⁺ T cell isolation kit (#130-091-155) or CD8+ MicroBeads (#130-045-201) respectively. T-cells were

loaded in LS columns (Miltenyi) and were separated by the MidiMACS™ separator (Miltenyi), following the suppliers instructions.

2.3.4 *Neutrophils*

Neutrophils were isolated by the laboratory of Dr. Lynne Prince (University of Sheffield) as described in ³¹², from peripheral blood; and were separated by Ficoll-Paque Plus (GE Healthcare) centrifugation. Cell purity was assessed by visual inspection following staining with Romanowski stain, as described in ³¹³, and fractions above 94% purity were used. Red blood cells in the purified pellet were lysed with red blood cell lysis solution (Miltenyi).

2.3.5 *Healthy and diseased fibroblasts*

Primary human dermal fibroblasts (HDFs) were obtained from healthy, non-palmar sites on Dupuytren's contracture patients (sourced from Prof Jagdeep Nanchahal; University of Oxford), and normal synovial fibroblasts (NSFs) were obtained from non-diseased patients undergoing limb amputation or joint biopsy (sourced from Dr Lynn Williams; University of Oxford), and waste tissue (synovium after joint replacement surgery) was obtained after signed, informed consent. Primary rheumatoid arthritis and osteoarthritis synovial fibroblasts (RA-SFs, OA-SFs) were obtained from Dr Lynn Williams; University of Oxford. Waste tissue (synovium after joint replacement surgery) was obtained after signed, informed consent following and local ethics approval^{43, 314}. All tissue samples were anonymized to protect individual identity. Fibroblasts were cultured in DMEM (Lonza) 10% (v/v) FBS and 1% (v/v) PS, and were passaged with trypsin-EDTA (Lonza) upon reaching 80% confluence. Fibroblast cultures were used in experiments between passages 0-6.

2.3.6 *Mouse Embryonic Fibroblasts*

Mouse embryonic fibroblasts (MEFs) were obtained from wild type (*wt*) and *TNC*^{-/-} animals, and were made available for this study by the Midwood lab. Cells were cultured in DMEM 10% (v/v) FBS and 1% (v/v) PS, and were passaged with trypsin-EDTA upon reaching 80% confluence.

2.3.7 *Human neuronal glioblastoma cell line (U-87 MG)*

Human neuronal glioblastoma cells U-87 MG (ATCC) were cultured with DMEM 10% (v/v) FBS and 1% (v/v) PS and were passaged every 4th day with trypsin-EDTA, before re-plating at 1:4 dilution.

2.3.8 Flow cytometry (FACS) analysis of cell purity

Protocols for monocyte preparation and macrophage and MDDC differentiation are well established in the Midwood lab, and routinely result in purities for each cell type exceeding 94%. The purity of each cell type was assessed routinely by FACS, and was performed by Dr. Claire Deligne (Kennedy Institute of Rheumatology, Oxford). For monocytes and macrophages, cell purity was assessed using anti-CD14, anti-CD33 and anti-CD11c antibodies. For MDDCs anti-CD45 and anti-CD11c antibodies were used; according to protocols described in ^{43, 311}. Cell populations above 94% purity were used in this study. FACS was performed using the LSR II FACS instrument (BD Biosciences) (Table 2.2).

CD3+, CD4+ and CD8+ T-cell population purity was assessed by me, using flow cytometry. 5×10^5 cells were suspended in 200µl ice cold FACS buffer, in a 96-well V-bottom plate (VWR) and were incubated with 5µl of FACS antibody in the dark at 4°C for 30 minutes; the list of FACS antibodies used to assess T-cell sub-population purity are listed in Table 2.2. T-cells were then washed in FACS buffer and fixed in 200µl 4% (v/v) paraformaldehyde (PFA) for 20 minutes at RT (shielded from light). Cells were washed and resuspended in 200µl PBS, and were analysed using the LSR II FACS instrument (BD Biosciences). Purity was typically 95%, and cells of purity less than 90% were discarded.

T-cell population	Antibody/Fluorophore	Catalog number	Dilution (1:x)
CD3+	Anti-CD45 FITC	11-0459-41 (BD biosciences)	40
	Anti-CD3 APC	130-091-373 (Miltenyi)	
CD4+	Anti-CD4 eFluor450	561844 (BD Biosciences)	40
	Anti-CD3 APC	130-091-373 (Miltenyi)	
CD8+	Anti-CD8 eFluor450	560273 (BD Biosciences)	40
	Anti-CD3 APC	130-091-373 (Miltenyi)	

Table 2.2. Antibodies used to assess the purity of the T-cell populations. The catalog number, supplier and the antibody dilution used for FACS are also included in the table.

2.4 Cell stimulation

In this study tenascin-C expression was characterised in monocytes, GM-CSF macrophages, M-CSF macrophages, MDDCs, neutrophils, CD3+ T-cells, CD4+ T-cells, CD8+ T-cells and HDFs that were resting or activated by cell specific stimuli; and tenascin-C expression was characterised in U87-MG, RA-SFs, OA-SFs and NSF s that were not stimulated. Table 2.3 shows the inflammatory stimuli used to activate cells in this study, while Table 2.4 shows the details of all the cell culture and stimulation conditions.

Cell Type	Inflammatory Stimuli	Reference
Monocytes	LPS	273, 301, 315
GM-CSF Macrophages	LPS	315
M-CSF Macrophages	LPS	273, 301, 315
MDDCs	LPS	273, 301
Neutrophils	LPS	316
	GM-CSF	317
CD3+/CD4+/CD8+ T-cells	anti-CD3, anti-CD28	318
HDFs	IL-1 β	319

Table 2.3. Cell specific inflammatory stimuli used in this study.

2.4.1 Monocytes, MDDCs, GM-CSF and M-CSF macrophages

Enriched monocytes, and differentiated GM-CSF macrophages, M-CSF macrophages and MDDCs were detached from the culture flasks using a cell scraper (Corning), were washed and counted with Trypan blue stain; to confirm viability >90%. Live cells were plated in RPMI-1640 5% (v/v) FBS and 1% (v/v) PS, at 1×10^6 cells/ml in a 6-well plate (Corning), and were rested for 16 hours prior to stimulation with 10ng/ml LPS from *E. coli*, serotype EH100 (Ra) (TLRgradeTM; Enzo, life sciences). Parallel wells were left unstimulated for each time point, and total RNA was isolated 0, 4, 8 and 24 hours from all cell types. For MDDCs, cell lysate (CL) and cell culture supernatant (SN) were also collected at these time points, and at additional 48 and 72 hour time points.

2.4.2 *Neutrophils*

Neutrophil stimulations were performed by the laboratory of Dr Lynne Prince (University of Sheffield). Neutrophils were plated at 5×10^6 cells/ml in 200 μ l volumes of RPMI-1640 5% (v/v) FBS and 1% (v/v) PS, and were stimulated without resting, with 100ng/ml LPS or 50 μ g/ml GM-CSF, or were left unstimulated. Total RNA, cell lysate and supernatant were isolated at 0, 4 and 6 hours.

2.4.3 *T-cells*

Following purification, T-cells were plated at 3×10^6 cells/ml in 1ml volume of RPMI-1640 5% (v/v) FBS and 1% (v/v) PS, 10nM β -mercaptoethanol, for 16 hours prior to stimulation with a 1:1 cell:bead ratio of anti-CD3/CD28 Dynabeads® T-cell activator (Invitrogen 111.31D) in a 24-well plate (Corning); parallel wells were plated where cells were left unstimulated. The Dynabeads® were magnetically removed from the T-cell culture after 3 days of stimulation. In CD3⁺ T-cells, total RNA was isolated after 0, 1.5, 4, 8, 24 and 48 hours; while in CD4⁺ and CD8⁺ T-cells, total RNA, cell lysate and supernatant was isolated at these time points as well as 72, 96, 168 and 240 hour time points.

2.4.4 *HDFs*

HDFs were plated at 2×10^5 cells/ml in 1.5ml DMEM 10% (v/v) FBS and 1% (v/v) PS, in a 6-well plate and were immediately stimulated with 10ng/ml Il-1 β (Peprotech), or were left unstimulated. Total RNA, cell lysate and supernatant were collected at 0, 4, 8, 24, 48, 72 and 96 hour time points and cell lysate and supernatant were collected at a further time point of 120 hours.

2.4.5 *Non stimulated cell types: Synovial fibroblasts and U-87 MG cell line*

NSFs, RA-SFs and OA-SFs were plated at 2×10^5 in 1.5ml DMEM 10% (v/v) FBS and 1% (v/v) PS, in a 6-well plate and were not stimulated. Total RNA, cell lysate and supernatant were collected at 72 hour time points. U-87 MG cells were plated at 6.6×10^5 cells/ml in 1.5ml DMEM 10% (v/v) FBS and 1% (v/v) PS, in a 6-well plate and were not stimulated. Total RNA, cell lysate and supernatant were collected at 0, 4, 8, 24, 48 and 72 hour time points.

Cell Type	Cell density (cells/ml)	Total Volume (ml)	Plate format	Stimulus
Monocyte				
GM-CSF Macrophage	1×10^6	1.5	6-well	$\pm 10 \text{ ng/ml LPS}$
M-CSF Macrophage				
MDDC				
Neutrophil	5×10^6	0.2	48-well	$\pm 100 \text{ ng/ml LPS}$ $\pm 50 \text{ ng/ml GM-CSF}$
CD3+ T-cell	3×10^6	1.0	24-well	$\pm \text{anti-CD3/CD28}$ Dynabeads® (cell to bead ratio 1:1)
CD4+ T-cell				
CD8+ T-cell				
HDF	2×10^5	1.5	6-well	$\pm 10 \text{ ng/ml IL-1}\beta$
Cells below were not stimulated				
U-87 MG	6.6×10^5	1.5	6-well	-
RA-SF				-
OA-SF	2×10^5	1.5	6-well	-
NSF				-

Table 2.4. Table displaying the cell plating and stimulation conditions used in this study.

2.5 **MTT cell viability assay**

The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromine (MTT) assay was performed on cells cultured in 96-well format, in triplicate, by adding 10% (v/v) MTT for between 3-16 hours at 37°C, 5% CO₂; until insoluble formazan crystals formed. Formazan was solubilised with 50% (v/v) 10% sodium-dodecyl sulfate (SDS), 0.01M HCl overnight, and optical density (OD)_{620nm} was quantified using the FluoStar Omega 96-well plate reader.

MTT assays were performed in parallel in separate plates for each experiment described in this study, with the exception of the cytokine expression assays, where the MTT assay was performed on the same cells from which supernatant was collected; and the cell adhesion assays and migration assays where MTT assays were not performed.

2.6 Quantifying tenascin-C gene expression

2.6.1 RNA extraction

All working surfaces and equipment were thoroughly cleaned and sprayed with RNaseZap® (SIGMA-ALDRICH) RNase inhibitor. Total RNA was extracted from cells using the RNeasy Mini kit (Qiagen), following the manufacturer's instructions. Briefly, cells were lysed in denaturing guanidine-thiocyanate buffer, and ethanol was added. Samples were then applied to a silica spin column, and after several washing steps, pure RNA was eluted with RNase-free water. RNA was stored at -80°C.

2.6.2 cDNA synthesis by reverse transcription of mRNA

RNA was reverse transcribed into cDNA using the AffinityScript cDNA synthesis kit and oligo(dT) primers (Agilent), following manufacturer's instructions. 500ng of RNA and 1µl oligo(dT) primer was added to 14.7µl dH₂O and heated to 65°C for 5 minutes and then cooled to 22°C for 10 minutes. 2µl 10x Affinity Script Buffer, 0.8µl dNTP (25nM), 0.5µl ribonuclease inhibitor and 1µl Affinity Script multiple temperature reverse transcriptase were then added, and the reaction tube was sealed and heated to 42°C for 5 minutes, 55°C for 55 minutes and 70°C for 15 minutes. cDNA was stored at -20° for short periods (<1 month), and at -80°C for longer periods (>1 month).

2.6.3 Quantification of nucleic acids

The concentration and total yields of RNA and DNA samples in RNase-free water, were quantified using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific). 1µl of RNA or DNA was added to the pedestal, and the absorbance was measured. The purity was determined by comparing the absorbance values at 260nm and 280nm wavelength (A260/280), and 260nm and 230nm (A260/230). Pure RNA or DNA samples had OD_{260/280nm} ratios of 2.0 and 1.8 respectively.

2.6.4 TaqMan® qPCR

The absolute abundance of tenascin-C mRNA was quantified by standard curve qPCR using FAM-conjugated TaqMan® primers and probes. TaqMan qPCR was performed in the Corbett life science Rotor-Gene 6000 instrument, or the ViiA7 instrument (Applied Biosystems) according to the manufacturer's qPCR thermocycle instructions (Table 2.5).

Reagent	Temperture (°C)	Time
1. Uracil-DNA glycosylase (UNG) incubation	50	2 minutes
2. AmpliTaq Gold activation	95	10 minutes
3. PCR Denature	92	15 seconds
4. Anneal/Extend	60	1 minute
Number of qPCR cycles (steps 3-4)	X40	-

Table 2.5. Thermocycle conditions required for TaqMan® qPCR assay

Individual 10µl reactions were prepared in triplicate, using the reagents as described in

Table 2.6. To each 7µl qPCR reaction mixture volume, 3µl of cDNA diluted 1:4 in water, or diluted purified plasmid at a known concentration was added. No template controls were included in triplicate for each TaqMan® assay.

Reagent	Volume added per reaction tube (µl)
TaqMan [®] Universal PCR Master Mix	5
TaqMan [®] gene expression assay	0.5
Sterile dH ₂ O	1.5
cDNA sample OR plasmid standard	3

Table 2.6. TaqMan qPCR gene expression analysis reaction mixture. The table details the reagent composition of the TaqMan qPCR reaction mixture, showing the volumes to add per 10µl reaction tube.

A list of FAM-conjugated TaqMan[®] primers (Invitrogen), and the corresponding I.M.A.G.E fully sequenced cDNA clone plasmids (SourceBioScience) used as standards in the TaqMan[®] qPCR assays are listed in Table 2.7.

Gene	TaqMan Gene Expression Assay (Cat N ^o)	I.M.A.G.E Fully Sequenced cDNA Plasmid (IMAGE ID)
TNC	Hs01115663_m1	IRCFp5008F0112D
RPLP0	Hs99999902_m1	IRAUUp969H0758D
HPRT1	Hs02800695_m1	IRAUUp969B109D
GNB2L1	Hs00272002_m1	IRAUUp969A041D
GAPDH	Hs02758991_g1	IRAUUp969E0824D
YWHAZ	Hs03044281_g1	IRATp970D09108D

Table 2.7. TaqMan assays and DNA plasmids utilised in gene expression analysis. Tenascin-C mRNA was quantified in resting and activated cells by standard curve qPCR. Assay and cell type dependent DNA standard curves were generated for control genes, so relative quantification could take place.

2.6.5 *Standard curves*

The concentration of individual I.M.A.G.E fully sequenced cDNA clones was quantified using the NanoDrop ND-1000 instrument, and plasmid copies/ μl was calculated as described below.

$$\text{Number of plasmid copies per } \mu\text{l} = \frac{(\text{plasmid concentration in ng per } \mu\text{l}) \times (6.022 \times 10^{23})}{(\text{size of plasmid in base pairs}) \times (650 \text{ g mol}^{-1}) \times (1 \times 10^9)}$$

Aliquots of each plasmid were stored at -80°C and fresh 10-fold serial standard dilutions were prepared for each qPCR reaction, ranging from 10^8 – 10^{-1} copies/ μl . Standard curve data analysis was performed using the Rotor-Gene 6000 Series Software 1.7 or ViiA7 software, to quantify the copies mRNA/ μl in the diluted cDNA sample. The final copies mRNA/cell were calculated by taking into account cDNA sample dilutions in the qPCR reaction, the total RNA yield and the number of cells isolated.

2.6.6 *Identification of stably expressed control genes for qPCR normalisation*

Endogenous control gene stability was assessed by delta Ct (ΔCt) qPCR methods, using the TaqMan qPCR protocol. Gene expression in basal and peak stimulated time points for each cell type was quantified in 1:4-1:10 diluted cDNA to determine the most stably expressed control genes with the lowest standard deviation of abundance across multiple donors. ΔCT analysis was performed using Rotor-Gene 6000 Series 1.7, ViiA 7 and Microsoft Excel software, with the least variably expressed genes selected as endogenous controls for 2-standard curve qPCR. In this study the endogenous control genes used were RPLP0, HPRT1, GNB2L1, GAPDH, YWHAZ or ACTB.

2.6.7 Calculations for standard curve qPCR

2.6.7.1 Absolute quantification

The values obtained from the standard curves for the gene of interest copies/ μ l were used to calculate the total copies/cell. Calculations were performed in the following order.

1. *number of copies/ μ l in undiluted cDNA sample* = $\frac{\text{copies}/\mu\text{l from PCR}}{\text{dilution factor } 0.075}$
2. *number of copies in 500ng cDNA* = (number of copies/ μ l in undiluted cDNA sample) $\times$ 20 μ l
3. *proportion of total RNA reverse transcribed to cDNA (500ng)* = $\frac{500\text{ng cDNA}}{(\text{RNA yield ng}/\mu\text{l}) \times 30\mu\text{l}}$
4. *RNA scaling up factor* = $\frac{1}{\text{proportion of total RNA reverse transcribed to cDNA (500ng)}}$
5. *Total copies in sample* = (number of copies in 500ng cDNA) $\times$ (RNA scaling up factor)
6. *copies/cell* = $\frac{\text{Total copies in sample}}{\text{Total number of cells}}$

The final copies/cell value was either then displayed as an absolute value, or was normalised to the absolute copies/cell value for an endogenous control gene.

2.6.7.2 Relative quantification

Relative quantifications were either calculated by normalising the absolute copies/cell values for the gene of interest, against the endogenous control gene, or were calculated using the delta-delta Ct method ($\Delta\Delta\text{Ct}$). The average threshold cycle (Ct) values were obtained for target genes, and were compared to an endogenous control gene, so that ΔCt and $\Delta\Delta\text{Ct}$ values could be calculated, to give fold gene expression values.

$$\Delta\text{Ct} = \text{average Ct gene of interest} - \text{average Ct endogenous control gene}$$

$$\text{followed by } \Delta\Delta\text{Ct} = \Delta\text{Ct treated sample} - \Delta\text{Ct untreated sample}$$

$$\text{Fold gene expression} = 2^{(-\Delta\Delta\text{Ct})}$$

2.7 Characterisation of tenascin-C protein

2.7.1 *Cell lysis for protein isolation*

Cell culture SN was aspirated from resting and activated cells and stored in aliquots at -20°C. Additional aliquots of unconditioned culture media were also stored, and this served as a media blank. Adherent cells were lysed in-well, and suspension cells were pelleted at 1500rpm for 5 minutes, prior to lysis with low-SDS RIPA buffer at ratio of 200µl per 5x10⁵ cells. Cells were lysed for 25 minutes on ice, and the cell lysate was transferred to fresh tubes for storage at -20°C for short periods (<1 month), and at -80°C for longer periods (>1 month).

2.7.2 *Total protein quantification by Bicinchoninic acid (BCA) assay*

Total protein concentration in cell lysate fractions was quantified with the PierceTM BCA protein assay kit, according to manufacturer's instructions. OD_{560nm} was measured using the FluoStar Omega plate reader, and the protein concentration in the cell lysate was calculated from the BSA standard curve.

2.7.3 Quantification of tenascin-C protein by USCN and FnIII-B ELISAs

USCN (USCN) and FnIII-B (IBL) tenascin-C ELISAs were performed according to manufacturer's instructions. Cell lysate and supernatant samples were quantified using assay and cell dependent optimized sample dilutions that were established by titrating dilutions of protein samples against the ELISA standard curves (see Table 2.8). OD_{450nm} was measured using the FluoStar Omega plate reader. Both ELISAs cross-reacted with serum tenascin-C present in the cell culture media, hence a culture media blank was included with the test samples, and its absorbance value subtracted from the supernatant samples only.

Cell Type	USCN ELISA		FnIII-B ELISA	
	Cell Lysate (1:x)	Supernatant (1:x)	Cell Lysate (1:x)	Supernatant (1:x)
Glioma	100	20-100	75	25
HDF	30	40	30-50	50-100
MDDC	10	100	1	1
NSF	20	20-40	2	20
OA-SF	20	20-40	2	20
RA-SF	20	20-40	20	20

Table 2.8. Assay and cell-type dependent dilutions of protein samples quantified on USCN and FnIII-B ELISAs. For samples where a concentration range is given, the higher dilution factor was used on early time point samples where tenascin-C was less abundant, and the lower dilution was used on late time points, where tenascin-C was more abundant.

2.7.4 Visualisation of tenascin-C by confocal immunofluorescence microscopy

18mm glass coverslips (VWR) were autoclaved and added to a 12-well tissue culture plate (Corning). 1ml of cell suspensions were seeded on the glass coverslips at cell dependent densities, and cells were rested or stimulated as described in Table 2.9.

Cell Type	Plating density (cells/ml)	Culture time $\pm$ stimulation (hours)	Stimulus
U-87 MG	5×10^5	0,8,24,48,72	None
HDF	2×10^5	0,4,8,24,48,72,96	+10ng/ml IL-1 β
MDDC	1×10^6	0,8,24,48,72	+10ng/ml LPS
Cells below were not stimulated			
NSF			
OA-SF			
RA-SF	2×10^5	48, 72	None
MEF wt			
MEF <i>TNC</i> ^{-/-}			

Table 2.9. Table detailing the cell specific seeding densities and culture times for cells cultured on glass coverslips for confocal immunofluorescence microscopy.

After culturing, the media was aspirated, and cells were washed with PBS 5 times, and fixed in 4% (v/v) PFA for 20mins at RT. Cells were either not permeabilised, or were permeabilised with 0.2% Triton X-100 for 10 minutes, prior to rinsing in 1xPBS and incubation with immunofluorescence blocking buffer for 1h at RT. Primary antibody solutions were prepared in antibody dilution buffer using dilutions described in Table 2.10, and coverslips were stained at 4°C for 16 hours.

Coverslips were washed in PBS and stained with 1:250 4',6-diamidino-2-phenylindole (DAPI) (ThermoFisher Scientific), with secondary antibodies diluted in antibody dilution buffer as described in Table 2.10; for 1 hour at RT. Coverslips were washed 5 times in PBS, were blotted dry, and then mounted on glass slides (VWR) with 1 drop ProLong® Antifade Gold (ThermoFisher Scientific). Coverslips were sealed with nail varnish, and were stored at

4°C. Slides were visualised with Olympus FV1200 IX83 Confocal System with a 60x objective lens, and 2x zoom. Z-stacked images were taken using the same microscope.

Alongside all experiments, no primary antibody controls (NPCs) were performed where the primary antibody was omitted from the staining solution; this confirmed the specificity of the secondary antibodies. Isotype control experiments were performed with the mouse immunoglobulin G1 (IgG1) isotype primary antibody control (ThermoFisher Scientific), to confirm the absence of non-specific mouse primary antibody binding.

Target protein	Primary Incubation		Secondary Incubation	
	Primary antibody	Dilution (1:x)	Primary antibody/ Dye	Dilution (1:x)
Tenascin-C	BC-24 (SIGMA)	100	Anti-mouse (488nm) DAPI	1000 250
Fibronectin	F3648 (SIGMA)	200	Anti-rabbit (488nm) DAPI	1000 250

Table 2.10. Optimised antibody dilutions for confocal immunofluorescence microscopy staining of tenascin-C and fibronectin. The antibody staining dilutions were optimised in U-87 MG.

2.7.5 Western blotting of tenascin-C

2.7.5.1 Tris-glycine SDS-polyacrylamide gel electrophoresis (PAGE)

Protein samples were denatured in 1x SDS-loading buffer at 95°C for 5 minutes and were sonicated using a Bioruptor water bath (Diagenode) for 5 minutes. Samples were heated again at 95°C with the tube caps off, to evaporate the sample volume to ~30µl. Tenascin-C bands were resolved on 5% SDS-PAGE gels. SDS-PAGE gels were prepared as described in Table 2.11.

Solution	5% separating gel (ml)	3.9% stacking gel (ml)
30% Bis-acrylamide solution	1.25	0.32
4x Tris-HCl (pH6.8/8.8)	1.87 (pH6.8)	0.62 (pH8.8)
H ₂ O	5.37	1.505
10% APS	0.02	0.0125
TEMED	0.005	0.001

Table 2.11. Reagents required for producing SDS PAGE gels to resolve tenascin-C protein.

Samples were run alongside 6µl Protein-plus molecular weight marker (ThermoFisher Scientific), and HiMark™ pre-stained protein ladder (ThermoFisher Scientific) standards. Gel tanks (ATTO) were loaded with 700ml 1xTris-glycine buffer and gels were run for approximately 2.5 hours at 20mA/gel; see Table 2.12 for cell lysate and supernatant sample volumes loaded from different cell types. The cell specific concentration of tenascin-C, meant that greater sample volumes were required where tenascin-C abundance was low.

Cell type	Cell lysate sample volume (µl)	Supernatant sample volume (µl)
U-87 MG	20	30
HDF	10	20
MDDC	100	100
NSF	50	100
RA-SF	30	30
OA-SF	30	30

Table 2.12. Table showing the volumes of cell lysate and supernatant from various cell types loaded onto 5% SDS-PAGE gels for Western blot.

2.7.5.2 *Electro-transfer and Western blotting for tenascin-C*

Proteins separated by SDS-PAGE were electrotransferred using the Blot®Turbo™ transfer system (BioRad) to Trans-Blot®Turbo™ Mini nitrocellulose membranes (BioRad) using the pre-set 180V high molecular weight program. Membranes were blocked with 5% BSA in PBS, 0.05% (v/v) Tween-20 (PBST) for 1 hour at RT on a roller. Membranes were placed in tubes on rollers, and incubated with primary antibodies in 2% BSA PBST, at 4°C for 16 hours, washed 3 times in PBST, and then incubated with secondary antibody in 2% BSA PBST for 2 hours at RT; as described in Table 2.13.

Primary antibody; 4°C, 16 hours		
Target	Name	Dilution
Tenascin-C	mAb1908 (Millipore)	1:1000
Vinculin	SPM227 (Novus)	1:1000
β-Actin	AC-15 (ab6276)	1:1000
6xHis-tag	Tetra-His (Qiagen)	1:1000
Secondary antibody/stain; RT, 2 hours		
Anti-mouse IgG	Anti-mouse IgG-HRP (BioRad)	1:20,000

Table 2.13. Table listing primary and secondary antibodies used for Western blot in this study.

2.7.5.3 *Enhanced chemo-luminescence (ECL) detection*

Nitrocellulose membranes were washed 4 times with PBST to remove all traces of the secondary antibody-HRP conjugate. Protein bands were visualised on the membranes using the ECL Plus (Amersham) kit. Membranes were incubated with 2ml reagent for 2 minutes at room temperature, then the membrane was secured in a photography-film cassette, and taken to a dark room where bands were visualised using X-ray film (Fuji), with a variety of exposure times.

2.7.5.4 *Re-blotting for loading control proteins*

After protein bands were visualized in cell lysate sample, Western blotting for vinculin or actin loading controls was performed. Membranes were stripped with RestoreTM Western Blot Stripping Buffer (ThermoFisher Scientific) following manufacturer's instructions, and were re-blocked, stained with primary and secondary antibodies, and bands were visualised as previously described.

2.7.5.5 *PNGase-F digestion*

30µl of U-87 MG SN was incubated with 3.3µl Glycoprotein Denaturing Buffer (10x; NEB), and boiled at 95°C for 10 minutes. After cooling, 6µl GlycoBuffer 2 (10x; NEB), 2µl 10% NP-40, 18µl dH₂O and 3µl PNGase-F (NEB) was added, and the tube was incubated at 37°C for 1 hour. Then, the tubes were stored on ice and loaded on to 5% SDS-PAGE gels to assess the extent of deglycosylation of tenascin-C by Western Blot with mAb1908.

2.8 Characterisation of alternatively spliced FnIII mRNAs by PCR

2.8.1 *Designing primers to characterize FnIII alternative splicing in tenascin-C*

Unless otherwise stated, all primers in this study were designed using the NCBI primer designing tool, with exon sequences to target genes freely available on the National Centre for Biotechnology Information database (NCBI) (see Table 2.14). All primers were checked for the absence of 3' self-complementarity, hairpin formation, self-annealing sites and non-specific binding regions with OligoCalc tool, *Homo sapiens* nucleotide BLAST, align sequences nucleotide BLAST and EMBOSS pairwise nucleotide sequence alignment. Lyophilized DNA-oligos were purchased from ThermoFisher Scientific, and were re-constituted in to a master stock of 100µM in 1xTE buffer; small 10µM aliquots were prepared and stored at -20°C.

Target	DNA sequence Accession number (NCBI)
Tenascin-C (full length molecule excluding domains FnIII AD2 and AD1)	X78565.1
Tenascin-C FnIII-AD2	U88892.1
Tenascin-C FnIII-AD1	EU295718.1
GAPDH	BC023632.2
RPLP0	BC107717.1
HPRT1	BC000578.2
GNB2L1	BC017287.2
YWHAZ	BC073141.1
RPL13A	BC015605.1
ACTB	BC113036.1

Table 2.14. The table lists the DNA exon sequence accession numbers against which primers in this study were designed

2.8.2 *Polymerase chain reaction (PCR)*

PCR was performed on cDNA samples using PuReTaqTM Ready-to-go-PCR beads (GE Healthcare) containing 1 U *Taq* DNA polymerase, according to the manufacturer's instructions; using the reagents listed in Table 2.15. For every PCR run, a no template control (NTC) was included. PCR was performed using the Biometra PCR thermocycler.

Reagent	Volume (1µL)
cDNA template	1
Forward primer (10µM)	1
Reverse primer (10µM)	1
dH ₂ O	22
PuReTaq TM Ready-to-go-PCR bead	1 bead

Table 2.15. Reagents that were required to perform PCR for agarose gel electrophoresis in this study

2.8.3 *Agarose gel electrophoresis*

Agarose gels comprised of 0.8% (w/v) TopVision agarose (ThermoFisher Scientific), 1x TAE buffer, 1:10,000 GelRed (VWR). Gels were inserted into gel tanks (BioRad) and submerged in 1xTAE buffer. 10µl amplified PCR products were mixed with 6x DNA loading buffer and were loaded alongside 6µl MassRulerTM DNA Ladder Mix (#SM0403; ThermoFisher Scientific) on 0.8% (w/v) TopVision agarose gels. DNA bands were resolved by electrophoresis at 135 volts for a length of time dependent upon agarose gel concentration, PCR product size, and gel length. PCR products were visualised on the G:BOX Chemi XRG gel doc instrument (Syngene).

2.8.4 T8F/T18R PCR

To replicate the PCR methods adopted by Garwood *et al.*, 2012⁷², to amplify all mRNA isoforms, PCR was performed with published primers annealing within exons 8 and 18 of tenascin-C (T8F/T18R), that span the entire alternatively spliced exons of tenascin-C. Primers annealed to constitutively expressed exons 20 and 23 (T20F/T23R) amplified FnIII 7-8, confirming the presence of tenascin-C mRNA transcripts. GAPDH primers (GAPDHf/GAPDHr) served as a positive control for the PCR reaction. PCR used the primer sets T8F/T18R, T20F/T23R and GAPDHf/GAPDHr shown in Table 2.16, to amplify template DNA from the purified tenascin-C plasmid pBS KS II +AD2 +AD1 (100ng), or from cDNA from U-87 MG, MDDC, HDF, NSF, RA-SF or OA-SF cells, using the PCR methods previously described in section 2.8.2.

FnIII domain name	Primer Name	Primer Sequence 5'-3'	Tm (°C)	Primer length (bp)	% GC content	Product size (bp)
4	T8F	5'-CAATCCAGCGACCATCAACG-3'	60.5	20	55	443-2903
6	T18R	5'-CGTCCACAGTTACCATGGAG-3'	60.5	20	55	
7	T20F	5'-CGGGAACACAGTGGAGTATG-3'	57.43	20	55	328
8	T23R	5'-GATCTTGGCTGTGTAGTGGG-3'	56.59	20	55	
GAPDH	GAPDHf	5'-AGAACATCATCCCTGCCTC-3'	55.95	19	53	347
	GAPDHr	5'-GCCAAATTCGTTGTCATACC-3'	56.44	20	45	

Table 2.16. Table showing sequences of primer pairs T8F/T18R, T20F/T23R, GAPDHf/GAPDHr.

All of the primer sets were amplified using the same PCR thermocycler conditions, over 40 cycles of PCR, as described in Table 2.17.

- PCR Thermocycle Step	T8F/T18R, T20F/T20R, GAPDHf/GAPDHr primer pairs	
	Temp (°C)	Time (secs)
1.Initial Denaturation	95	90
2.Denaturation	95	45
3.Annealing	58	60
4.Extension/elongation	72	240
5.Termination	72	600
N° of PCR cycles (steps 2-4)	x40	

Table 2.17. PCR thermocycler conditions for amplifying template DNA using T8F/T18R, T20F/T23R and GAPDHf/GAPDHr primer sets

PCR product DNA amplicons were resolved by agarose gel electrophoresis, using the electrophoresis method described in 2.8.3. DNA bands were resolved with 100 V for ~2.5 hours, and were visualised with UV using the G:BOX Chemi XRG gel doc instrument (Syngene).

2.8.5 *Absolute quantification of alternatively spliced FnIII mRNAs by standard curve qPCR*

2.8.5.1 *SYBR Green compatible primer sequences*

Primer sequences were designed to anneal within exons corresponding to tenascin-C FnIII A1, A2, A3, A4, B, AD2, AD1, C, D, Fn7-8, and to control genes GAPDH, RPLP0, HPRT1, GNB2L1 and YWHAZ; using the NCBI exon sequences as previously described in 2.8. Amplicons for all primers were between 70-132bp in length and were compatible with SYBR Green qPCR methods (Table 2.18).

FnIII domain name	Primer Name	Primer Sequence 5'-3'	Tm (°C)	Amplicon size (bp)	Primer Efficiency (%)
A1	SYBR-A1F	GCCGAGAAAGGCAGACACAAG	61.8	92	92.58
	SYBR-A1R	ACGGTGAGGTTTTCCAGCTCAG	62.76		
A2	SYBR-A2F	CCCAGAACCTCACCGTCCCA	63.66	105	91.10
	SYBR-A2R	TGAAGTCCTGAGTGACCCCGC	64.09		
A3	SYBR-A3F	GAGGCTCACAATCTCACGGT	59.75	80	87.08
	SYBR-A3R	ACTGTGTAAGGAGTGCCAGC	59.96		
A4	SYBR-A4F	ACAGCTGGGAGATTTAGCCG	61.65	108	90.86
	SYBR-A4R	ACCTCCTGCACCTGAATGAC	60.12		
B	SYBR-BF	TGCTGAACGAACTGCCATA	59.68	96	86.67
	SYBR-BR	ATGGTTTTGGTCCGGATGCT	59.96		
AD2	SYBR-AD2F	GTCCATCTCGTGGGAAGCTC	60.18	85	88.29
	SYBR-AD2R	CATGGTCTCATGGGGGTGAT	59.15		
AD1	SYBR-AD1F	CACAGTTGGGCACGCTAATC	59.55	73	93.45
	SYBR-AD1R	CCAGCTTGAGTGGTCCATGA	59.67		
C	SYBR-CF	AGTTTCCTGGATGGCATCGG	60.11	72	85.51
	SYBR-CR	AGCAGCTTCCCAGAATCCAC	60.03		
D	SYBR-DF	AGCTGATGAAGGGGTCTTCG	59.46	94	87.54
	SYBR-DR	TTCGGGGGCAAGTAGGGTTA	60.55		
7-8	SYBR-Fn7,8F	CCCCAGAAGAGCTCAACCAT	59.38	99	94.33
	SYBR-Fn7,8R	AAGGAGGGCAGTTTCCGACT	61.13		
RPLP0	SYBR RPLP0-F	GCGTCCTCGTGGAAGTGAC	60.73	102	92.43
	SYBR RPLP0-R	GTAGTTGGACTTCCAGGTCGC	60.67		
HPRT1	HPRT1-F 1	GGCGAACCTCTCGGCTTTCC	63.46	132	93.21
	HPRT1-R 1	TGCGGGTCGCCATAACGGA	64.24		
GNB2L1	GNB2L1-F	CTCTGGGATCTCACAACGGG	59.82	84	91.68

FnIII domain name	Primer Name	Primer Sequence 5'-3'	T_m (°C)	Amplicon size (bp)	Primer Efficiency (%)
	GNB2L1-R	GTCAGAGGAGAAGGCCACAC	60.04		
YWHAZ	YWHAZ-F1	CGAGCAGGCTGAGCGATATG	60.20	139	92.67
	YWHAZ-R1	CCAAGATGACCTACGGGCTC	59.70		

Table 2.18. Sequences of primers designed for the quantitative analysis of alternatively spliced FnIII mRNAs by standard curve qPCR with SYBR Green

2.8.5.2 Optimising the primers for SYBR Green qPCR

The efficiency and specificity of the primers used to anneal to alternatively spliced and constitutive tenascin-C FnIII domains was performed on 8, 4-fold serial dilutions of purified tenascin-C plasmid pBS KS II hTNC+AD2+AD1 from a top concentration of 10^5 copies/ μ l. For endogenous control genes, primer efficiency and specificity were validated on 8, 4-fold serial dilutions of I.M.A.G.E fully sequenced cDNA clone plasmids (SourceBioScience) (listed in Table 2.7) from a top concentration of 10^5 copies/ μ l. qPCR optimisation of the primers was also performed on 1:4 serially diluted U-87 MG cDNA, to ensure efficient amplification of cDNA from cell samples. To ensure the levels of cDNA template in the cell sample lay within the dynamic range of the qPCR assay, samples of cDNA from all cell types were run alongside the serial plasmid and pooled cDNA dilutions. NTCs were included in every PCR reaction.

2.8.5.3 SYBR Green qPCR

The SYBR Green standard curve qPCR assay master mix was prepared according to the manufacturer's instructions, for 10 μ l reaction volumes. 7 μ l of the qPCR reaction mixture was prepared per well; containing 5 μ l PowerUp™ SYBR® Green Master Mix (2x) (Invitrogen), 1 μ l of forward (10 μ M) and 1 μ l of reverse primers (10 μ M). Each sample was quantified in duplicate, and 7 μ l of the mix was pipetted into wells of a 384-well plate. To each well, 3 μ l of

neat cDNA (MDDC, GM-CSF macrophage, M-CSF macrophage, CD4⁺ and CD8⁺ T-cell), or 1:10 diluted cDNA (HDF, RA-SF, OA-SF, NSF) was added in duplicate. Standard curves were generated by adding 3µl of the purified tenascin-C plasmid pBS KS II hTNC+AD2+AD1, or purified I.M.A.G.E fully sequenced cDNA clone plasmids at 10-fold serial dilutions from 10⁶-10⁻¹ copies/µl (as described in Table 2.19).

Reagent	Volume (µl)
PowerUp™ SYBR® Green Master Mix (2x)	5
Forward primers (10µM)	1
Reverse primers (10µM)	1
cDNA sample OR plasmid standard	3

Table 2.19. Reagents required to prepare a 10µl reaction well for SYBR Green qPCR.

The qPCR was performed on a ViiA™ instrument (ABI) using the manufacturer recommended standard cycling mode thermocycle conditions, with ROX selected as a passive reference. Melt curves were always performed immediately following the PCR (Table 2.20). The absence of non-specific PCR products and presence of amplicons of the predicted size was confirmed by resolving PCR products by agarose gel electrophoresis as previously described in section 2.8.3.

SYBR Green qPCR thermocycler step	Temperature (°C)	Time
1. UDG activation	50	2 minutes
2. Dual-Lock™ DNA polymerase	95	2 minutes
3. Denature	95	15 seconds
4. Anneal/Extend	60	1 minute
Number of qPCR cycles (steps 3-4)	x40	-
5. Melt curve step 1: Ramp rate 1.6°C/second	95	15 seconds
6. Melt curve step 2: Ramp rate 1.6°C/second	60	1 minute
7. Melt curve step 3: Ramp rate 0.15°C/second	95	15 seconds

Table 2.20. Thermocycle conditions used for SYBR Green® qPCR assay

2.8.5.4 Absolute and relative quantification of FnIII mRNA copies/cell

The absolute number of copies/cell was calculated for the alternatively spliced and constitutively expressed FnIII, and for the control genes using the method previously described in section 2.6.7.1. The relative abundance of FnIII relative to control genes was calculated by comparing the absolute copies/cell of the FnIII gene of interest with the normaliser gene as previously described in section 2.6.7.2.

2.9 Cloning the FnIII AD2AD1, BCD and BAD2AD1CD domains of tenascin-C

2.9.1 Designing cloning primers

Primers were manually designed to the exon boundaries of 5'-B, 3'-D, 5'-AD2, and 3'-AD1, of the alternatively spliced tenascin-C FnIII domains; so that tenascin-C domains FnIII AD2-AD1 (AD2AD1), FnIII B-C-D (BCD) and FnIII B-AD2-AD1-C-D (BAD2AD1CD) could be cloned. The tenascin-C and tenascin-C FnIII-AD2 and AD1 exon sequences against which the primers were designed were obtained from NCBI (tenascin-C; NM_002160) and are shown in Table 2.14. Primers were designed to include a 6xHis N-terminal-tag, and contained palindromic DNA restriction enzyme sites in the forward primer for *NdeI*, and in the reverse primer for *NotI*. The 5'-*NdeI* site (CATATG) contained a start codon, followed by a 6xHis-tag, while the 3'-*NotI* site (GCGGCCGC) was preceded by a TTA stop codon. The 5' end of all forward and reverse primers were stabilised by a CCG codon (Table 2.21).

The restriction sites in the tenascin-C exon were mapped with RestrictionMapper version 3 software. All primers were checked for the absence of 3' self-complementarity, hairpin formation, self-annealing sites and non-specific binding regions with OligoCalc tool. Lyophilized DNA-oligos were purchased from ThermoFisher Scientific, and were re-constituted in to a master stock of 100µM in 1xTE buffer; small 10µM aliquots were prepared and stored at -20°C.

FnIII domain	Uniprot Accession number	Primer Name	Sequence (5' to 3')	Amplicon size (bp)
B	X78565.1	C-B.F	GGC CAT ATG <u>CAT CAT CAT</u> <u>CAT CAT CAT</u> GAA ATT GGA AAC TTA AAT GTT TCT G	1077 (BCD) 1356 (BAD2AD1CD)
D	X78565.1	C-D.R	GGC GCG GCC GCT <u>TAG</u> CCC ATG GCT GTT GTT GCT ATA G	
AD2	U88892.1	C-AD2.F	GGC CAT ATG <u>CAT CAT CAT CAT</u> <u>CAT CAT</u> GTA GCC AAA CCT CTT CTT AGC CAC	546 (AD2AD1)
AD1	EU295718.1	C.AD1.R	GGC GCG GCC GCT <u>TAT</u> GTA ATG ACA AAG GCA GTG AGG G	

Table 2.21. Details of the recombinant domains to be cloned, and their corresponding cloning primers. Each forward primer sequence contains a 6xHis-tag (underlined), and a restriction site in the forward primers for *NdeI* (bold), and in the reverse primers for *NotI* (bold).

2.9.2 *High fidelity Phusion® touchdown PCR*

High fidelity Phusion® touchdown PCR was performed using 400nM of each primer, 1µg of cDNA template, 200µM dNTPs, 1x Phusion HF buffer and 0.02units/µl of Phusion Hot Start II DNA Polymerase in a final volume of 50µl. Two tenascin-C cDNA templates were used, and were gifted from Dr Gertraud Orend. The first was pBS KS II hTNC which contained full length tenascin-C without FnIII-AD2 or -AD1; the second was pBS KS II hTNC+AD2+AD1 which contained full length tenascin-C with FnIII-AD2 and -AD1 situated between FnIII-B and -C. The touchdown PCR was performed as described in Table 2.22, using a Biometra PCR thermocycler instrument.

Step	Temperature	Time	Cycles
Denaturation	94°C	10 min	1
1	94°C	1 min	3
	71°C		
	72°C		
2	94°C	1 min	6
	69°C		
	72°C		
3	94°C	1min	20
	67°C		
	72°C		
4	94°C	1min	4
	65°C		
	72°C		
5	94°C	1min	4
	63°C		
	72°C		
Elongation	72°C	10 min	1

Table 2.22. Details of the touchdown PCR protocol for cloning primers

2.9.3 *DNA extraction from agarose gel*

20µl of cloning PCR products were separated by DNA gel electrophoresis using the protocol previously described in section 2.8.3. The correct size of the DNA bands was confirmed following visualisation with the G:BOX Chemi XRG gel doc instrument, and bands were excised from the agarose gel with a scalpel, following a short 2-second UV lamp exposure.

Cloning PCR products were purified from the agarose gel using the GeneJET Gel extraction kit (QIAGEN), and were eluted in 20µl volumes of elution buffer. DNA concentration was assessed with the nanodrop ND-1000 instrument as previously described in section 2.6.3.

2.9.4 *TOPO cloning*

5µl of PCR products obtained in section 2.9.3, were ligated into TOPO pCRBlunt vector (ThermoFisher Scientific) following the protocol described in the Zero Blunt TOPO PCR cloning kit (ThermoFisher Scientific). Inserts were ligated over 10 minutes at RT, and samples were placed ice in preparation for TOP10 chemical transformation.

TOP10 Competent OneShot® *E.coli* were thawed on ice prior to transformation. 45µl of bacteria were mixed with 5µl aliquots of TOPO cloning reactions for 10 minutes, and were then heat shocked, by immersing the tubes into a 42°C waterbath for 30 seconds, before being immediately transferred to ice for 2 minutes. Pre-warmed SOC media was added to the cells and they were incubated at 37°C for 1 hour with shaking at 220rpm.

Transformed bacteria were plated on selective 50µg/ml kanamycin LB agar plates at 37°C overnight. Individual colonies were inoculated into 3ml LB media with 50µg/ml kanamycin and cultured overnight to scale up the TOPO plasmid. 2.5ml of the bacterial suspension were pelleted and the TOPO plasmids containing inserts were purified using the MiniPrep kit (QIAGEN), according to the manufacturer's instructions. The remaining 0.5ml of bacterial suspension was used to prepare 50% glycerol bacteria stocks, which were stored at -80c.

All TOPO plasmid clones were sequenced using the M13F/R primers included in Zero Blunt TOPO PCR cloning kit, as described in section 2.9.8. DNA sequencing confirmed the identity and sequence integrity of the insert and flanking regions, ensuring no errors had been introduced during the cloning stages.

2.9.5 ***Restriction enzyme digestion***

Reactions comprised 1µl of *NdeI* and *NotI* restriction enzymes (NEB), 1µg of DNA in 5µl volume, 4µl 10x CutSmart 3.1 digestion buffer (NEB) and 29µl water, to a final volume of 40µl. Reactions were incubated at the required temperature overnight.

Digested products were resolved by 1.5% (w/v) agarose gel electrophoresis as previously described in section 2.8.3, and inserts were extracted from the gel using the GeneJET Gel extraction kit as described in section 2.9.3.

2.9.6 ***DNA ligation and pET32b cloning***

Inserts were ligated into pET32b vector (Millipore) linearized with *NdeI* and *NotI* restriction enzymes as described in 2.9.5. Insert and vector were ligated at 10:1 molar ratios that were calculated as follows,

$$ng\ of\ insert\ DNA = \left(\frac{(ng\ vector) \times (insert\ size\ Kb)}{vector\ size\ Kb} \right) \times molar\ ratio\ insert\ to\ vector$$

The ligation used 100ng of vector, Xng insert, 2µl 10x ligation buffer (Fermentas), 10mM ATP, 1 U T4 DNA ligase and water up to 20µl volume. The reaction was incubated overnight at RT, and then at 65°C for 10 minutes to heat-inactivate the T4 ligase enzyme.

2.9.7 ***Transformation of chemically competent BL21 (DE3) pLysS E.coli***

45µl *E.coli* BL21 (DE3) pLysS were thawed on ice, and mixed with 5µl of the ligation reactions from sections 2.9.6 for 10 minutes. Cells were heat shocked at 42°C for 30 seconds, then immediately transferred to ice for 2 minutes. Pre-warmed SOC media was added to the cells and they were incubated at 37°C for 1 hour with shaking at 220rpm.

Bacteria were plated on selective carbenicillin LB agarose plates with at 37°C overnight, to select transformed colonies. Single transformed colonies were grown overnight in LB media

+ 100µg/ml carbenicillin at 37°C, and the plasmids were purified using the MiniPrep plasmid purification kit according to the manufacturer's instructions. The identity of the pET32b plasmid inserts was verified by DNA sequencing. Glycerol stocks were made of single bacterial colonies containing the pET32b vector and insert for recombinant protein expression; and were stored at -80°C.

2.9.8 *DNA sequencing*

DNA sequencing was performed by Eurofins. Sequencing required 25ng template/kb. 1µl of forward, or reverse primers (10µM) were added, and the reaction was topped up to 17µl total volume with sterile water. DNA sequencing data was analysed using Chromas software to confirm the presence of a single emission peak; and then DNA sequences were analysed using DNADynamo software to compare against published DNA sequences in order to verify 100% sequence identity to the target sequence.

2.10 Expression of recombinant FnIII proteins in *E.coli*

2.10.1 *Proteins used in cell-based assays*

In this study, some protein had previously been cloned, expressed or purified and were made available to this study by the Midwood lab. Tenascin-C was purified from tenascin-C overexpressing HEK cells, and FnIII 3-5 was cloned, expressed and purified as previously described in ³²⁰. Fibronectin was purified from human plasma as previously described in ³¹. The FnIII 5-7 protein had previously been cloned by the Midwood Lab³²⁰, and in this study was purified by myself using the methods later described. The proteins AD2AD1, BCD and BAD2AD1CD were cloned and purified by myself, for this study; using protocols that had previously been published by the Midwood lab⁴³.

2.10.2 *Small scale expression in E.coli*

Starter cultures of BL21 (DE3) pLysS *E.coli* containing the pET32b vectors including FnIII5-7, AD2AD1, BCD or BAD2AD1CD inserts, were prepared in 2 mL of LB media with 1% glucose and 100 µg/mL carbenicillin, and grown overnight at 37 °C, 220 rpm. Cells were centrifuged for 10 min, 3000 rpm and were re-suspended in 1 mL of fresh LB media. This was used to inoculate 20 mL of LB + 100 µg/mL carbenicillin. Bacterial cultures were incubated at 37 °C, 220 rpm until OD₆₀₀ between 0.6-0.8 was reached. IPTG concentration, and protein expression temperature and expression time were all titrated in order to promote the expression of soluble recombinant proteins. The titrations resulted in the following conditions being used for all expressions; 1mM of IPTG was added to induce protein expression for 3h at 37 °C, 220 rpm. A sample of 100 µL was taken after 3 hours to monitor protein expression.

The bacterial suspension was centrifuged 15 min, 3000 rpm, and the pellet was washed in TBS. Pellets were lysed by sonication using a Bioruptor water bath (Diagenode) for 4 cycles (30 sec on; 30 sec off). Bacterial solution was centrifuged at 13000 rpm for 5 min at 4°C. Supernatant containing solubilised proteins was collected and the pellet containing insoluble inclusion bodies was resuspended in 1 mL of TBS buffer. 10 µL of each sample with 6x SDS loading buffer were boiled and separated on 12% (w/v) SDS-PAGE gels as described in section 2.11. Gels were stained with coomassie blue stain as described in section 2.11.2.

2.10.3 *Large scale expression*

Starter cultures for *E.coli* BL21 (DE3) pLysS cells containing the pET32b vectors including FnIII 5-7, AD2AD1, BCD or BAD2AD1CD inserts were prepared as previously described in section 2.10.2. This was used to inoculate 1 L of LB + 100 µg/mL carbenicillin. The culture was incubated at 37 °C, 220 rpm until bacterial growth reached an OD₆₀₀ between 0.6-0.8. 1mM of IPTG was added to induce protein expression for 3h at 37 °C, 220 rpm.

Cell suspensions were centrifuged for 15 min, 3500 rpm and pellets were washed in TBS. The pellet was re-suspended in 40ml TBS, and cells were sonicated for 4 minutes, at 40% amplitude, for cycles of 30 secs on; 30 secs off (Cole-Parmer). The solution was centrifuged 10,000 rpm for 15 min at 4°C. For bacteria expressing FnIII5-7, BCD and BAD2AD1CD, the supernatants were collected. For bacteria expressing AD2AD1, the supernatant was discarded and the pellet was stored at -20°C until protein solubilisation.

2.10.4 *Protein solubilisation*

For the purification of AD2AD1, bacterial inclusion bodies were resuspended in 40ml 8M urea buffer + 5mM β-mercaptoethanol. The solution was left overnight on a rotator at 4°C.

2.10.5 ***Protein purification by Ni²⁺-NTA chromatography under denaturing conditions***

5 mL of Ni²⁺-NTA beads were placed in a Poly-Prep chromatography column (Bio-Rad). Beads were washed with 5 column volumes of ultrapure water and equilibrated with 5 column volumes of 8M urea buffer. 40 mL of solubilised AD2DA1 protein was loaded. Columns were washed with 5 column volumes of urea buffer, followed by 20 column volumes of 0.1 % triton-X114 in urea buffer, and 20 column volumes of urea buffer. AD2AD1 was eluted with 300 mM imidazole in urea buffer, and 5 fractions of 5 mL were collected.

2.10.6 ***Protein re-folding***

Fractions of AD2AD1 enriched in section 2.10.5, were pooled and the concentration was assessed at OD₂₈₀ by Nanodrop ND-1000. Protein was diluted to <100µg/ml in urea buffer + 20mM cystamine to prevent precipitation of protein during dialysis. The diluted protein was dialysed for 4 days, and dialysis buffers were changed daily.

Day 1 and 2: Dialysed 24 hours against TN buffer + 5mM β-ME, 1mM 2-hydroxyethylthiodylsulfide at 4 °C on a rotator.

Day 3 and 4: Dialysed 24 hours against TN buffer at 4 °C on a rotator.

The refolded protein solution was filtered through a pyrogen-free 0.45µm Corning® vacuum filter (CN membrane), to remove precipitate. Once AD2AD1 was refolded, it could be purified by Ni²⁺-NTA chromatography under native conditions.

2.10.7 ***Protein purification by Ni²⁺-NTA chromatography under native conditions***

1.5 mL of Ni²⁺ beads (Bio-Rad) were placed in a Poly-Prep chromatography column (Bio-Rad). Beads were washed with 5 column volumes of ultrapure water and were equilibrated

with 5 column volumes of TN buffer. Supernatant protein fractions FnIII5-7, BCD, BAD2AD1CD), and refolded AD2AD1 were loaded onto the column. Columns were washed with 7.5ml TN buffer followed by 750ml 0.1 % triton-X114 in TN buffer and 450ml TN buffer. 25mM imidazole (in TN buffer) was added to remove unspecific bound proteins. Proteins were eluted with 300mM imidazole in TN buffer, and 8 fractions of 1 mL were collected in total. Fractions were dialyzed against TN buffer using Float-A-Lyzer (SIGMA-ALDRICH) tubes, to remove imidazole. The concentration of the protein was measured at OD₂₈₀ by Nanodrop ND-1000.

FnIII5-7, BCD and BAD2AD1CD proteins underwent 2 successive rounds of native Ni²⁺-NTA chromatography.

2.11 Characterisation of purified proteins

2.11.1 *Tris-glycine SDS-PAGE*

Protein samples were prepared and separated by SDS-PAGE on 12% (w/v) gels as previously described for 5% (w/v) gels in section 2.7.5.1. 12% (w/v) polyacrylamide gels were prepared in-house, as described in Table 2.23.

Solution	12% separating gel (ml)	3.9% stacking gel (ml)
30% Bis-acrylamide solution	3	0.32
4x Tris-HCl (pH6.8/8.8)	1.87 (pH6.8)	0.62 (pH8.8)
H ₂ O	3.62	1.505
10% APS	0.02	0.0125
TEMED	0.005	0.001

Table 2.23. Reagents required for producing 12% SDS PAGE gels to resolve tenascin-C protein.

2.11.2 *Coomassie staining*

SDS-PAGE gels were stained with 40ml coomassie blue solution for 30 min at RT. Gels were rinsed and de-stained with coomassie destainer solution for 16 hours at RT or until protein bands were clearly defined above the background.

2.11.3 *Silver staining*

1µg of purified protein was separated by SDS-PAGE as described in section 2.11 and samples were fixed with 50% methanol, 5% acetic acid solution. Gels were stained with 0.1 % silver nitrate solution and bands were developed using 0.04% formalin and 2 % sodium carbonate solution. The reaction was stopped using 5 % acetic acid, once bands were visualised.

2.11.4 *Western blotting*

Proteins purified in section 2.10.7, were separated by 12% SDS-PAGE, and were then electro transferred and stained with the tetra-His primary antibody, and the anti-mouse-HRP secondary antibody as previously described in 2.7.5.2. Protein bands were visualized using ECL plus (Amersham) on X-ray film (Fuji Film)

2.11.5 *Circular dichroism (CD) spectra*

200µg/ml samples of purified proteins from section 2.10.7, were analysed using the J-815 Circular Dichroism Spectrometer (Jasco). The CD spectrum was measured in the “far UV” spectral region (190-250 nm) in samples at 20°C or following boiling at 90°C for 10 minutes. Five repeated measures were taken and the mean averages were plotted and analysed using the Spectra Manager™ II software and Microsoft Excel.

2.11.6 *Endotoxin test*

The level of endotoxin contamination was measured in duplicate, in recombinant protein samples that were diluted to 1µM. The assay was performed using the Endpoint Chromogenic LAL Assay (Lonza) according to the manufacturer’s instructions. The endotoxin units (EU)/ml were converted to the equivalent concentration in LPS using the rate of 1 EU/ml $\approx$ 0.1ng/ml LPS (ThermoFisher Scientific).

2.12 Cell based assays with recombinant proteins

2.12.1 Cytokine expression assays

2.12.1.1 M-CSF macrophage stimulations

Differentiated M-CSF macrophages were plated at 5×10^5 cells/ml in 200 μ l RPMI-1640 5% FBS, 1% P/S in a 96-well plate. Cells were rested for 16 hours prior to stimulation with 1ng/ml LPS, 1 μ M FBG-C, 1 μ M FnIII5-7 or 1, 0.5, 0.25, 0.1, 0.01 μ M AD2AD1, BCD and BAD2AD1CD proteins. Controls included boiling stimuli at 90°C for 30 minutes; pre-incubating stimuli with 1 μ g/ml polymyxin-B (PMB) (ThermoFisher Scientific) for 30 minutes; or pre-incubating stimuli with 3 μ M TAK242 (Millipore) or 0.2% DMSO for 6 hours. Proteinase-K (PK; SIGMA-ALDRICH) digestion controls were included for protein stimuli; a 1 μ g protein stimulus was incubated with 1 U PK at 37°C for 1 hour, according to manufacturer's instructions. Total SN was collected 24 hours following stimulation. Cell viability was assessed by performing an MTT assay, as previously in section 2.5.

2.12.1.2 TNF α , IL-6 and IL-8 quantification by ELISA

High-binding Nunc-Immuno 96-well plates (SIGMA-ALDRICH) were coated with capture antibodies for IL-6 (1 μ g/ml), IL-8 (2 μ g/ml), or TNF α (4 μ g/ml), at 4°C for 16 hours. Plates were washed in PBST and blocked in 2% BSA/PBS for 1 hour at RT. Plates were washed in PBST and samples/standards were diluted in 0.5% BSA/PBS.

Standards were a 1:3 serial dilution of recombinant human IL-6, IL-8 and TNF α , from 10 - 0.01ng/ml. SN samples were diluted 1:10 for IL-6, 1:400 for IL-8 and 1:2 for TNF α ELISAs. Samples and standards were incubated on plate for 2 hours at RT. Biotinylated mAbs to IL-6/IL-8 or TNF α were added at 0.5 μ g/ml and incubated for 1 hour at RT. Wells were washed with PBST and incubated with streptavidin-HRP antibody diluted 1:400 for 1 hour at RT. The plate was washed in PBST and the assay was developed with TMB/H₂O₂ for 10-20

minutes. The colorimetric reaction was stopped with 6% sulfuric acid. OD_{450nm} was read on the FluoStar Omega plate reader, and data was analysed using the Omega software.

2.12.2 *Equal coating with recombinant proteins*

96-well tissue culture (TC) plates were coated with 50µl of 5µg/ml and 0.2µM concentrations of tenascin-C, FnIII 3-5, FnIII 5-7, AD2AD1, BCD or BAD2AD1CD at 4°C for 16 hours, in triplicate. Protein solutions were then aspirated, and the wells were immediately blocked with 10% BSA/PBS, for 2 hours at RT, and washed 3 times with PBST. His-tagged proteins bound to the plate were detected by staining with a tetra-His antibody (Qiagen; 1:1,000) in 2% BSA PBST for 1 hours at RT. Wells were washed with PBST and were stained with secondary antibody anti-mouse IgG HRP in 2% BSA/PBS, for 1 hour at RT. Wells were washed 3 times in PBST, and the plate was developed by incubation with TMB/H₂O₂ for 10-20 minutes. The reaction was stopped with 6% sulfuric acid. OD_{450nm} was read on the FluoStar Omega plate reader, and data was analysed using the Omega software.

2.12.3 *Cell adhesion assay*

2.12.3.1 *Optimisation of cell plating density, blocking conditions and adhesion time for HDFs and MDDCs*

HDF and MDDC plating density, BSA blocking conditions and adhesion times were optimised on 1µM fibronectin coatings, and measured using the crystal violet staining methods described in 2.12.3.4.

Cell plating density: 100µl HDF cell suspension was added at 8, 4 and 1x10⁵ cells/ml in serum free media; and 100µl MDDC suspension was added at 5, 2, 1 and 0.5 x10⁶ cells/ml in serum free media.

Blocking conditions: 96-well TC plates were coated with 50µl 1µM fibronectin 4°C for 16 hours. Wells were blocked with either 0%, 1%, 5% or 10% BSA/PBS at 37°C for 2 hours.

Adhesion times: Cells adhered to the plates at 37°C/5% CO₂ for 30, 60 or 120 minutes.

2.12.3.2 Protein coating protocol for adhesion assay

96-well flat bottom TC plates were coated with 50µl volumes of protein diluted in PBS at 4°C for 16 hours, at the concentrations listed in

Table 2.24.

Protein coating	HDFs	MDDCs
Fibronectin	1, 0.5, 0.2, 10 ⁻² , 10 ⁻³ , 10 ⁻⁴ , 10 ⁻⁵ , 10 ⁻⁶ , 10 ⁻⁷ , 10 ⁻⁸ , 10 ⁻⁹ µM	10 ⁻² , 10 ⁻³ , 10 ⁻⁴ , 10 ⁻⁵ , 10 ⁻⁶ , 10 ⁻⁷ µM
Tenascin-C	5, 1, 0.5, 0.2, 10 ⁻² , 10 ⁻³ , 10 ⁻⁴ , 10 ⁻⁵ , 10 ⁻⁶ , 10 ⁻⁷ , 10 ⁻⁸ µM	1, 0.2, 10 ⁻² , 10 ⁻³ , 10 ⁻⁴ , 10 ⁻⁵ , 10 ⁻⁶ , 10 ⁻⁷ µM
FnIII 3-5	5, 1, 0.5, 0.2, 10 ⁻² , 10 ⁻³ , 10 ⁻⁴ , 10 ⁻⁵ , 10 ⁻⁶ , 10 ⁻⁷ , 10 ⁻⁸ µM	1, 0.2, 10 ⁻² , 10 ⁻³ , 10 ⁻⁴ , 10 ⁻⁵ , 10 ⁻⁶ , 10 ⁻⁷ µM
FnIII 5-7	5, 1, 0.5, 0.2, 10 ⁻² , 10 ⁻³ , 10 ⁻⁴ , 10 ⁻⁵ , 10 ⁻⁶ , 10 ⁻⁷ , 10 ⁻⁸ µM	5, 1, 0.2, 10 ⁻² , 10 ⁻³ , 10 ⁻⁴ , 10 ⁻⁵ , 10 ⁻⁶ , 10 ⁻⁷ µM
AD2AD1	5, 1, 0.5, 0.2, 10 ⁻² , 10 ⁻³ , 10 ⁻⁴ , 10 ⁻⁵ , 10 ⁻⁶ , 10 ⁻⁷ , 10 ⁻⁸ µM	5, 1, 0.2, 10 ⁻² , 10 ⁻³ , 10 ⁻⁴ , 10 ⁻⁵ , 10 ⁻⁶ , 10 ⁻⁷ µM
BCD	5, 1, 0.5, 0.2, 10 ⁻² , 10 ⁻³ , 10 ⁻⁴ , 10 ⁻⁵ , 10 ⁻⁶ , 10 ⁻⁷ , 10 ⁻⁸ µM	5, 1, 0.2, 10 ⁻² , 10 ⁻³ , 10 ⁻⁴ , 10 ⁻⁵ , 10 ⁻⁶ , 10 ⁻⁷ µM
BAD2AD1CD	5, 1, 0.5, 0.2, 10 ⁻² , 10 ⁻³ , 10 ⁻⁴ , 10 ⁻⁵ , 10 ⁻⁶ , 10 ⁻⁷ , 10 ⁻⁸ µM	5, 1, 0.2, 10 ⁻² , 10 ⁻³ , 10 ⁻⁴ , 10 ⁻⁵ , 10 ⁻⁶ , 10 ⁻⁷ µM
BSA	10% (w/v)	10% (w/v)

Table 2.24. Concentration of coating proteins (µM) for HDF and MDDC cell adhesion assays.

For GM-CSF and M-CSF macrophages, the plates were coated with 0.01µM protein concentrations. After coating, the wells were washed twice in PBS, and blocked with 10% BSA/PBS for 2 hours at RT. The wells were washed with PBS, and 100µl of serum free media was added to each well, prior to the addition of cells.

2.12.3.3 *Crystal violet cell adhesion assays in HDFs, MDDCs, GM-CSF and M-CSF*

macrophages

HDFs were harvested from cell culture. MDDCs, GM-CSF and M-CSF macrophages were harvested following differentiation as previously described; and were washed in serum free media twice. HDFs were resuspended at 4×10^5 cells/ml, and MDDCs, GM-CSF and M-CSF macrophages were resuspended at 1×10^6 cells/ml in serum free media. 100µl of cell suspensions were added to the 100µl of serum free media already present in each well, and cells were allowed to adhere at 37°C/5% CO₂ for 60 minutes.

2.12.3.4 *Crystal violet staining protocol*

Following adhesion of the cells, the 96-well plate wells were gently washed with PBS. Cells were fixed in 4% PFA for 20 minutes at RT, and were stained with 0.1% crystal violet solution for 60 minutes. Excess dye was removed with MES buffer, and the stained cells were lysed in 100µl 10% SDS for 5 minutes. Solubilised dye was measured at OD_{590nm} using the FluoStar Omega plate reader; data was analysed using the Omega software. Photographs were taken using the Nikon Eclipse Ts2R light microscope with a 20x, and a 40x objective lens.

2.12.4 ***Morphological assessment and immunofluorescence microscopy***

Lab-Tek® 8-well chamber slides (ThermoFisher Scientific) were coated with 150µl, 0.01µM fibronectin, tenascin-C, FnIII 3-5, FnIII 5-7, AD2AD1, BCD, BAD2AD1CD and 10% BSA at 4°C for 16 hours. Coated slides were washed in PBS and blocked in 10% BSA/PBS for 2 hours at RT. After blocking, slides were washed twice with PBS and 100µl serum free cell culture media was added to each well. HDFs and MDDCs were harvested and 100µl of cell suspensions were added to each chamber, where they were left to adhere as previously described in section 2.12.3.3.

Cells were stained with mouse anti-human vinculin primary antibody (SPM227; diluted 1:400) at 4°C for 16 hours. Secondary antibody staining was performed with goat anti-mouse AlexaFluor® 488nm (1:1000), AlexaFluor® 568nm phalloidin (1:400), and DAPI (1:250) for 2 hours at room temperature (shielded from light). Slide mounting and sample preparation was performed as previously described in section 2.7.4. Confocal microscopy images were obtained using the Olympus FV1200 confocal microscope, with a 60x objective lens and a 2x zoom.

2.12.5 *Cell migration assay*

2.12.5.1 *Optimisation of cell density and FBS concentration*

MDDC cell plating density and FBS chemoattractant concentrations were optimised on Transwell® inserts with 5µm pores (SIGMA-ALDRICH). These were either coated with 0.01µM fibronectin, 10% BSA/PBS or were uncoated, at 4°C for 16 hours. Inserts were blocked with 10% BSA/ PBS for 1 hour at RT, and washed twice with PBS. Cell migration was quantified using the methods described in section 2.12.5.2.

Cell density optimisation: 100µl of MDDC suspension at 5, 1, or 0.2x10⁶ cells/ml were plated per well in serum free media.

Titration FBS concentration: The chamber underneath the insert was filled with 600µl RPMI-1640 1% (v/v) PS containing 0, 1, 5 or 10% FBS (v/v).

MDDCs were incubated for 3 hours at 37°C/5% CO₂, to permit cell migration.

2.12.5.2 *Transwell® migration assay*

Transwell® inserts with 5µm pores, were coated with 10⁻²µM fibronectin, 10⁻¹, 10⁻², 10⁻³µM tenascin-C and FnIII 3-5, or 1, 10⁻¹, 10⁻², 10⁻³µM FnIII 5-7, AD2AD1, BCD, BAD2AD1CD or 10% BSA at 4°C for 16 hours. The inserts were washed with PBS and blocked with 10%

BSA/PBS for 1 hour at RT. The chamber underneath was either filled with 600µl serum free media RPMI-1640 1% (v/v) PS, or 600µl RPMI-1640 10% FBS (v/v) 1% PS (v/v).

MDDCs were harvested, washed twice in serum free media, and resuspended at 1×10^6 cells/ml in serum free media. 100µl MDDC suspension was added to the upper chamber of each transwell[®] insert. MDDCs were incubated for 3 hours at 37°C/5% CO₂, to permit cell migration.

The media in the transwell[®] inserts was aspirated, and cells in the upper chamber were removed using a cotton bud. Migrated MDDCs on the underside of the transwell[®] membranes were fixed in 4% paraformaldehyde solution for 20 minutes at RT, and were stained with 0.1% crystal violet solution for 60 minutes. The excess dye was removed with MES buffer, and each membrane was photographed in 3 locations using the Nikon Eclipse Ts2R light microscope with a 20x, and a 40x objective lens. Staining and cell counting was performed as described in ³²².

2.12.5.3 Migration index (MI)

The cell count derives from the mean number of positively stained cells present in a 40x field, and the MI was calculated by dividing the mean number of migrated cells in response to stimulus, by the mean number of cells passively migrating through the transwell[®] pores; as described in ³²³.

MigrationIndex (MI)

$$= \frac{\text{Mean number of migrated cells on Substrate X towards RPMI 1640 + 10\% FBS}}{\text{Mean number of migrated cells on Substrate X towards serum free RPMI 1640}}$$

2.13 Statistical analysis

The data means + SEM, and statistical analysis were calculated using GraphPad Prism 7 software. Multiple group means of more than 3 samples, were subject to one-way analysis of

variance by ANOVA; and where appropriate, the analysis of variance of two different independent variables were assessed by two-way ANOVA. Two-tailed paired and un-paired t-tests were used for experiments involving only two groups where those samples came from different or identical populations respectively. *P* values less than 0.05 were considered significant.

Chapter 3 Characterising Tenascin-C Expression

3.1 Introduction

Tenascin-C is a large, multimodular, extracellular matrix protein, which is expressed in a strict spatio-temporally regulated manner. High tenascin-C expression is observed during embryogenesis near migrating cells, and at sites of epithelial-mesenchymal interaction and branching morphogenesis, while expression is absent in most healthy adult tissues, where it is restricted to sites of high cell turnover and tissue remodelling; such as in stem cell niches, regions of the central nervous system (CNS) and areas of high tensile stress including tendons.²⁴⁸ However, the expression of tenascin-C is down-regulated in healthy adults, and becomes restricted to a limited number of tissues including tendon, thymus, spleen, lymph nodes and bone marrow. In other tissues, the expression of tenascin-C is tightly regulated, being induced at sites of inflammation caused by a range of insults^{149, 324}; such as during sterile injury^{242, 243} and infection^{244, 245, 246}; while persistent expression is associated with chronic pathological states, including cancer and atherosclerosis, in addition to fibrotic and autoimmune disorders such as RA²⁴⁸. The elevated levels of tenascin-C expression in these locations in response to inflammation suggest that tenascin-C serves a role that is shared in global inflammatory responses. Further examples of associations between tenascin-C expression and inflammation are reviewed by Udalova *et al.*,⁶¹.

Previous work by our group found that tenascin-C has remarkable immunomodulatory properties. For example, it induced synovial inflammation when injected into mouse joints *in vivo*, and promoted chronic inflammation in experimental arthritis, as knockout of the tenascin-C gene in mice, protected them from erosive joint disease⁴³. Additionally, the synovial levels of pro-inflammatory cytokines such as TNF, IL-6 and IL-17 were reduced in

the joints of *TNC* $-/-$ mice²⁶¹. *TNC* $-/-$ mice are also less susceptible to LPS induced sepsis³⁰¹, and are protected from experimentally induced asthma due to an impaired Th2 response⁵³.

In vitro studies provide further evidence that tenascin-C directly impacts immune cell behaviour. It is an endogenous activator of TLR4, driving TNF α , IL-6 and IL-8 synthesis by primary human myeloid cells⁴³, and it is required for the polarization of Th17 cells by dendritic cells²⁶¹.

Despite these studies, very little is known of how tenascin-C is expressed or during inflammation or in inflammatory diseases; as no systematic characterisation of tenascin-C expression or alternative splicing has been performed.

In this chapter I systematically characterise the expression of tenascin-C in primary human myeloid and lymphoid cells, and HDFs, when the cells are resting, and following stimulation with inflammatory signals. I also characterise the expression of tenascin-C in primary human synovial fibroblasts isolated from non-arthritic joints (NSFs), and from the joints of people with rheumatoid arthritis (RA-SFs) and osteoarthritis (OA-SFs). The absolute abundance and fold changes in tenascin-C mRNA levels were quantified using standard curve qPCR and $\Delta\Delta\text{Ct}$ qPCR respectively, tenascin-C protein was quantified by ELISA, and the cellular distribution of tenascin-C was observed by confocal immunofluorescence microscopy.

Together these data comprise the first comparison of tenascin-C expression in immune cells and fibroblasts, and reveal the extent to which tenascin-C expression is regulated by cell type and stimulation in cells of the immune system, and by disease in synovial fibroblasts.

3.2 Basal expression of tenascin-C is cell lineage specific

Absolute levels of tenascin-C mRNA were quantified by standard curve qPCR in resting primary human cells; HDFs, GM-CSF macrophages, M-CSF macrophages, monocyte derived dendritic cells (MDDCs), monocytes, CD3⁺ T-cells, CD4⁺ T-cells, CD8⁺ T-cells and neutrophils. As a positive control the human glioma cell line (U-87 MG) was used, due to its reported high levels of tenascin-C expression³²⁵. Basal tenascin-C mRNA copies/cell were highest in HDFs, with significantly higher levels than U-87 MG cells, which in turn contained significantly higher levels than all of the myeloid and lymphoid cell types assayed. GM-CSF macrophages contained significantly higher levels than M-CSF macrophages, MDDCs, monocytes, T-cells and neutrophils, while the levels present in M-CSF macrophages, MDDCs and monocytes were not significantly different. CD3⁺, CD4⁺ and CD8⁺ T-cells contained ~100-fold less tenascin-C than M-CSF macrophages and MDDCs, and there was no difference in mRNA abundance between lymphocyte subsets. Neutrophils did not contain any detectable tenascin-C mRNA at rest (Figure 3.1).

These data provide the first absolute quantification of tenascin-C mRNA, allowing direct comparison of tenascin-C abundance across different cell types, and revealing significant differences in tenascin-C expression amongst stromal, myeloid and lymphoid cell lineages.

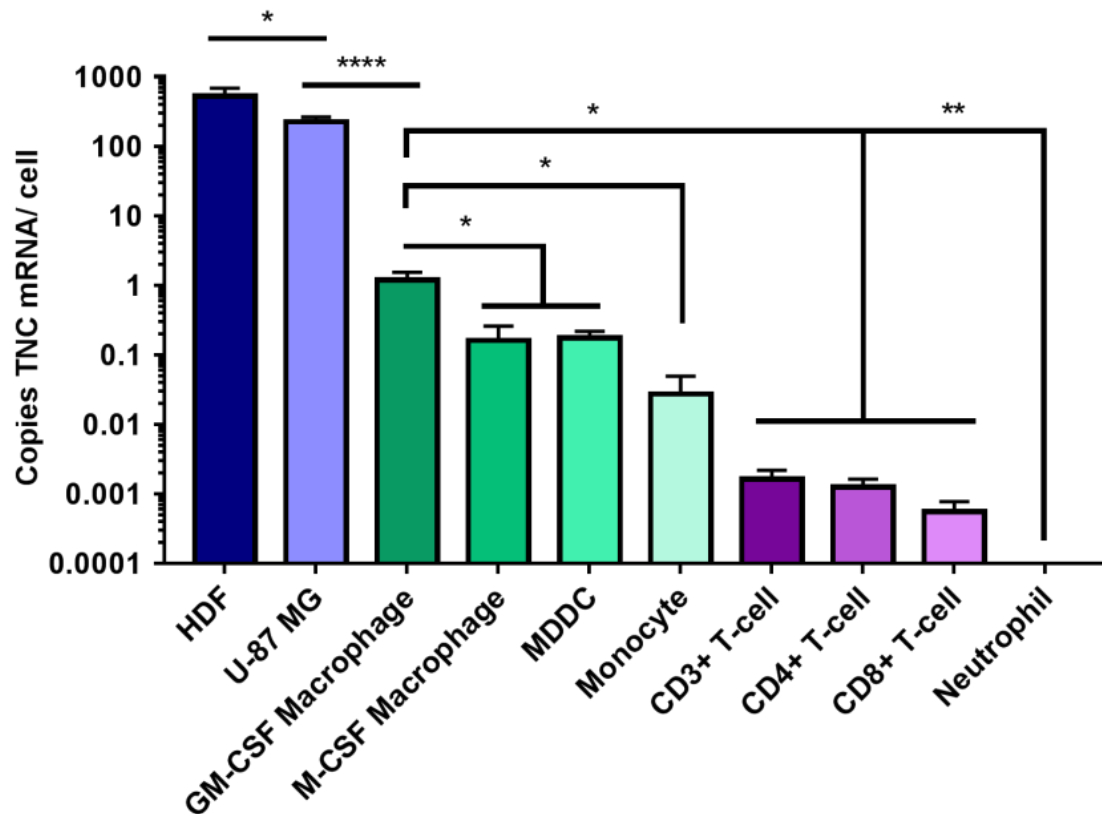


Figure 3.1. Basal levels of tenascin-C mRNA are higher in tumour cells and fibroblasts than in cells of myeloid or lymphoid lineage. Absolute levels of tenascin-C mRNA (TNC) were quantified as copies mRNA/cell using standard curve qPCR in resting cells, HDF (n=6), U-87 MG (n=6), GM-CSF macrophages (n=8), M-CSF macrophages (n=4), monocyte-derived dendritic cells (MDDCs) (n=8), monocytes (n=5), CD3+ T-cells (n=5), CD4+ T-cells (n=7), CD8+ T-cells (n=4) and neutrophils (n=5). Data are shown as mean + SEM. Un-paired t-tests were performed and statistical significance is displayed as *p<0.05, **p<0.01, and ****p<0.0001.

3.3 Relative abundance of tenascin-C mRNA to endogenous control genes

The abundance of tenascin-C mRNA was compared to a number of genes which are vital for maintaining cell homeostasis, and which were used to normalize tenascin-C expression in later sections of this chapter. For each cell type, genes which were stably transcribed following cell activation were selected, including 60s acidic ribosomal protein (RPLP0), guanine nucleotide-binding protein subunit beta-2-like 1 (GNB2L1), hypoxanthine phosphoribosyltransferase 1 (HPRT1), glyceraldehyde-3 phosphate dehydrogenase (GAPDH), beta-actin (ACTB) and 14-3-3 protein zeta/delta (YWHAZ). Here I quantified the abundance of these genes in resting cells to give an idea of the relative abundance of tenascin-C mRNA to that of other cellular proteins.

In U-87 MG cells tenascin-C mRNA levels were significantly lower than RPLP0, higher than HPRT1 and comparable to GNB2L1 levels (Figure 3.2.A). In HDFs, RPLP0, GAPDH and ACTB were more abundant than tenascin-C, although levels for ACTB were not significantly different (Figure 3.2.B). In GM-CSF macrophages, MDDCs and CD4⁺ T-cells, tenascin-C mRNA levels were the least abundant mRNA quantified but were not significantly lower than HPRT1 in GM-CSF macrophages and MDDCs, nor ACTB in CD4⁺ T-cells (Figure 3.2.C-E).

In wild type mouse embryonic fibroblasts (MEF *wt*), tenascin-C mRNA levels were confirmed to be significantly higher than in the tenascin-C knockout mouse embryonic fibroblasts (MEF *TNC*^{-/-}), where tenascin-C mRNA was absent. In the *wt* and *TNC*^{-/-} MEFs, RPLP0 mRNA was more abundant than TNC mRNA, and RPLP0 levels were not affected by the absence of tenascin-C mRNA (Figure 3.2.F).

These data show that in U-87 MG cells and HDFs, tenascin-C mRNA was transcribed at levels comparable to key homeostatic genes; but that in myeloid and lymphoid cells, tenascin-C mRNA levels were lower than for other genes assessed. It also confirmed that tenascin-C

mRNA is constitutively expressed in wt MEFs, but is absent in the tenascin-C knockout controls.

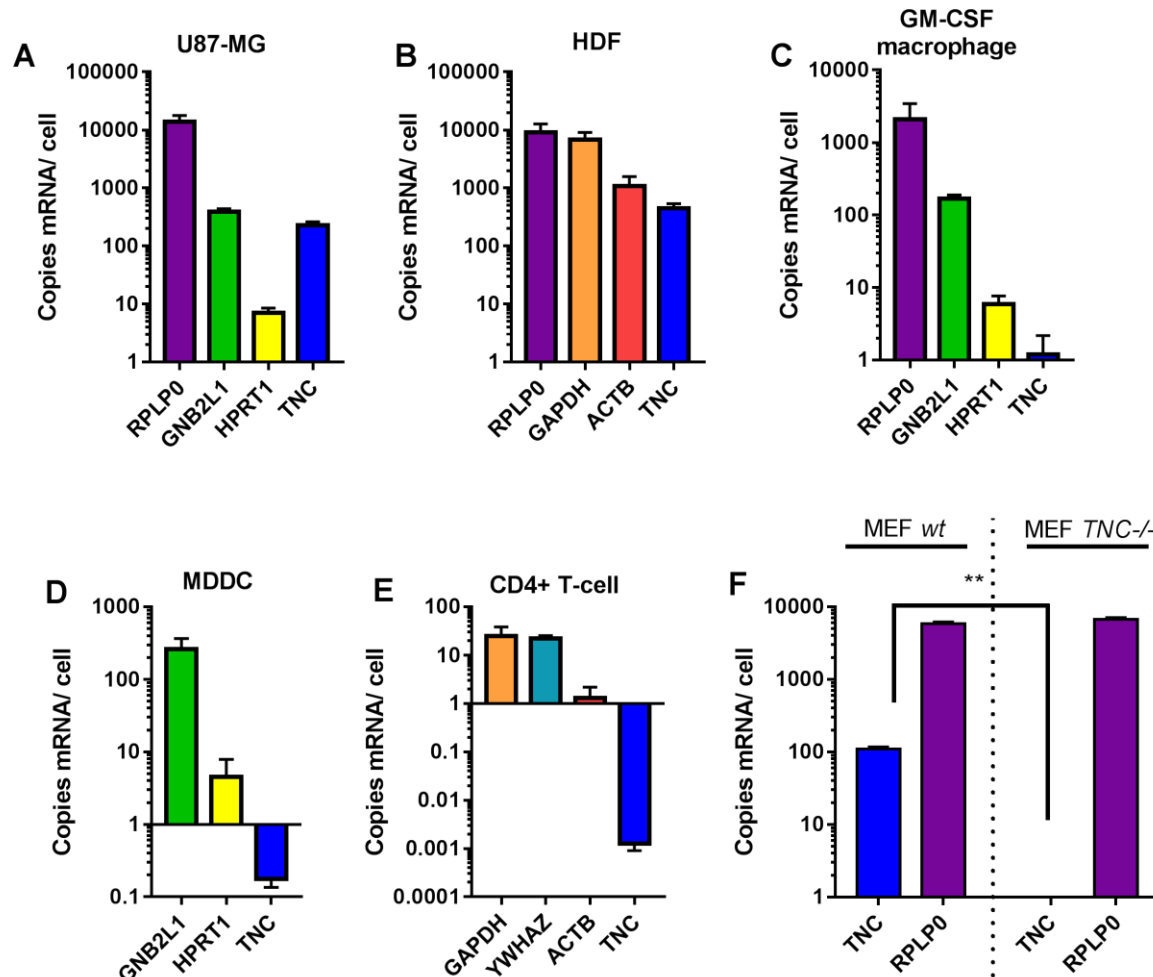


Figure 3.2. Basal tenascin-C mRNA levels are comparable to house keeping genes in U-87 MG and HDFs. The basal copies/cell of mRNA for tenascin-C and the endogenous control genes RPLP0, GNB2L1, HPRT1, GAPDH, ACTB and YWHAZ, were quantified by standard curve qPCR. Data are shown for U-87 MG (n=3) (A), HDFs(n=4) (B), GM-CSF macrophages (n=4) (C), MDDCs (n=6) (D), CD4+ T-cells (n=4) (E), and in wild type mouse embryonic fibroblasts (MEF wt) (n=3), and tenascin-C knockout mouse embryonic fibroblasts (MEF TNC -/-) (n=3) (F). Data are shown as mean + SEM. Un-paired t-tests were performed and statistical significance is displayed as **p<0.01.

3.4 Optimizing qPCR, ELISA and immunostaining methods in U-87 MG cells

Having established basal tenascin-C mRNA abundance in a range of primary human cell types, the next aim was to examine tenascin-C expression, at both mRNA and protein level, in these cells in response to inflammatory activation. However, there are no published protocols for quantifying tenascin-C mRNA using standard curve qPCR, and few reports quantifying tenascin-C protein by ELISA, or staining tenascin-C in cells using immunofluorescence. Therefore these methods were optimized using U-87 MG cells, prior to analysis of other cell types. The high levels of tenascin-C mRNA and protein expression in U87-MG, coupled with a rapid rate of cell proliferation and ease of cell culture make these ideal for validation. These methods are now published (Giblin *et al.*, 2018, *Methods in Cell Biology*: *in press*); and a copy of this publication is included at the end of this thesis.

3.4.1 *Tenascin-C* qPCR

Tenascin-C mRNA and control gene RPLP0 were quantified using standard curve qPCR in U87-MG cells that were cultured for up to 24h. The copy number/cell for RPLP0 was then used to normalise tenascin-C mRNA levels, and determine the fold change in abundance at 0h, 4h, 8h and 24h time points. Tenascin-C mRNA was detectable in U-87 MG cells at 0h and these levels remained unchanged for 24 hours following plating (Figure 3.3.A). RPLP0 mRNA levels were higher than for tenascin-C, and were stable over 24 hours following plating (Figure 3.3.B). Normalisation of tenascin-C mRNA to RPLP0 revealed no overall increase in tenascin-C mRNA over 24 hours (Figure 3.3.C).

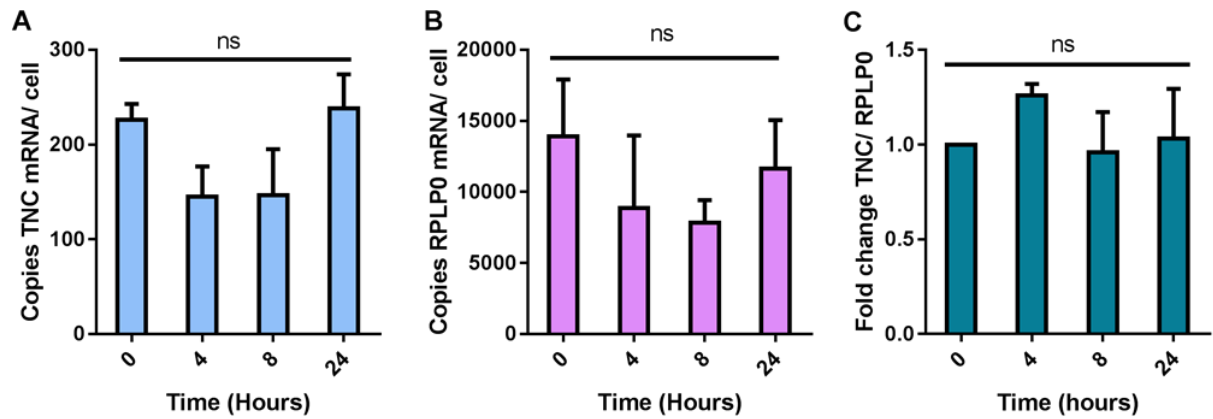


Figure 3.3. Tenascin-C mRNA is present at constitutively high levels in U-87 MG cells. U-87 MG cells were plated for 0, 4, 8 and 24 hours and the abundance of tenascin-C mRNA (A) and RPLP0 (B) mRNA was quantified by standard curve qPCR. Normalisation of tenascin-C mRNA copies mRNA/cell by RPLP0 revealed the fold change in tenascin-C mRNA abundance over the time course (C). Data are shown as mean + SEM (n=3). One way Anova was performed and statistical significance is displayed as, non-significant (ns).

3.4.2 *Tenascin-C ELISA*

To quantify tenascin-C protein in cell associated (cell lysate), or secreted (supernatant) fractions from U-87 MG cells, two commercially available ELISA kits were utilised, each with different limitations. The first ELISA (USCN/Cloud-Clone Corp.) contained a detection antibody raised against amino acid residues Cysteine 174 – Serine 466 of human tenascin-C, corresponding to the constitutively expressed EGF-Like repeats, meaning all tenascin-C isoforms may be detected. This kit had a low reported detection limit (31pg/ml), and was reportedly highly sensitive, but use of this ELISA was not widely reported in the literature. The second ELISA (IBL) detected an epitope within the alternatively spliced FnIII-B domain of human tenascin-C, so does not detect all tenascin-C isoforms. It was also reported to have a higher limit of detection (12.5ng/ml), with lower sensitivity for detecting tenascin-C, although it was better characterised and more widely published than the USCN ELISA, exhibiting cross reactivity with mouse and rat tenascin-C. Furthermore, use of the FnIII-B ELISA is widely reported in the literature in a range of samples, for example blood plasma, urine and cell culture samples.

The specificity of the USCN ELISA was confirmed by quantifying tenascin-C present in the conditioned media of wild type (*wt*) and *TNC*^{-/-} MEFs; as *wt* MEFs have previously been reported to express high levels of tenascin-C, while tenascin-C is absent in *TNC*^{-/-} MEF cultures³²⁶. The USCN ELISA detected a significantly greater concentration of tenascin-C in the *wt* MEF, than in the *TNC*^{-/-} MEF supernatant samples; however, detection of tenascin-C was not completely absent in the *TNC*^{-/-} MEF samples (Figure 3.4.A).

To optimize detection of tenascin-C in human cell lysate and supernatant, samples were isolated from U-87 MG cells cultured for 0 and 24 hours and were titrated against the USCN ELISA standards to determine the correct dilution factor, to bring the samples within the

dynamic range of the standards to permit accurate quantification. Samples were diluted 1:20, 1:100, 1:500 and 1:1000 and all samples except the 0 hour supernatant produced a dilution dependent decrease in absorbance when quantified, with the 1:100 diluted samples within the dynamic range (Figure 3.4.B), a representative standard curve from the USCEN ELISA is also shown (Figure 3.4.C). Cell lysate and supernatant samples of U-87 MG glioma cultured for 0, 4, 8 and 24 hours revealed significant increases in tenascin-C protein between 0 and 24 hours in the cell lysate and supernatant, with most of this increase occurring between 8 and 24 hours after plating. Additionally, the USCEN ELISA detected a greater abundance of tenascin-C in the U-87 MG supernatant than the cell lysate (Figure 3.4.D-E).

The specificity of the FnIII-B ELISA kit was also validated using MEF conditioned media. The FnIII-B ELISA detected significantly more tenascin-C in the *wt* MEF, than the *TNC*^{-/-} MEF samples; where the tenascin-C concentration was negligible (Figure 3.4.F); this ELISA detected more tenascin-C in the *wt* MEF supernatant than the USCEN ELISA.

To determine the correct dilution factor for accurately quantifying U-87 MG cell lysate and supernatant samples with the FnIII-B ELISA, samples from U-87 MG cells cultured for 24 hours were quantified after dilution to 1:25, 50, 75, 100, and 1:5, 10, 25, 50 respectively. Both samples displayed dilution dependent decreases in absorbance (Figure 3.4.G); and a representative standard curve for the FnIII-B ELISA is shown (Figure 3.4.H). Cell lysate was diluted 1:50 and supernatant 1:10 for subsequent FnIII-B quantifications. The FnIII-B ELISA detected a significant increase in cell lysate and supernatant tenascin-C between 0 and 24 hours after plating. The total amount of tenascin-C measured in the cell lysate was higher than in the supernatant (Figure 3.4.I-J), contrasting with the results obtained using the USCEN ELISA kit.

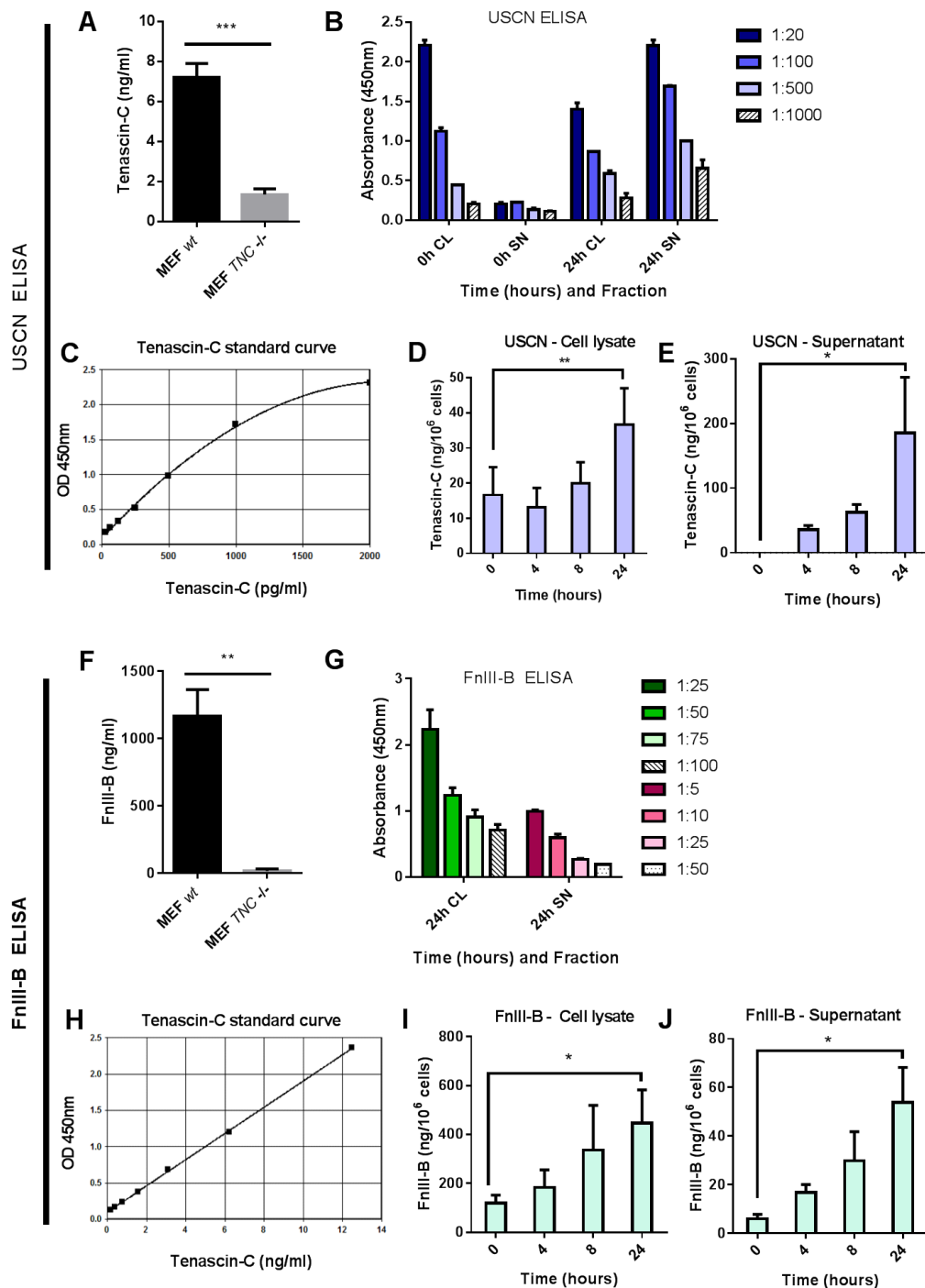


Figure 3.4. Validation of the commercially available USCN and FnIII-B ELISA kits using *wt*, *TNC*^{-/-} MEFs and U-87 MG cells. Tenascin-C was quantified in samples of *wt* MEF and *TNC*^{-/-} MEF conditioned media after culture for 72 hours, using the USCN ELISA kit (A) and the FnIII-B ELISA kit (F). Cell lysate (CL), and supernatant (SN) from U-87 MG cells cultured for 0 and 24 hours were diluted as stated, and quantified with the USCN ELISA (B), or the FnIII-B ELISA (G). U-87 MG cells were cultured for 0, 4, 8 and 24 hours, and were diluted 1:100 or 1:50 for quantification by USCN and FnIII-B ELISAs respectively in the cell lysate (CL) (D,I), and 1:100 or 1:10 for quantification in the supernatant (SN) (E,J). Representative tenascin-C standard curves produced by the USCN (C), and FnIII-B (H) ELISAs are shown. Data are shown as mean + SEM, (n=3). Un-paired t-tests were performed and statistical significance is displayed; *p<0.05, **p<0.01 and ***p<0.001.

These data show that the USCN ELISA exhibited non-specific binding and had a smaller linear range compared to the FnIII-B ELISA. This meant that using the USCN ELISA to quantify tenascin-C was not ideal, despite its ability to detect all tenascin-C isoforms. These data also showed that the FnIII-B ELISA kit was more specific, and had a larger linear range, making it more accurate for quantifying tenascin-C from different samples, despite the limitation of only being able to detect FnIII-B containing tenascin-C isoforms. I use both kits in the subsequent chapters of this study, but propose that data from the FnIII-B kit is more likely to be accurate, especially when assaying cell types expressing low levels of tenascin-C.

3.4.3 *Tenascin-C immunostaining using confocal fluorescence microscopy*

The cellular distribution of tenascin-C protein was assessed by confocal immunofluorescence microscopy. I adapted protocols for the staining of tissue sections using the mouse anti-human tenascin-C antibody BC-24^{296, 327} in order to be able to visualize tenascin-C in cell cultures. BC-24 binds to the constitutively expressed N-terminal EGF-L repeat region of tenascin-C. Fibronectin, a ubiquitously expressed ECM component, served as a positive control for the immunostaining protocol, co-staining with the rabbit anti-human fibronectin antibody F3648, using methods previously optimized in the Midwood lab³²⁰.

Firstly in order to confirm that BC-24 specifically bound to tenascin-C, with no non-specific binding to endogenous Fc receptors, both BC-24 and an IgG1 isotype antibody were used to stain wt MEFs and *TNC*^{-/-} MEFs cultured for 48 hours. The F3648 antibody was used to stain for fibronectin which is abundantly expressed by both cell types. Corresponding no-primary control (NPC) slides where cells were stained in the absence of the primary antibody were prepared, but with secondary antibody as normal, to determine the presence of non-specific secondary antibody staining.

wt MEFs exhibited tenascin-C immunostaining that was diffuse throughout the cytosol, while there was a complete absence of staining in the *TNC*^{-/-} MEFs. In both the *wt* MEFs and *TNC*^{-/-} MEFs, staining of fibronectin was abundant, with *wt* MEFs exhibiting concentrated, fibrillar tenascin-C staining at the edges of the cells, and diffuse cytosolic staining. In contrast the *TNC*^{-/-} MEFs displayed punctate fibronectin staining at the edges of the cells, comprising dense short foci with little cytosolic staining observed. IgG1 immunostaining was absent in both cell types, as was secondary antibody fluorescence in the BC-24, F3648 and IgG1 NPC slides (Figure 3.5).

Next, the concentrations of BC-24 and F3648 primary antibodies required for optimal immunostaining were assessed by titration in U-87 MG cells cultured for 48 hours. Immunostaining with BC-24 and F3648 was performed at 1:100, 200, 500 and 1000 antibody dilutions, alongside the NPC. BC-24 immunostaining in U-87 MG was detected at all antibody dilutions between 1:100 and 1:1000, and was absent from the NPC. The intensity of tenascin-C staining was dose dependent and reached a peak when diluted 1:200, where tenascin-C was observed in the regions between cells, with faint staining of the cytosol. The F3648 exhibited strong staining at 1:100 and 1:200 dilutions, which decreased in intensity at 1:500 and was barely detectable at the 1:1000 dilutions. At the optimal 1:200 dilution, the fibronectin distribution was punctate and spread over the outside of the cells with a subset of cells positively stained in the cytosol. There was no detectable immunostaining in the F3648 NPC (Figure 3.6).

To observe the intracellular and extracellular tenascin-C and fibronectin distribution at a higher resolution, Z-stack images were compiled. U-87 MG cells were cultured for 72 hours and immunostained with optimised BC-24 and F3648 antibody dilutions, and were permeabilised. Z-stack composite images had a 0.4µm step size, and are 120x zoom.

Tenascin-C staining was present at low levels over almost all of the non-permeabilised cells, but with more intense staining at regions of cell contact. The Z-stack cross sectional slice images confirmed this, and revealed that tenascin-C was predominantly present in-between the cells and not distributed above or below the cell monolayer (Figure 3.7.A). Fibronectin localised to the cell surface in a uniformly distributed fashion but was hardly observed on the basal side of the cells (Figure 3.7.B).

Permeabilisation of U-87 MG cells, revealed that tenascin-C was present intracellularly, as higher intensity of tenascin-C staining was observed in the permeabilised cells relative to the non-permeabilised cells (Figure 3.7.C). The images show a punctate distribution of fibronectin that was evenly distributed over all of the cells, but with some regions of intense staining at cell-cell contacts, and at the cell periphery (Figure 3.7.D).

To summarize, here I optimised a protocol for the immunostaining of tenascin-C and fibronectin in cell cultures using *wt* MEFs, *TNC*^{-/-} MEFs and U-87 MG cells. I established that the BC-24 primary antibody recognizes both mouse and human tenascin-C, and that there is no detectable non-specific binding of the BC-24 antibody, or the secondary antibodies to *wt* MEFs, *TNC*^{-/-} MEFs and U-87 MG cell cultures. I also showed similarities in the pattern and distribution of tenascin-C and fibronectin immunostaining in U-87 MG cells, and these similarities are most apparent in permeabilised cells.

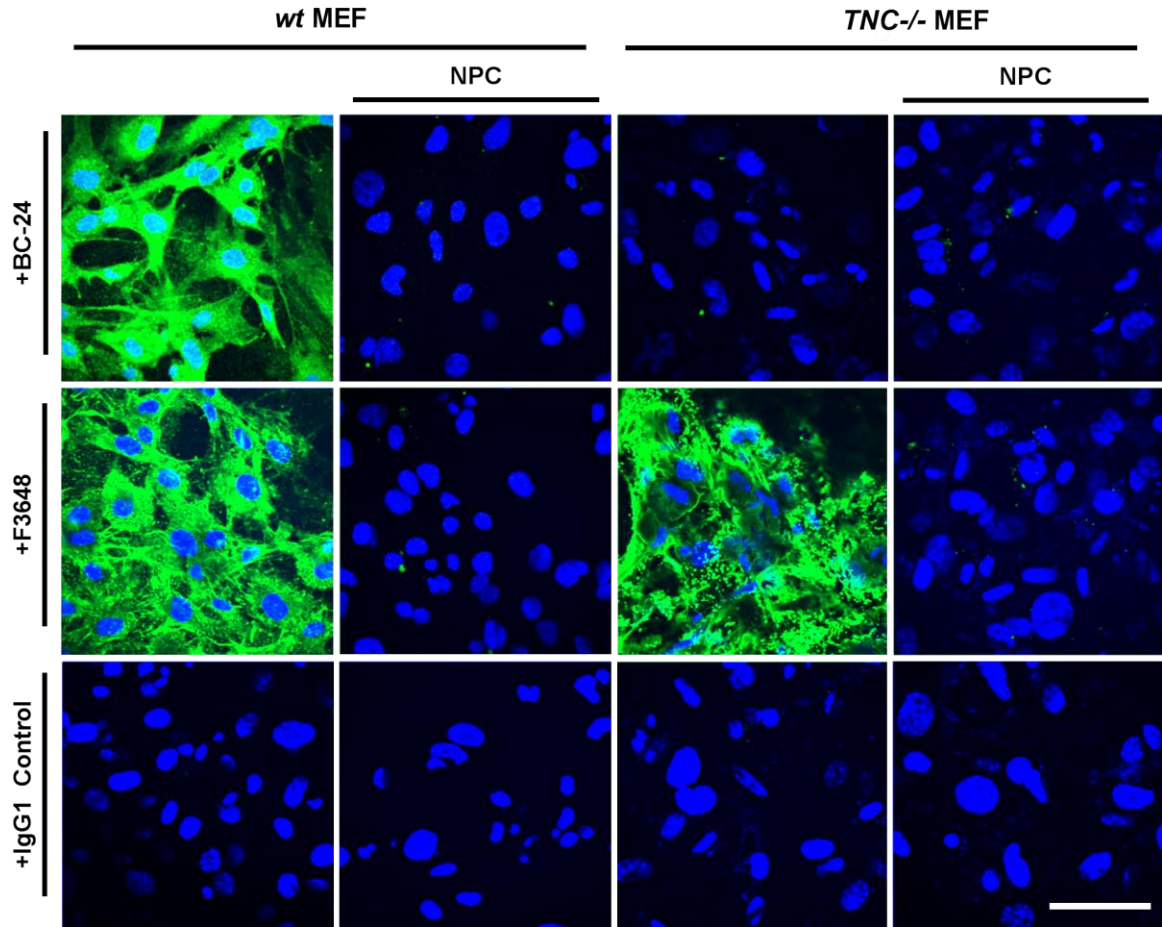


Figure 3.5. Confirming BC-24 specificity to tenascin-C in *wt* and *TNC*^{-/-} MEFs for confocal immunofluorescence microscopy. *wt* and *TNC*^{-/-} MEFs were cultured for 72 hours, fixed, permeabilised and stained for tenascin-C with BC-24 and goat anti-mouse AlexaFluor488 secondary (top row: green), for fibronectin with F3648 and goat anti-rabbit AlexaFluor488 secondary (middle row: green) or with an IgG1 isotype control antibody and goat anti-mouse AlexaFluor488 secondary (bottom row: green). No-primary controls (NPCs) were included for each secondary antibody type. All slides were stained with DAPI (blue) alongside the secondary antibodies. Representative images from 3 experiments are shown. Images were visualized with the Olympus FV1200 IX83 Confocal System, with a 60x objective and 2x zoom. Scale bar is 20µm.

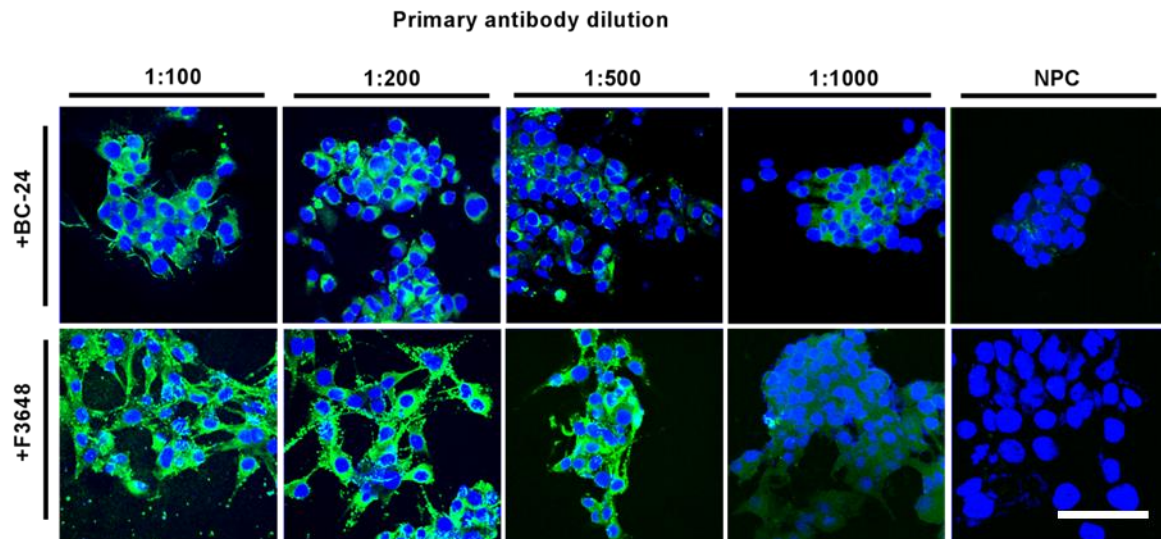


Figure 3.6. Titration of primary antibody staining dilutions for tenascin-C and fibronectin immunostaining in U-87 MG cells. U-87 MG cells were cultured for 48 hours, then fixed and permeabilised prior to staining for either tenascin-C with BC-24 (top row: green), or fibronectin with F3648 (bottom row: green) using primary antibodies diluted as indicated, alongside a no primary control (NPC). All slides were stained with DAPI (blue) alongside the respective secondary antibodies. Representative images from 3 experiments are shown. Images were visualized with the Olympus FV1200 IX83 Confocal System, with a 60x objective and 2x zoom. Scale bar is 20µm.

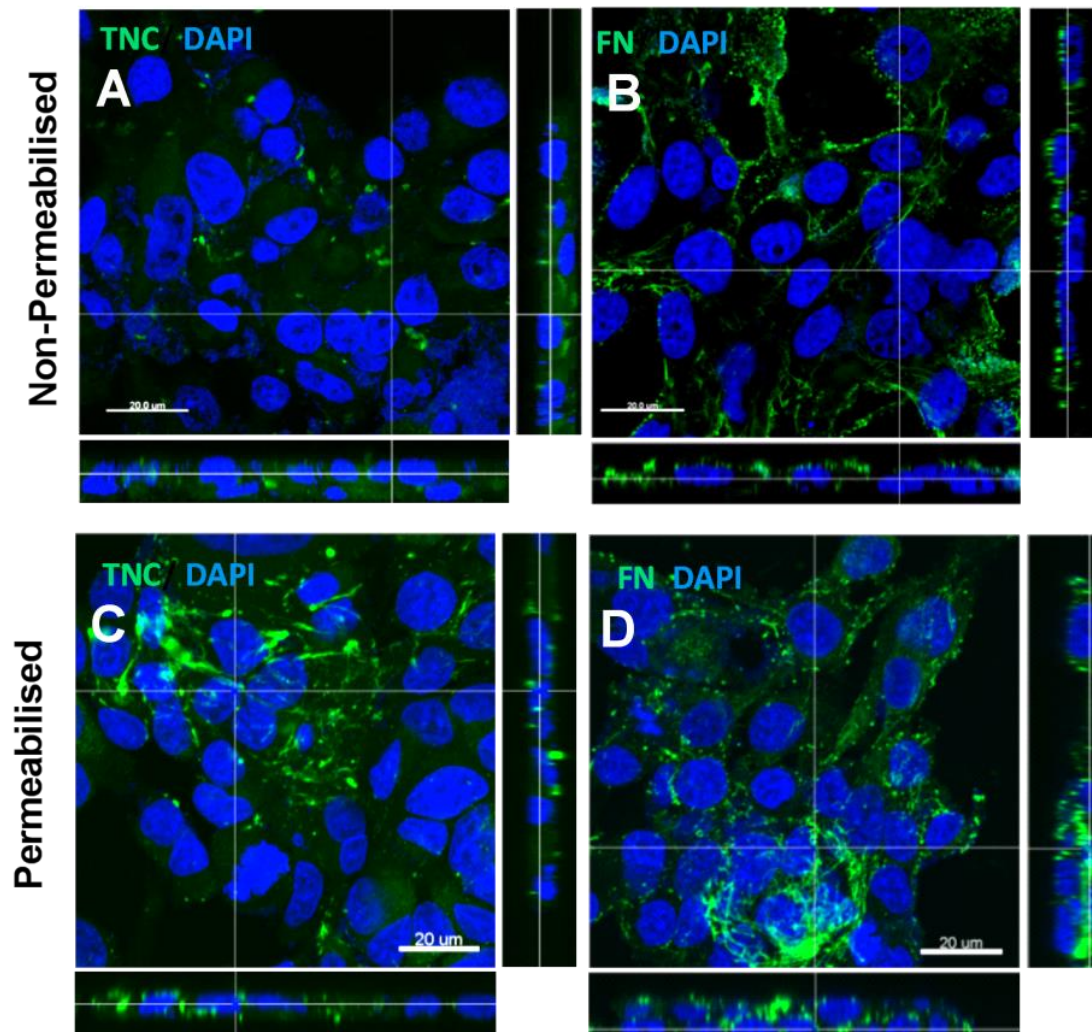


Figure 3.7. Cellular distribution of tenascin-C and fibronectin in U-87 MG cells. U-87 MG cells were cultured for 72 hours prior to fixing and were either not permeabilised (A,B), or were permeabilised with 0.02% Triton X-100 (C,D). Cells were then immunostained for tenascin-C with BC-24 mAb (green) (A,C) fibronectin with F3648 mAb (green) (B,D). All slides were stained with DAPI diluted (blue) alongside the respective secondary antibodies. Representative images from 3 experiments are shown. Images were visualized with the Olympus FV1200 IX83 Confocal System, with a 60x objective and 2x zoom. Z-stacked cross sections were compiled from $n=45$ $0.4\mu\text{m}$ step size scans. Scale bar is $20\mu\text{m}$.

Together these data show that glioma cells express high basal levels of tenascin-C mRNA and protein; and that tenascin-C protein is both retained in the cell associated fraction, and is secreted following biosynthesis. Furthermore, tenascin-C is detectable in U-87 MG by immunofluorescence where it exhibits a punctate distribution pattern over the cell surface, and localises at regions of cell contact within clusters of cells.

3.5 Tenascin-C expression in primary human myeloid cells

Having optimized methods for assessing tenascin-C mRNA by qPCR and protein by ELISA and immunofluorescence in glioma cell lines, the next aim was to apply these methods to primary human immune cells, both at rest and following stimulation. Tenascin-C expression is detected in myeloid cell populations isolated from RA synovium²⁷³, and is known to impact the behaviour and activity of myeloid cells, for example dendritic cells from *TNC*^{-/-} mice exhibit an impaired ability to polarize Th17 cells²⁶¹. Therefore in this section I systematically characterised tenascin-C biosynthesis in myeloid cells to determine whether the expression of tenascin-C by these immune cells might be functionally relevant. Previous work by our group has shown that tenascin-C mRNA is induced in human monocyte derived dendritic cells (MDDCs) by stimulation with 10ng/ml LPS²⁷³. Here I confirm these data, and further examine the kinetics of mRNA and protein synthesis, and assess tenascin-C expression in a wider range of myeloid cell types.

3.5.1 *Tenascin-C mRNA levels are induced in activated monocytes, macrophages and dendritic cells*

The abundance of tenascin-C mRNA was quantified in primary human monocytes, GM-CSF macrophages, M-CSF macrophages and MDDCs at 0, 4, 8 and 24 hour time points following stimulation with 10ng/ml LPS, or in cells that were left unstimulated. Additionally, the fold change in tenascin-C mRNA levels relative to the initial t=0 hour time point was normalised to endogenous control genes that were shown to be stably expressed in each cell type following LPS stimulation, so that the profile of tenascin-C mRNA induction could be assessed. Tenascin-C mRNA was normalised against GAPDH in monocytes and HPRT1 in GM-CSF and M-CSF macrophages, and against GNB2L1 in MDDCs.

In all cell types low basal expression of tenascin-C was observed over time in the absence of stimulation, and LPS induced transient tenascin-C expression. In primary human monocytes tenascin-C mRNA induction peaked at 8 hours following stimulation, and decreased by 24 hours but not to the unstimulated baseline levels (Figure 3.8.A). By contrast in GM-CSF macrophages, induction was faster than in monocytes, peaking between 4 and 8 hours following stimulation, reducing to below baseline levels by 24 hours. GM-CSF macrophages exhibited the highest total number of tenascin-C mRNA copies/cell that peaked 4 hours following stimulation (Figure 3.8.B). M-CSF macrophages exhibited similar transient tenascin-C induction kinetics to GM-CSF macrophages, with a statistically significant induction at 4 hours following stimulation, reducing to the baseline by 24 hours, and a corresponding peak in the number of TNC mRNA copies/cell; although the total levels of mRNA are not as high as in GM-CSF macrophages (Figure 3.8.C). MDDCs exhibited the largest fold induction of tenascin-C mRNA, which is statistically significant at 4 and 8 hours following stimulation, peaking at 8 hours and returning the baseline by 24 hours. The absolute TNC mRNA copies/cell detected in MDDCs peaked at 4 hours following stimulation, and levels were higher than peak levels observed in monocytes and M-CSF macrophages, but at just over half of the peak levels recorded in GM-CSF macrophages (Figure 3.8.D).

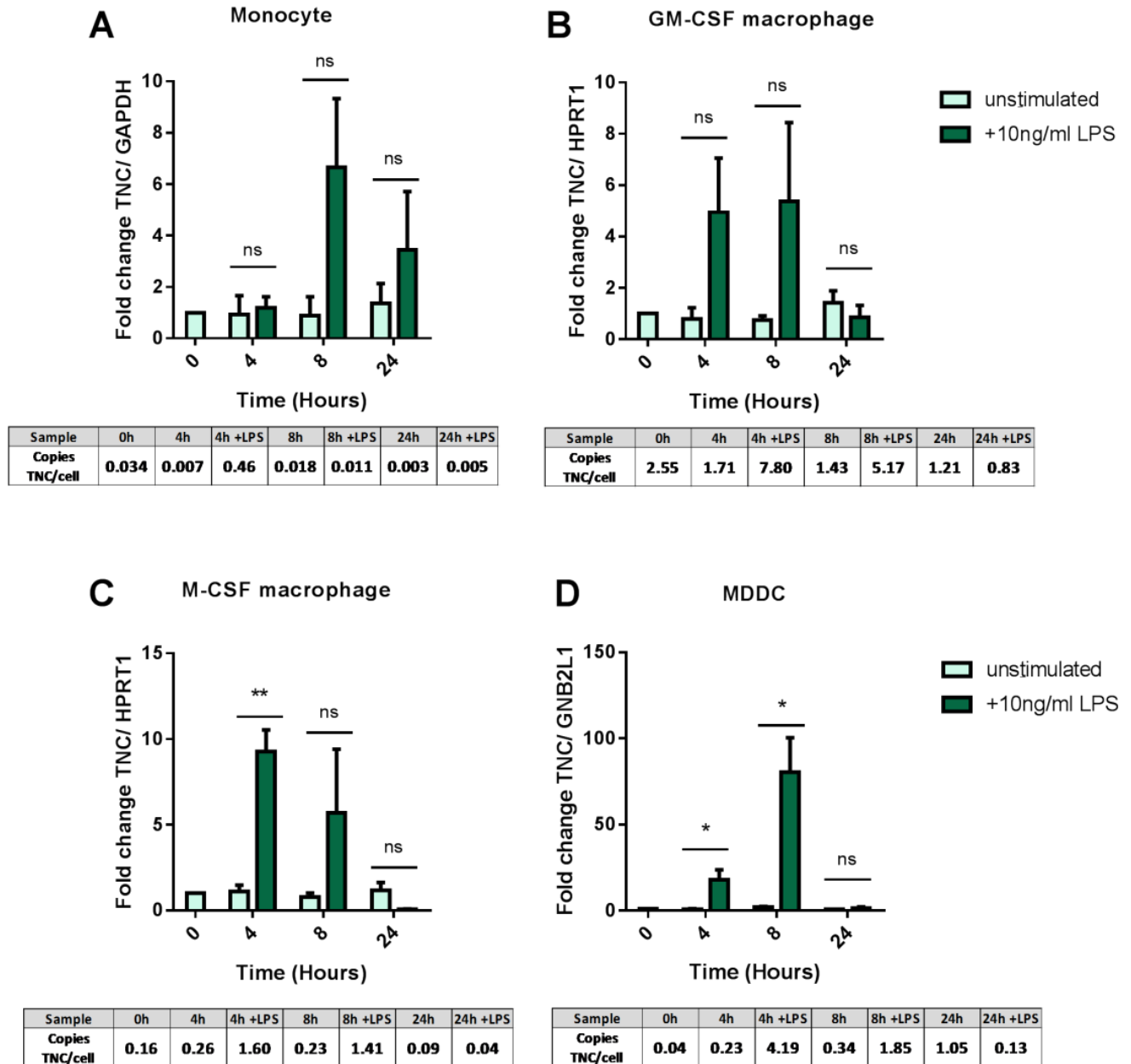


Figure 3.8. Tenascin-C mRNA levels in primary human monocytes, macrophages and MDDCs are induced by LPS treatment. Primary human monocytes, GM-CSF macrophages, M-CSF macrophages and MDDCs were plated and rested for 16 hours prior to stimulation with 10ng/ml LPS. RNA was extracted at 0, 4, 8 and 24 hours post stimulation and tenascin-C mRNA was quantified by standard curve qPCR. The fold induction of tenascin-C mRNA relative to the initial t=0 hour time point was assessed and normalised in monocytes (n=4) to GAPDH (A), in GM-CSF macrophages (n=3) to HPRT1 (B), in M-CSF macrophages (n=3) to HPRT1 (C) and in MDDCs (n=4) to GNB2L1 (D). The absolute quantification of tenascin-C mRNA copies/cell in each cell type is marked in the tables underneath the relevant graphs. Data are shown as mean + SEM. Un-paired t-tests were performed and statistical significance is displayed as *p<0.05, **p<0.01 and non-significant (ns).

3.5.2 *Characterisation of tenascin-C protein biosynthesis in activated dendritic cells*

MDDCs exhibited the largest fold induction of tenascin-C mRNA in response to LPS stimulation compared to primary human monocytes, GM-CSF and M-CSF macrophages. However the expression of tenascin-C protein in these cells is not well understood. Here, I examined the biosynthesis of tenascin-C in MDDCs by ELISA and immunofluorescence.

Levels of tenascin-C protein were quantified in the cell lysate and supernatant fractions of MDDCs that were stimulated with 10ng/ml LPS 0, 4, 8, 24, 48 and 72 hour time points, or were left unstimulated; using the two commercially available anti-tenascin-C ELISA kits; USCN and FnIII-B. The total protein detected is shown as ng/10⁶ cells in order to compare absolute levels of expression across cell types. For the USCN ELISA, tenascin-C protein in the MDDC cell lysate fractions was below the limit of detection for the kit at all time points, but was higher than the background lysis buffer control. By contrast, in the supernatant, tenascin-C accumulated steadily up to 24 hours independent of LPS stimulation, before decreasing slightly at 48 and 72 hours, though this trend was not statistically significant. (Figure 3.9.A-B).

The FnIII-B/IBL ELISA kit detected different expression kinetics and cellular distribution of tenascin-C in MDDC cell lysate and supernatant than the USCN ELISA kit. In MDDC cell lysate, the FnIII-B ELISA detected a statistically significant and transient increase in tenascin-C levels following LPS stimulation, beginning with a small increase by 4 hours that peaked at 8 hours and gradually decreased to 72 hours where it remained elevated compared to the unstimulated MDDCs. This spike in cell associated tenascin-C quickly follows the peak in TNC mRNA copies/cell present at 4 hours following stimulation. In the supernatant there was no increase in tenascin-C levels until 8 hours following LPS stimulation, where there was a statistically significant LPS dependent increase in tenascin-C levels from 8 hours

until 72 hours that was still increasing by the 72 hour time point (Figure 3.9.C-D). Both the USCN and FnIII-B ELISA kits detected higher absolute peak levels of tenascin-C in the supernatant than the cell lysate of MDDCs.

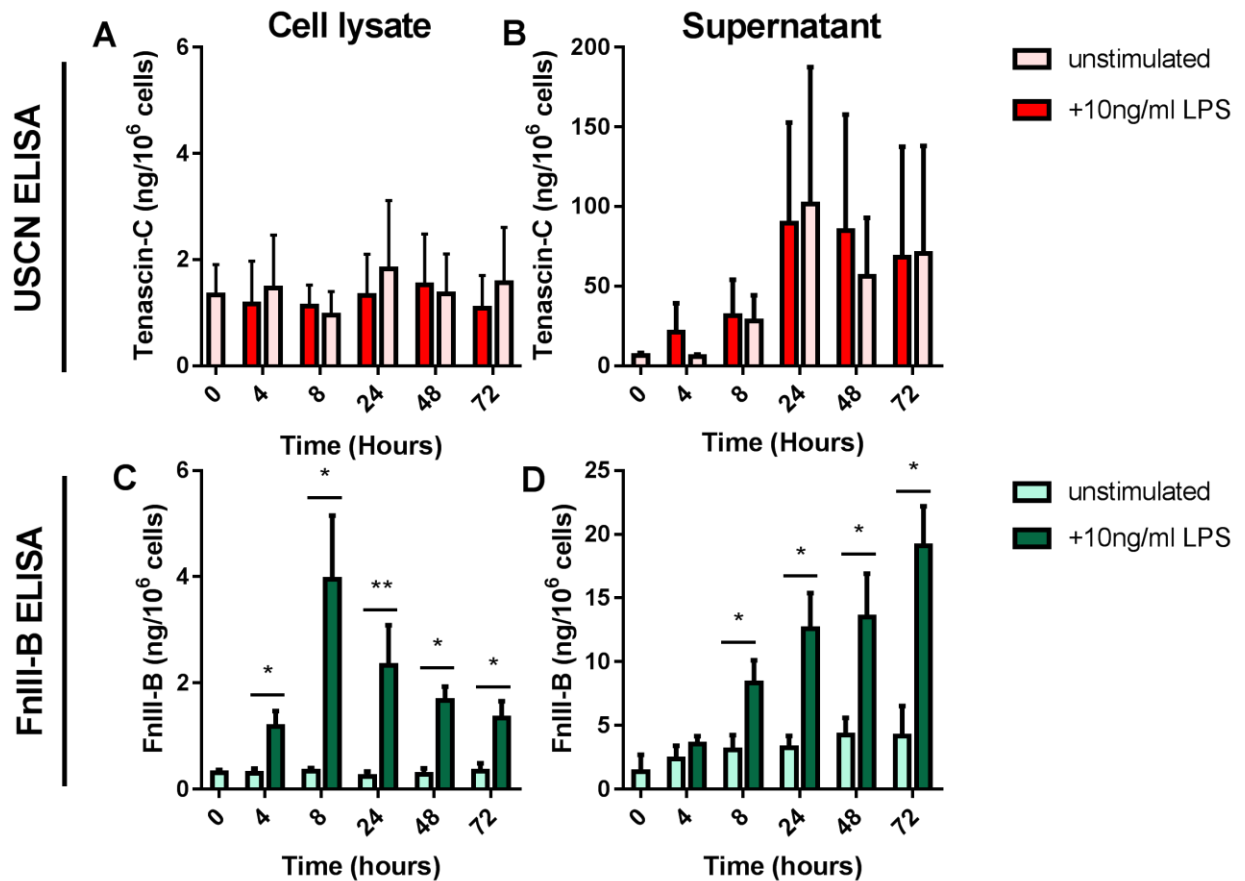


Figure 3.9. MDDCs transiently express tenascin-C protein following stimulation with LPS. Primary human MDDCs were plated and rested for 16 hours prior to stimulation with 10ng/ml LPS for 0, 4, 8, 24, 48 and 72 hours where total tenascin-C was quantified in cell lysate (A) and supernatant (B) by USCN ELISA, and FnIII-B containing tenascin-C was quantified in cell lysate (C) and supernatant (D) using the FnIII-B ELISA. Data are shown as mean + SEM (n=3). Un-paired t-tests were performed and statistical significance is displayed; *p<0.05, **p<0.01 and non-significant (ns).

Due to the absence of any information about tenascin-C immunostaining in myeloid cells in the literature, the optimal conditions required to observe tenascin-C immunofluorescence in MDDCs were determined using an LPS dose response experiment where MDDCs were fixed, and either permeabilised or not permeabilised, prior to staining at t=8 hours. This time point

was chosen as it yielded the highest levels of cell associated FnIII-B containing tenascin-C; determined by ELISA in Figure 3.9.C.

Stimulation of MDDCs with 10, 100 and 500ng/ml LPS revealed a dose dependent increase in tenascin-C immunostaining. In permeabilised cells, there was a small dose dependent increase in tenascin-C staining, with none detected in unstimulated MDDCs. The tenascin-C staining at all LPS doses is predominantly cytosolic, with dense foci of staining observed at the cell boundary in 10-20% of the cells imaged (Figure 3.10.A-D). Fibronectin staining increased in permeabilised cells in a dose dependent manner, and was strongest with 500ng/ml LPS, where high levels of cytosolic staining was observed, with intense staining appearing all around the outline of the cell under the membrane (Figure 3.10.E-H).

In non-permeabilised cells, tenascin-C staining in MDDCs was absent in when unstimulated, but faint detection was observed when stimulated with 10ng/ml LPS, which increased in a dose dependent manner to 500ng/ml LPS. Here, the staining for tenascin-C was limited to the boundary of the cells (Figure 3.10.I-L). In contrast, staining for fibronectin in non-permeabilised MDDCs, revealed no dose dependent increase in response to LPS stimulation; instead the fibrillar structure of extracellular fibronectin that was observed in unstimulated MDDCs became disrupted following the addition of 10-500ng/ml LPS. After adding LPS, the fibronectin clustered around the cell boundary and did not form long fibrils (Figure 3.10.M-P).

During the same experiment as displayed in Figure 3.10, MDDCs were stained with an IgG1 isotype antibody, and NPCs were performed. Both the IgG1 immunostaining controls and the NPCs lacking BC-24 and F3648 were negative for immunostaining (Figure 3.11).

In order to better understand tenascin-C distribution in activated MDDCs, cells rested for 16 hours were either not stimulated, or stimulated with 100ng/ml LPS for 8, 24 or 48 hours prior

to fixing and permeabilisation. The MDDCs were then co-stained for tenascin-C and fibronectin. 100ng/ml LPS was used to stimulate MDDCs when observing tenascin-C protein due to the comparatively lower sensitivity of the confocal microscopy method in observing tenascin-C compared to the qPCR and ELISA methods for absolute quantification. Stimulating MDDCs with 100ng/ml LPS produced more intense tenascin-C immunofluorescence staining and permits a more accurate observation of tenascin-C cellular and subcellular distribution.

Tenascin-C immunostaining was absent in unstimulated MDDCs over the 8, 24 and 48 hour time course, but was detected in permeabilised MDDCs 8 hours following stimulation with LPS; where individual cells exhibited very intense cytosolic staining that was almost completely abolished in non-permeabilised cells. At 24 and 48 hours following LPS stimulation, this strong cytosolic stain decreased to a faint staining that was mainly observed in permeabilised cells, with several small regions of tenascin-C and fibronectin co-localisation at 24 hours. In contrast fibronectin immunostaining was not induced by LPS stimulation. Fibronectin staining was most intense at 8 hours following plating and decreased from 24 to 48 hours. At 8 hours, fibronectin staining was predominantly extracellular as intense staining was observed in both permeabilised and non-permeabilised MDDCs. NPCs were negative for non-specific secondary antibody staining (Figure 3.12).

With the optimised stimulus of 100ng/ml LPS, and an appropriate 8 hour time point at which to observe the tenascin-C and fibronectin distribution in MDDC cultures in greater detail, confocal immunofluorescence microscopy was performed and Z-stacks were collated. Compiling the 0.2µm step-size scans, permitted the construction of a 3-dimensional image of the MDDC culture to establish where the tenascin-C is located. Co-staining of tenascin-C and fibronectin in unstimulated MDDCs at 8 hours, showed no detectable tenascin-C immunofluorescence, but fibronectin was present in both permeabilised and non

permeabilised cells (Figure 3.13.A-B) Fibronectin staining took two forms, the first was a diffuse cytosolic staining, and the second is distributed over the cell surface in patches of intense staining. When non-permeabilised MDDCs were stimulated with 100ng/ml LPS, no tenascin-C immunostaining was visible (Figure 3.13.C); however the fibronectin staining was increased relative to the unstimulated MDDCs, but followed the same pattern of intense patches of staining on the cell surface. When LPS stimulated MDDCs were permeabilised, intense cytosolic immunostaining of tenascin-C was visible (see white arrow: Figure 3.13.D). This staining pattern appeared to take the form of dense foci within the bounds of the cell. Intracellular fibronectin staining was also increased relative to the unstimulated MDDCs, and appeared as a diffuse stain across the cytosol, with more intense patches at the edges of the cells. The tenascin-C observed was not distributed solely on the top or bottom of the cell layer, and at 8 hours post stimulation there was no detectable tenascin-C bound to the exterior of the MDDCs or the glass slides.

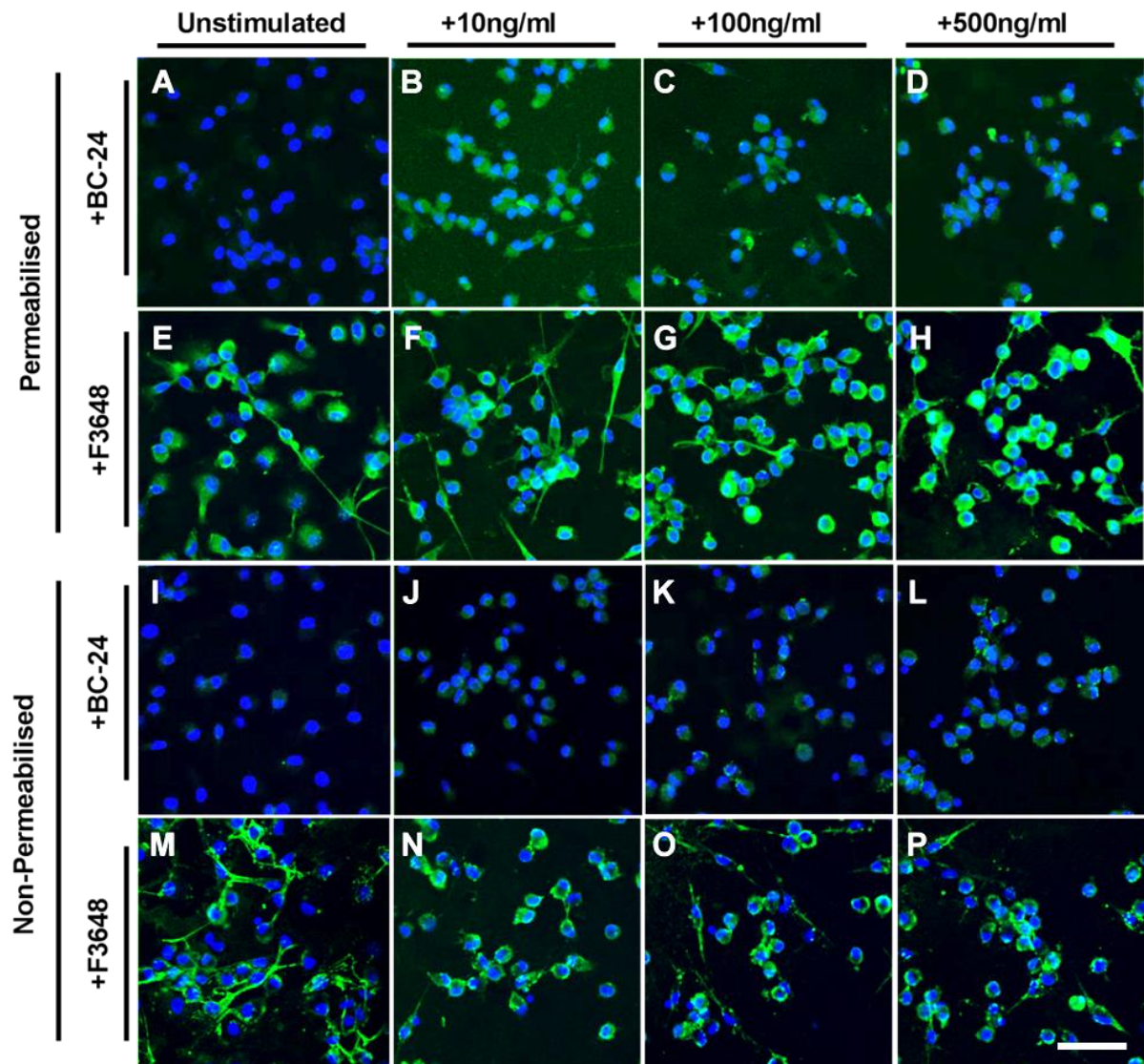


Figure 3.10. LPS induces dose dependent accumulation of cell associated tenascin-C. Primary human MDDCs were plated and rested for 16 hours prior to stimulation with 0, 10, 100 or 500ng/ml LPS for 8 hours. Cells were permeabilised (A-H), or non-permeabilised (I-P), and were immunostained for tenascin-C with BC-24 mAb (A-D, I-L), or for fibronectin with F3648 mAb (E-H, M-P), as indicated on the left of the figure. All slides were stained with DAPI (blue) alongside the secondary antibodies. Representative images from 3 experiments are shown. Images were visualized with the Olympus FV1200 IX83 Confocal System, with a 60x objective. Scale bar is 20µm.

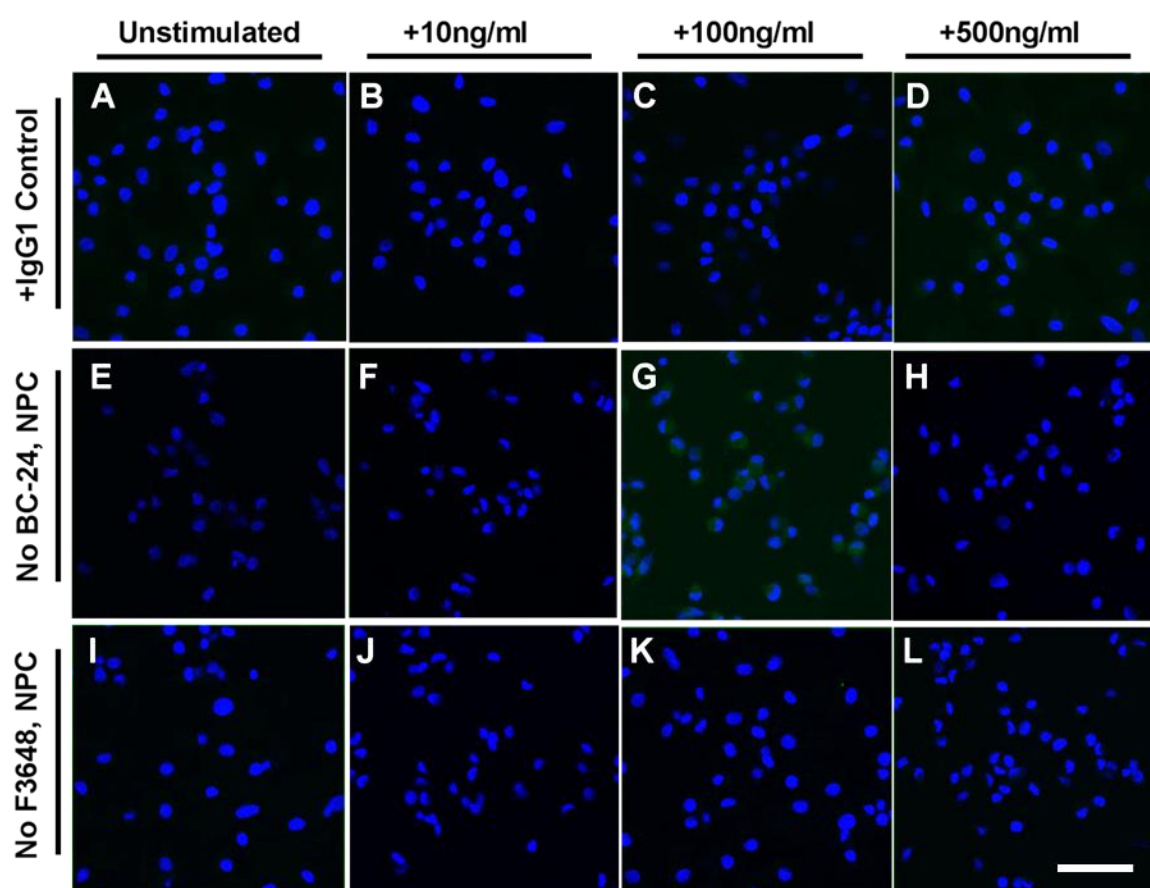


Figure 3.11. Negative controls confirming the specificity of primary and secondary immunostaining antibodies for MDDCs. Primary human MDDCs were plated and rested for 16 hours prior to stimulation with 0, 10, 100 or 500ng/ml LPS for 8 hours. The cells were permeabilised immunostained with IgG1 isotype control antibody (A-D), and NPCs were stained in the absence of BC-24 mAb (E-H), and F3648 mAb (I-L) primary antibodies, but with the corresponding secondary antibodies. All slides were stained with DAPI (blue) alongside the secondary antibodies. Representative images from 3 experiments are shown. Images were visualized with the Olympus FV1200 IX83 Confocal System, with a 60x objective.

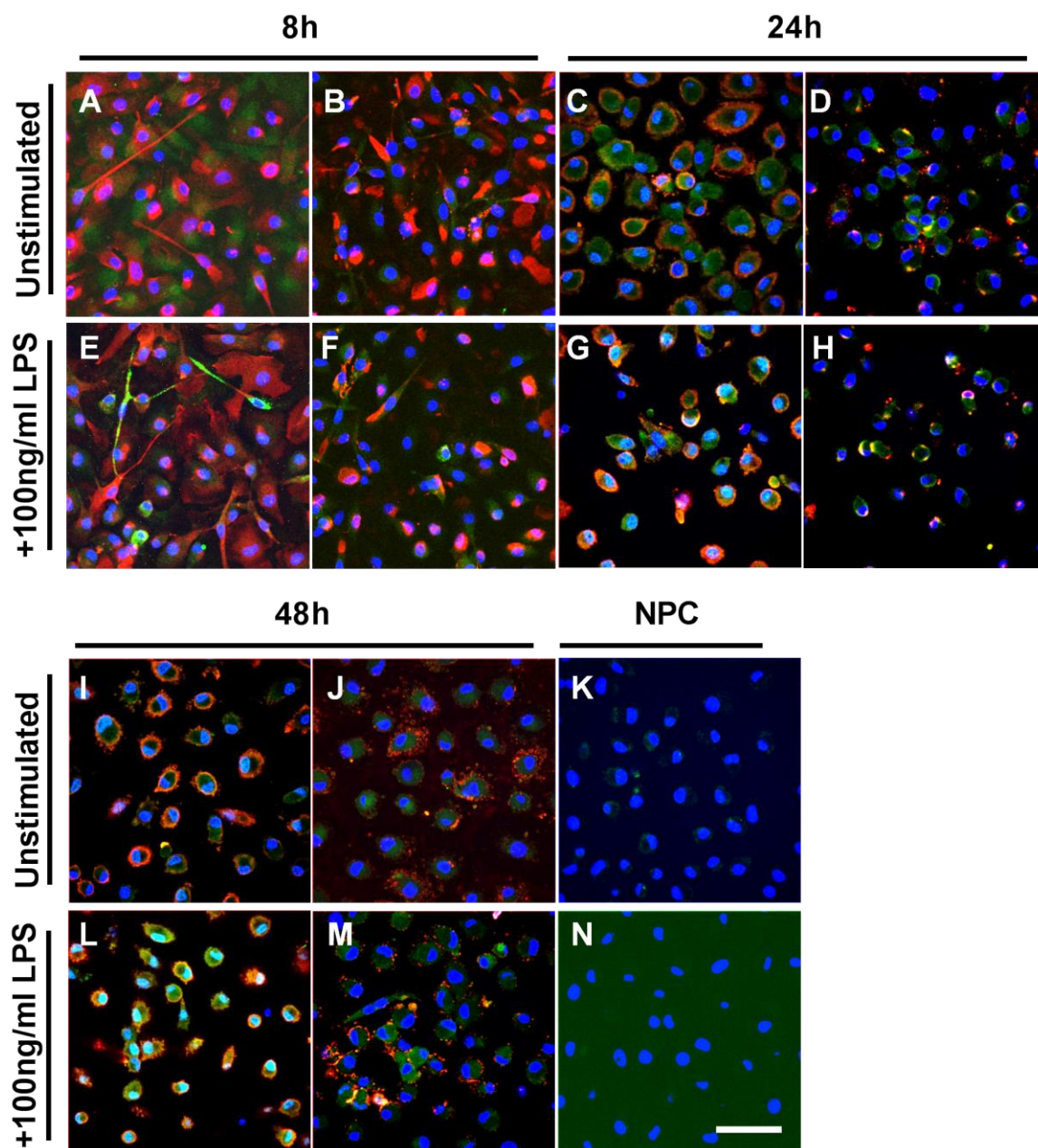


Figure 3.12. Tenascin-C transiently localises to MDDCs 8 hours following LPS stimulation. The cellular distribution of tenascin-C and fibronectin was assessed in primary human MDDCs that were plated and rested for 16 hours prior to stimulation with 100ng/ml LPS for 8, 24 and 48 hours. Cells were fixed, and were either permeabilised (A,C,E,G,I,K,L,N) or non-permeabilised (B,D,F,H,J,L,M) and co-stained for tenascin-C with BC-24 mAb (green), and fibronectin with F3648 mAb (red) in unstimulated cells (A-D,I,J), or 100ng/ml LPS stimulated cells (E-H,L,M). NPCs were performed on unstimulated (K) and 100ng/ml LPS stimulated 8h MDDC slides (N). All slides were stained with DAPI (blue) alongside the secondary antibodies. Representative images from 3 experiments are shown. Images were visualized with the Olympus FV1200 IX83 Confocal System, with a 60x objective. Scale bar shows 20µm.

Figure 3.13. Tenascin-C is trafficked through the MDDC cytosol 8 hours after LPS stimulation. See next page for figure legend.

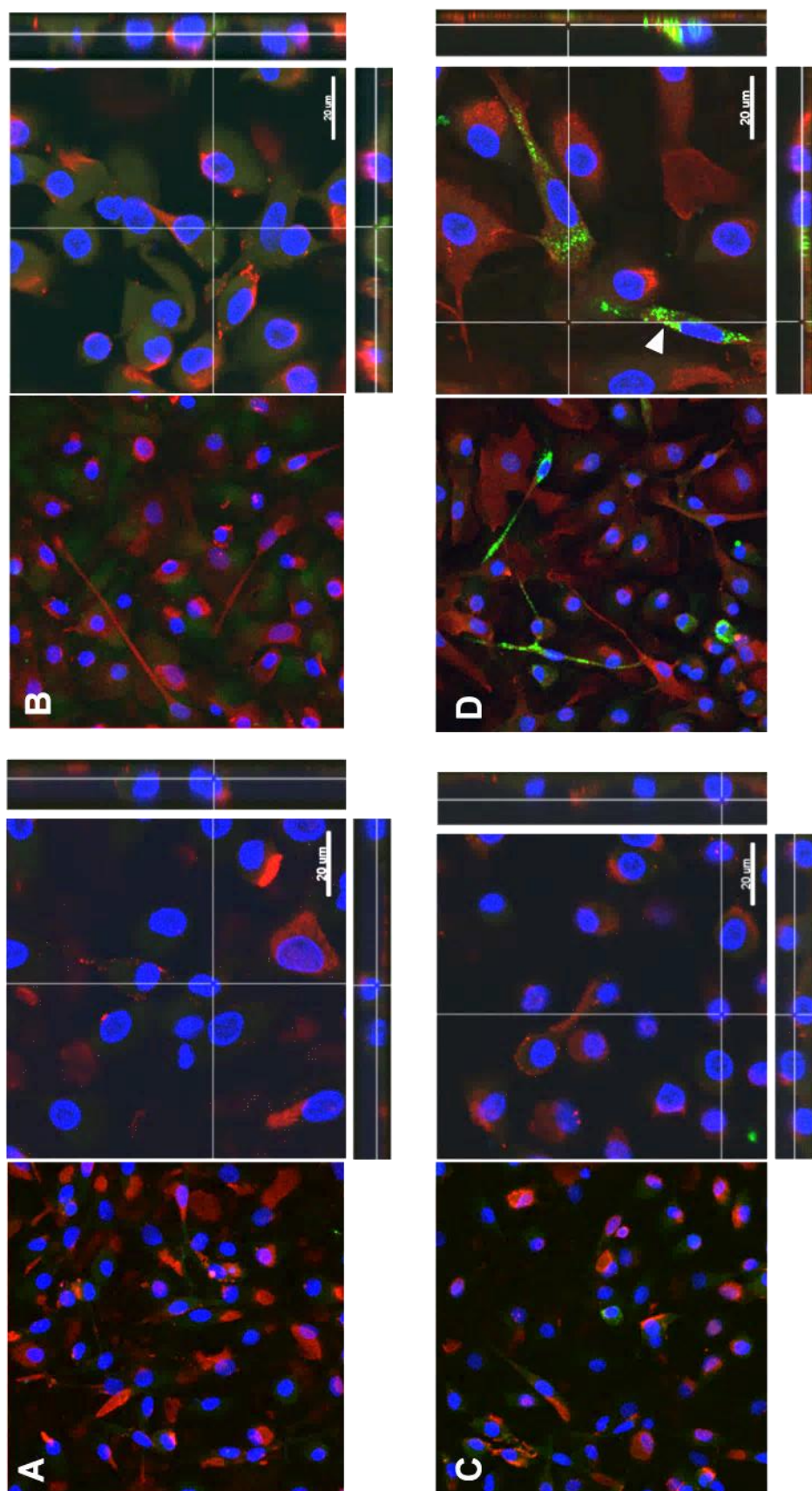


Figure 3.13 Tenascin-C is trafficked through the MDDC cytosol 8 hours after LPS stimulation. The cellular distribution of Tenascin-C and fibronectin was assessed in primary human MDDCs that were plated and rested for 16 hours prior to stimulation with 100ng/ml LPS for 8 hours. Cells were fixed and co-stained for tenascin-C (green) and for fibronectin (red) in unstimulated and non-permeabilised (A), unstimulated and permeabilised (B), stimulated +100ng/ml LPS, non-permeabilised (C), stimulated +100ng/ml LPS permeabilised (D) MDDCs. All slides were stained with DAPI (blue) alongside the secondary antibodies. For each condition, the figure shows a 60x objective image on the left, and a 120x z-stack cross section on the right. Representative images were visualised using the Olympus FV1200 IX83 Confocal System. Z-stack cross sections were compiled from 0.2µm step size scans. Images shown are representative of images acquired from 5 experiments. The scale bar is 20µm.

Together these data show that transcription of tenascin-C mRNA is transiently induced following cell activation in MDDCs, GM-CSF and M-CSF macrophages in response to cell activation. Tenascin-C protein in these MDDCs is transiently trafficked through the cytosol, and is predominantly secreted in a soluble form where it does not become cell associated.

3.6 Tenascin-C expression in primary human neutrophils

Previously a report has proposed tenascin-C as a mediator of pathogenic events in liver ischemia/reperfusion injury, where tenascin-C induced MMP-9 expression in neutrophils via TLR4 activation²⁶⁵. To determine whether neutrophils can make tenascin-C, or if they only respond to exogenous tenascin-C the biosynthesis of tenascin-C was investigated in primary human neutrophils.

Tenascin-C mRNA levels were quantified in primary human neutrophils immediately following isolation, and at 4 and 6 hours following stimulation with well characterised neutrophil activators LPS³¹⁶ and GM-CSF³²⁸. Neutrophil mRNA and protein could only be isolated for 0, 4 and 6 hour time points due to the rapid deterioration of neutrophil viability in cell culture beyond 6 h.

Neutrophils contained no detectable tenascin-C mRNA at any time point following stimulation with LPS or GM-CSF, but did show low constitutive expression of endogenous control gene HPRT1, demonstrating that cDNA quality was sufficient for gene expression analysis. Interleukin-1 receptor antagonist (IL-1RN) mRNA was previously reported to be upregulated by LPS and GM-CSF stimulation in neutrophils, and here its induction was quantified relative to HPRT1, revealing a large and statistically significant upregulation at 4 and 6 hours following LPS and GM-CSF treatment, confirming neutrophil activation by both stimuli (Figure 3.14.A-C).

Due to the lower levels of detection achieved using the USCN ELISA in MDDCs, the FnIII-B ELISA was used to quantify tenascin-C in neutrophil cell lysate and supernatant. However, the tenascin-C levels were below the limit of detection for the FnIII-B ELISA kit, even in undiluted samples. In the cell lysate, the neutrophil samples are compared against a U-87 MG positive control sample, while in the supernatant the concentration of tenascin-C detected

falls beneath what is usually observed in cell culture media supplemented with 10% foetal bovine serum (FBS), represented in the figure as 'Blank' (Figure 3.14.D-E).

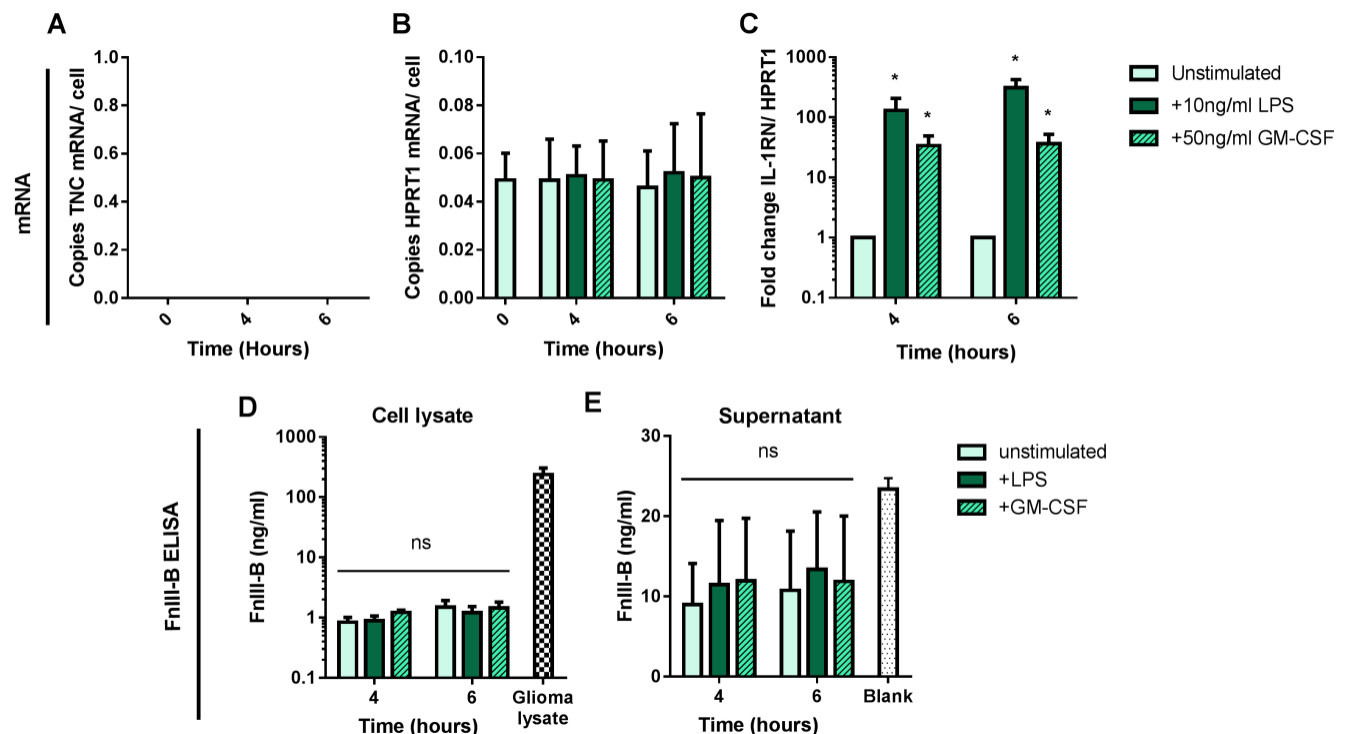


Figure 3.14. Tenascin-C mRNA and protein is not detected in resting or LPS/GM-CSF activated neutrophils. Primary human neutrophils were isolated and stimulated with 10ng/ml LPS, 50ng/ml GM-CSF or were left unstimulated for 0, 4 and 6 hours by the laboratory of Dr. Lynne Prince, University of Sheffield. Here I performed the quantification of A) tenascin-C mRNA copies/cell (A), HPRT1 mRNA copies/cell (B) and the fold induction of IL-1RN (C) using standard curve qPCR and $\Delta\Delta C_t$ qPCR. Tenascin-C protein was quantified using the FnlII-B ELISA kit in cell lysate where it is shown alongside a sample of U87-MG glioma cell lysate (D), and supernatant, where it is shown alongside a sample of neutrophil cell culture media (E). Data are shown as mean + SEM (n=5). Un-paired t-tests were performed and statistical significance is displayed *p<0.05 and non-significant (ns).

Together these data show that neutrophils do not biosynthesise tenascin-C at rest, or following cell activation with LPS or GM-CSF. Therefore any impact on neutrophil behaviour mediated by tenascin-C is likely to be due to exogenously expressed tenascin-C.

3.7 Characterizing tenascin-C biosynthesis in primary human T-cells

Previous studies have shown that tenascin-C is required for adequate Th17²⁶¹ and Th2⁵³ immune responses *in vivo*, and that tenascin-C modifies T-cell behaviour directly by supporting adhesion and rolling²⁹³, and by inhibiting anti-CD3 induced T-cell proliferation and cytokine expression¹⁵⁵. Previous work by our group also detected tenascin-C mRNA in total lymphocyte populations isolated from RA synovial membranes²⁷³, and therefore the extent of tenascin-C mRNA and protein biosynthesis in resting and activated primary human T-cells was assessed in this study.

Firstly, tenascin-C mRNA was quantified in CD3+ T-cells that were resting or stimulated with the anti-CD3/CD28 Dynabead® T-cell activator for 0, 1.5, 4, 8, 24 or 48 hours. CD3+ T-cells were initially selected for tenascin-C mRNA screening due to the broad expression of this marker across T-cell subsets. The Dynabead® cell activator was utilised as a stimulus, due to its ability to closely mimic the physiological activation of T-cells by antigen presenting cells (APCs) by co-presenting a T-cell with an anti-CD3 and anti-CD28 co-receptor stimulus on an APC sized magnetic bead, which is well described in the literature³¹⁸. Tenascin-C mRNA was expressed at low basal levels in CD3+ T-cells and was increased following stimulation with anti-CD3/CD28 after 24 hours, with a slight reduction by 48 hours post stimulation (Figure 3.15.A).

In order to identify which T-cell subset is responsible for synthesising tenascin-C in the CD3+ population, and to extend the time points examined, tenascin-C mRNA was quantified in CD4+ and CD8+ T-cells that were either unstimulated or stimulated with anti-CD3/CD28 Dynabead® T-cell activator for 0h, 1.5h, 4h, 8h, 1d, 2d, 3d, 4d, 7d, and 10 days. CD4+ T-cells contained negligible tenascin-C mRNA levels basally, but these levels increased significantly at day 1, and peaked at day 2 when stimulated with anti-CD3/CD28. The fold-

induction in tenascin-C mRNA relative to the initial 0h time point, and normalised to the endogenous control gene YWHAZ, was large and statistically significant; but was transient and gradually reduced to levels below the baseline by day 7 (Figure 3.15.B-C).

In CD8⁺ T-cells the basal tenascin-C mRNA expression was also negligible, but increased following stimulation with anti-CD3/CD28 at day 1 and peaked at day 2 post stimulation. The absolute level of tenascin-C mRNA was significantly higher than the baseline at day 2 and 3 following stimulation, but decreased gradually between days 3 and 10. The fold induction in tenascin-C mRNA relative to the 0h time point, and normalized to YWHAZ is larger than in CD4⁺ T-cells, and peaked at day 2 before gradually decreasing to day 10, but started from a lower absolute baseline level, meaning that CD4⁺ T-cells biosynthesised a higher total amount of tenascin-C mRNA copies/cell than CD8⁺ T-cells (Figure 3.15.D-E).

Tenascin-C protein was assessed in the cell lysate and supernatant of resting and activated CD4⁺ T-cells using the FnIII-B ELISA kit but could not be accurately quantified due to the low abundance of tenascin-C in the samples, meaning the concentration was below the ELISA's limit of detection (Figure 3.15.F-G). Similarly the tenascin-C concentration in CD8⁺ T-cell cell lysate and supernatant samples was below the limit of detection of the FnIII-B and USCN ELISA kits so could not be accurately quantified (Figure 3.15.H-K).

Figure. 3.15. Tenascin-C mRNA but not protein is upregulated in activated T-cells. See next page for figure legend

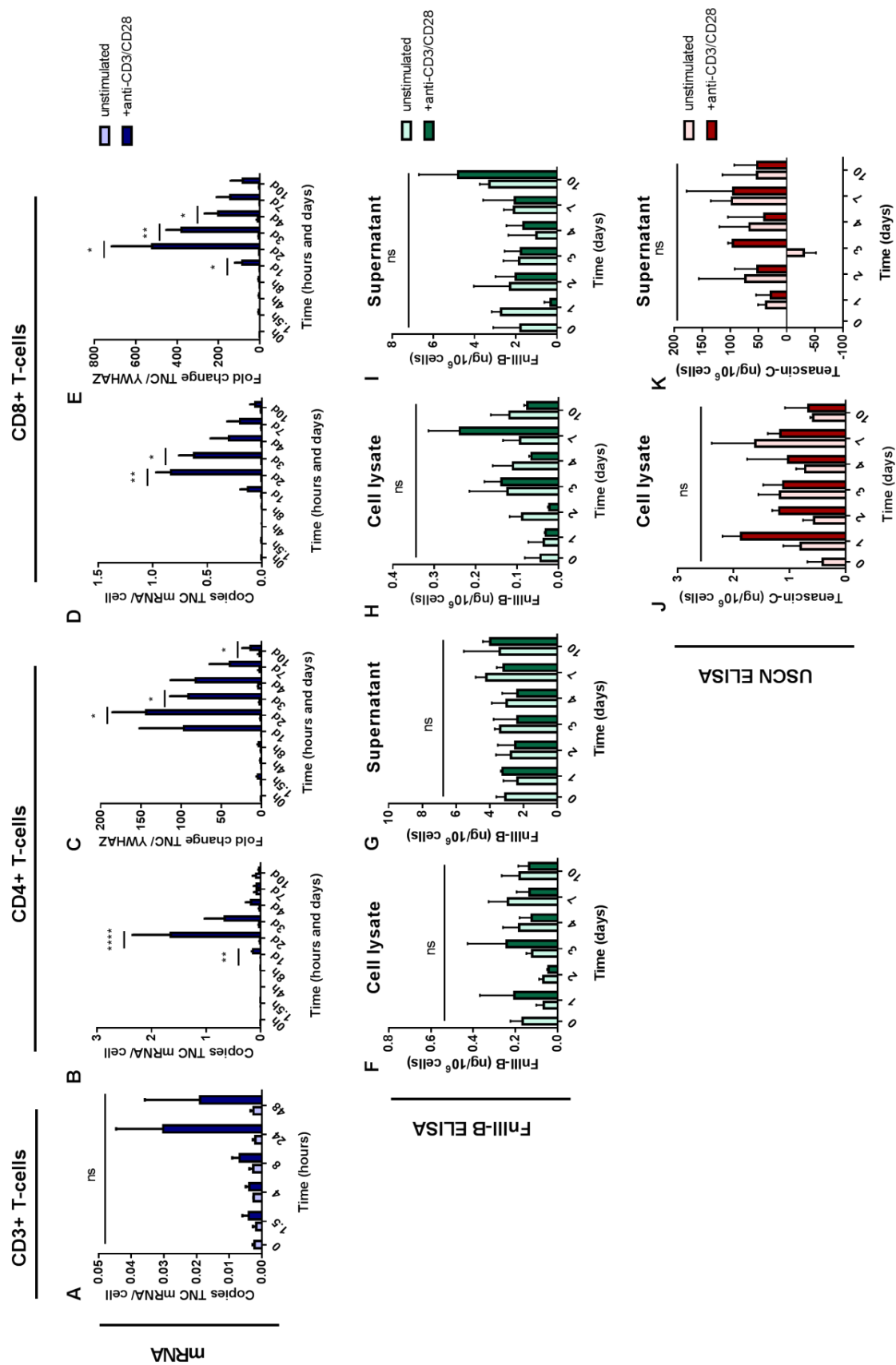


Figure 3.15 Tenascin-C mRNA but not protein is upregulated in activated T-cells. Primary human T-cells were purified from peripheral blood, plated and rested for 16 hours and were either not stimulated or stimulated with anti-CD3/CD28 DynaBeads® T-cell activator at a 1:1 cell:bead ratio. Tenascin-C mRNA was quantified by standard curve qPCR in CD3+ T-cells (A) stimulated for t=0, 1.5, 4, 8, 24 and 48 hours (n=3). Tenascin-C mRNA was quantified, and the fold induction of tenascin-C mRNA/YWHAZ was calculated in CD4+ (B-C) and CD8+ (D-E) T-cells, stimulated for t=0, 1.5, 4, 8 hours, and 1, 2, 3, 4, 7 and 10 days (n=5). Tenascin-C protein was quantified by FnIII-B ELISA in CD4+ T-cell cell lysate (F), supernatant (G), and in CD8+ T-cell cell lysate (H), supernatant (I) (n=3). Tenascin-C protein was quantified using the USCN ELISA in CD8+ T-cell cell lysate (J), and supernatant (K) (n=3). Data are shown as mean + SEM. Un-paired t-tests were performed between unstimulated and stimulated time points and statistical significance is displayed; *p<0.05, **p<0.01, ***p<0.0001 and non-significant (ns).

These data have shown T-cells contain low resting levels of tenascin-C mRNA that are transiently increased following activation; however despite detecting tenascin-C, resting and activated T-cells did not express detectable levels of tenascin-C protein.

3.8 Inducible expression of tenascin-C by IL-1 β in HDFs

Having examined tenascin-C synthesis in a range of different immune cells, the next aim was to compare expression in these cell lineages to stromal cells. Previously, tenascin-C mRNA has been detected in dermal fibroblasts by Northern blotting^{329, 330, 331}, and is up-regulated in HDFs in response to stimulation by IL-1 β ³¹⁹. However, no systematic analysis of tenascin-C biosynthesis has been performed on HDFs following cell activation with stimuli associated with inflammatory states.

Firstly to better characterise tenascin-C expression induced by IL-1 β , HDFs were cultured and either not stimulated or stimulated for 0, 4, 8, 24, 48, 72, 96 and 120 hours with 10ng/ml IL-1 β , as was previously reported to induce tenascin-C mRNA synthesis³¹⁹. Basal tenascin-C levels in HDFs were higher than in U-87 MG cells, and were significantly elevated at 24, 48 and 72 hours post stimulation with IL-1 β , although they remained elevated above baseline levels for 120 hours. For normalisation of TNC mRNA induction, the endogenous control gene RPLP0 was selected due to its stable abundance over the experimental conditions. Tenascin-C mRNA induction was found to be statistically significant and transient from 4 to 48 hours, decreasing 72 hours post stimulation (Figure 3.16.A-B).

Previously I showed that LPS stimulation induced tenascin-C expression in monocytes, macrophages and MDDCs. Tenascin-C mRNA was quantified following LPS stimulation of HDFs at 0, 4, 8 and 24 hour time points, but no induction was observed (Figure 3.16.C). As a positive control for LPS activation of HDFs, the fold induction of IL-8 mRNA relative to the stably expressed control gene ACTB was quantified 8 hours post stimulation with 0, 1, 10 and 100ng/ml LPS. This stimulation revealed a statistically significant LPS dependent increase in IL-8 mRNA, confirming the HDFs were activated by LPS (Figure 3.16.D).

Tenascin-C protein levels were assessed by FnIII-B ELISA, and revealed a steady accumulation of tenascin-C protein in the cell lysate from 0 to 120 hours, although slightly more tenascin-C accumulated at the 72 to 120 hour time points in IL-1 β stimulated HDFs. In the supernatant, there was no change in tenascin-C protein levels between 0 and 8 hours following stimulation, and between 24 to 120 hours there was a steady accumulation of tenascin-C in the unstimulated HDF supernatant. Stimulation of HDFs with IL-1 β induced an additional increase in the accumulation of tenascin-C in the supernatant between 24 and 120 hours that was significantly higher than in unstimulated cells. The FnIII-B ELISA reveals that at peak expression in HDFs, tenascin-C is predominantly secreted to the supernatant (Figure 3.16.E-F).

Quantification of tenascin-C protein by USCEN ELISA in stimulated and unstimulated HDF cell lysates revealed no overall change in tenascin-C abundance between 0 and 48 hours post stimulation, with a slight IL-1 β independent increase at 72 hours. In the supernatant, tenascin-C steadily accumulated over time, peaking at 72 hours in an IL-1 β dependent manner. The USCEN ELISA also revealed that in HDFs the tenascin-C is predominantly secreted to the supernatant (Figure 3.16.G-H).

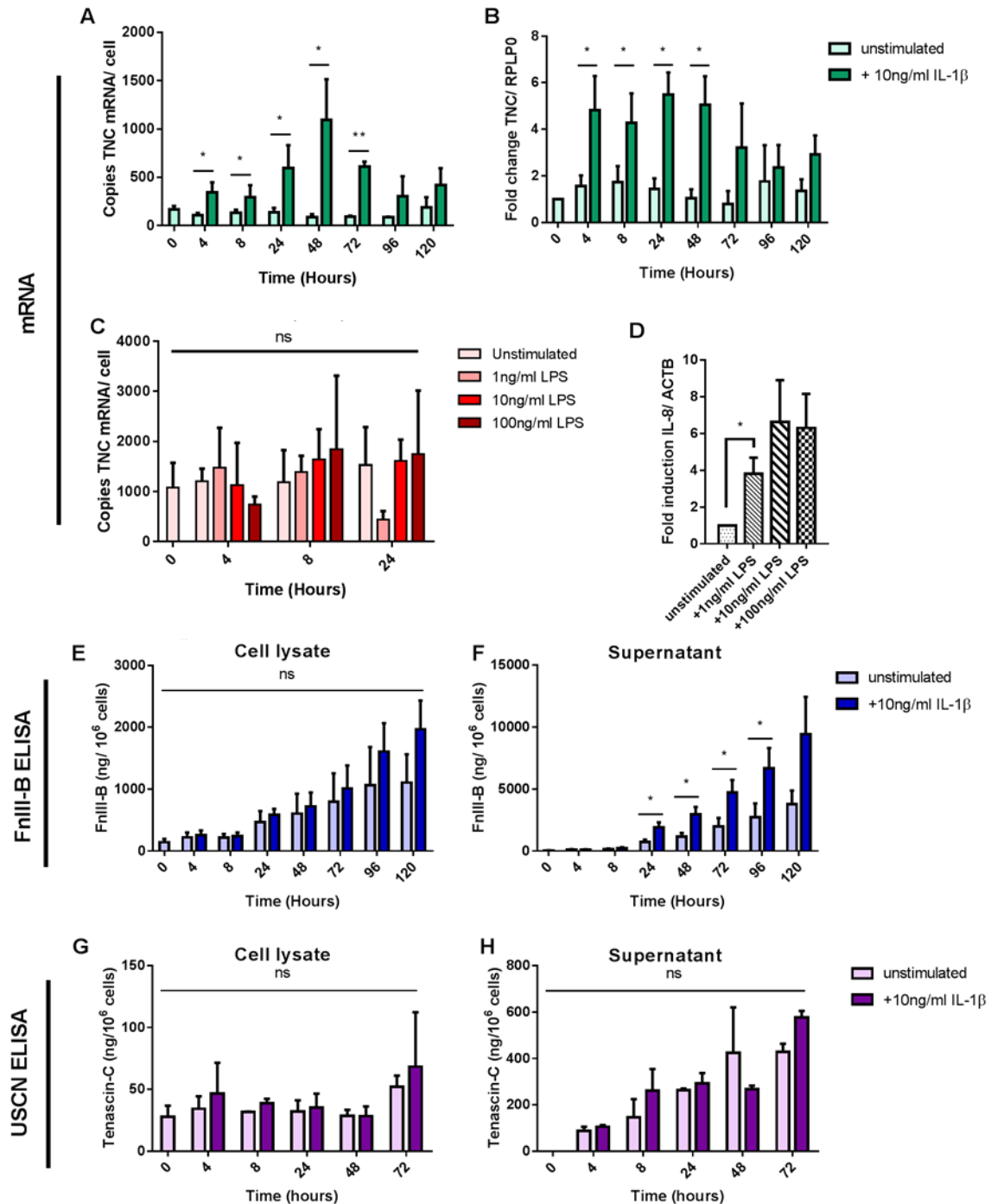


Figure 3.16. IL-1 β induces tenascin-C expression and secretion in HDFs. (A,B) HDFs were plated and either left unstimulated, or were stimulated with 10ng/ml IL-1 β for 0, 4, 8, 24, 48, 72, 96 and 120 hours. At each time point tenascin-C mRNA was quantified by standard curve qPCR (n=5) (A), and fold induction of TNC mRNA relative to RPLP0 was calculated (n=5) (B). (C,D) HDFs were plated and stimulated with 1, 10 or 100ng/ml LPS for 0, 4, 8 and 24 hours. Tenascin-C mRNA was quantified by standard curve qPCR in HDFs stimulated with 100ng/ml LPS for 0, 4, 8 and 24 hours (C). The fold induction of IL-8 mRNA relative to ACTB at 8 hours was calculated by $\Delta\Delta C_t$ qPCR (D). (E-H) Tenascin-C protein was quantified in HDFs stimulated with 10ng/ml IL-1 β over time by FnIII-B ELISA in cell lysate (E) and supernatant (F) (n=5), and by USCN ELISA in cell lysate (F) and supernatant (G) (n=3). Data are shown as mean + SEM. Un-paired t-tests were performed between unstimulated and stimulated time points and statistical significance is displayed *p<0.05 or non-significant (ns).

There were no previous reports of tenascin-C immunolocalisation in fibroblast cell cultures in the literature, and therefore little is known of its cellular distribution. Here for the first time, tenascin-C and fibronectin are co-stained in HDF cell cultures over time, in the presence or absence of IL-1 β stimulation.

Fibronectin immunostaining was detected in permeabilised and non-permeabilised HDFs, and increased over time. At 4 hours, fibronectin staining was predominantly extracellular and was not regulated by IL-1 β stimulation. Between 24 and 72 hours, fibronectin staining intensity increased independently of permeabilisation or IL-1 β stimulation, and formed extracellular fibrils that run along the length of the HDFs, forming a striated pattern determined by HDF cell polarity. The strongest fibronectin staining was observed in the IL-1 β stimulated, 72 hour permeabilised cell culture where intensely stained extracellular fibrils and fainter intracellular staining was observed. Tenascin-C staining was detected in HDFs from 4 hours, and in non-permeabilised cells was independent of IL-1 β stimulation. However, IL-1 β treatment increased cytosolic tenascin-C staining which co-localised with fibronectin. Stimulation of HDFs with IL-1 β increased tenascin-C staining in permeabilised and non-permeabilised cells at 48 and 72 hour time points. The tenascin-C staining here was predominantly extracellular and co-localised to fibronectin in extracellular fibrillar structures in images taken of HDF cultures beyond 24 hours. The only regions of tenascin-C staining that are not co-localised to fibronectin are faint intracellular regions in permeabilised cells. This suggests that the majority of cell associated tenascin-C expressed by HDFs is incorporated into extracellular matrices rich in fibronectin (Figure 3.17).

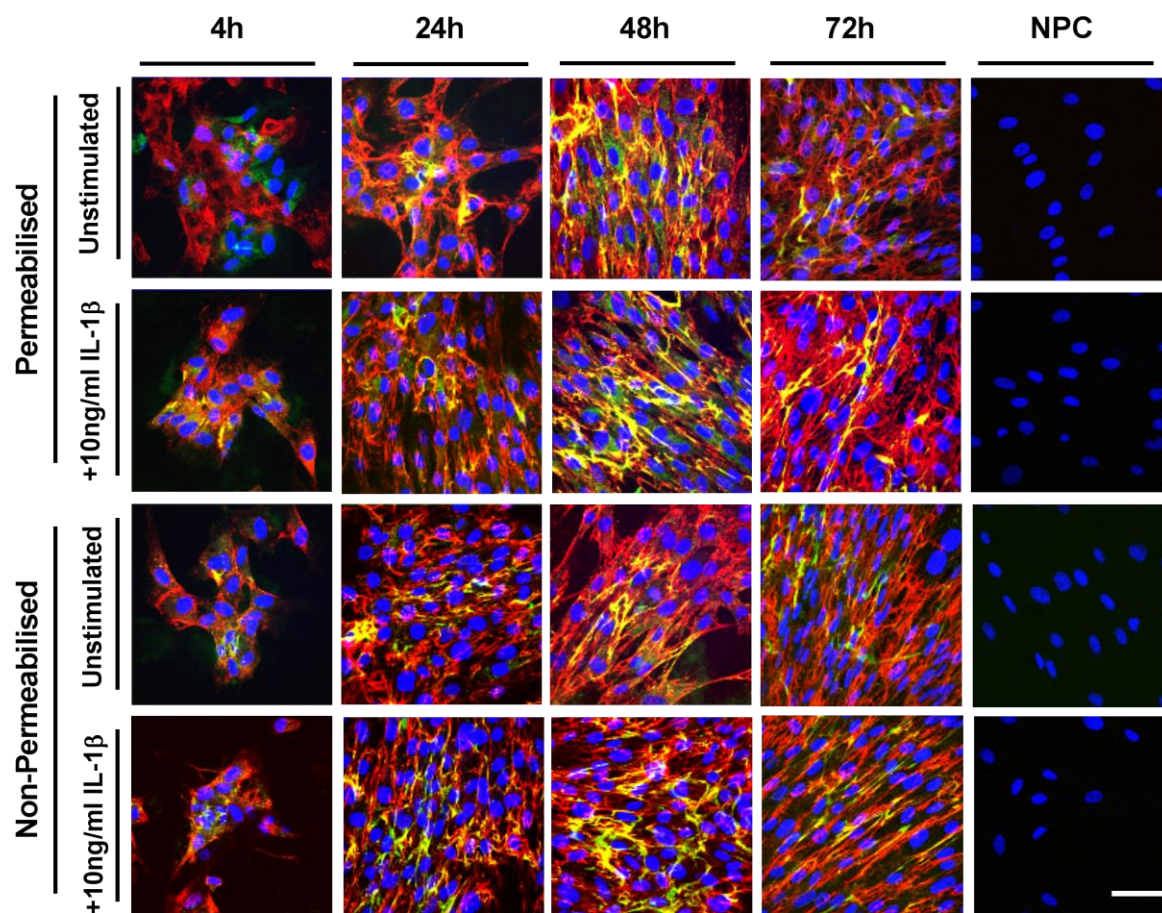


Figure 3.17 Tenascin-C accumulation in the extracellular matrix of HDFs is increased by IL-1 β stimulation. Tenascin-C and fibronectin were immunolocalized in cell cultures that were either not stimulated, or stimulated with 10ng/ml IL-1 β for t=4, 24, 48 and 72 hours (indicated above top row). These slides were fixed and either not-permeabilised or permeabilised prior to co-staining for tenascin-C with BC-24 mAb (green), and fibronectin with F3648 mAb (red). All slides were stained with DAPI (blue) alongside the secondary antibodies. The conditions for cell resting, stimulation and permeabilisation are marked on the left of the image. NPCs were performed for each experiment to confirm the specificity of the secondary antibodies; see the far right column labelled NPC. Representative images from 3 donors (n=3) were visualised using the Olympus FV1200 IX83 Confocal System, with a 60x objective. Scale bar is 20 μ m.

To further examine the details of the tenascin-C rich matrix in HDFs, further observation with Z-stack confocal microscopy was performed using the 48h time point as this permitted the best visualisation of tenascin-C and fibronectin co-localisation. The Z-stack technique assembles a 3D image of the tenascin-C and fibronectin distribution around the HDF.

In non-permeabilised HDFs that were not stimulated, fibronectin and tenascin-C staining strongly co-localised in extracellular fibrils. Fibronectin staining was more widely distributed than tenascin-C, and tenascin-C staining was stronger at regions of intense fibronectin staining. Regions of tenascin-C deposition lay on the upper surface of the cells (Figure 3.18.A-B). Following stimulation with IL-1 β , the overall distribution of fibronectin and tenascin-C staining did not change, but tenascin-C staining was relatively stronger (Figure 3.18.C-D).

Permeabilised HDFs that were not stimulated displayed fibronectin staining in short fibrils, and faint cytoplasmic staining. Tenascin-C staining here did not co-localise with fibronectin, and appears as small extracellular foci of very intense staining (Figure 3.18.E-F). Following stimulation with IL-1 β , fibronectin and tenascin-C were co-localised in fibrils, with few regions where tenascin-C staining is detected alone. As was previously observed in unstimulated, non-permeabilised HDFs, the tenascin-C fibrils are deposited on the upper surface of the cells, and no strong tenascin-C immunostaining is detected on the bottom side (Figure 3.18.G, H).

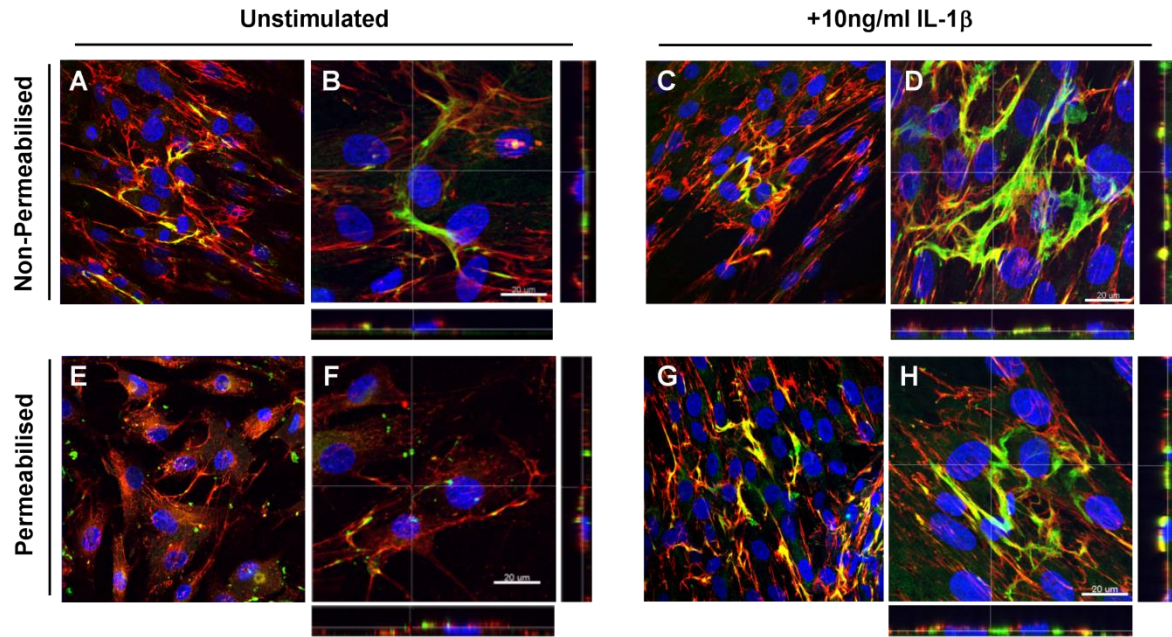


Figure 3.18. Tenascin-C incorporation into the HDF fibronectin matrix is increased following stimulation with IL-1 β . HDFs were left unstimulated, or were stimulated with 10ng/ml IL-1 β for 48 hours. Cells were fixed without permeabilisation (A-D), or were fixed and permeabilised (E-H) prior to staining for tenascin-C with BC-24 mAb (green), or fibronectin with F3648 mAb (red). All slides were stained with DAPI (blue) alongside the secondary antibodies. Representative images from 3 experiments were visualised using the Olympus FV1200 IX83 Confocal System, and for each condition, the figure shows a representative 60x objective image on the left, and a 120x Z-stack cross section on the right with two adjacent bars displaying Z-axis slices. Z-stack cross sections were compiled from n=45, 0.2 μ m step size scans.

These data show that HDFs exhibit constitutively high basal expression of tenascin-C, that was further induced by stimulation with IL-1 β , but not LPS. Over time, resting HDFs expressed tenascin-C protein, which accumulated in the cell lysate, but was predominantly secreted to the supernatant. The accumulation of cell associated tenascin-C was visible by immunofluorescence microscopy, where tenascin-C organised in fibrils and was incorporated into the fibronectin rich extracellular matrix.

3.9 Tenascin-C expression is higher in RA-SF relative to NSFs

It has been widely reported that tenascin-C mRNA and protein levels are elevated in disease. Previous findings have shown that tenascin-C expression is elevated in inflamed synovium³¹⁹, that tenascin-C accumulates in RA synovium *in vivo*²⁷³, and that circulating levels of tenascin-C are also elevated in RA patients³⁰⁷. Here I examined tenascin-C biosynthesis in cultured primary human rheumatoid arthritis synovial fibroblasts (RA-SFs), osteoarthritis synovial fibroblasts (OA-SFs) and in non-diseased NSFs, and compare to levels observed in HDFs. These fibroblast types were removed from their respective donors and were cultured *in vitro* up to passage 4, and therefore are no longer truly normal or diseased cell types, as they have been removed from their physiological and pathological milieus.

HDFs contained significantly higher tenascin-C mRNA levels than RA-SFs, OA-SFs and NSFs. RA-SFs contained more tenascin-C mRNA than OA-SFs but this was not statistically significant. Tenascin-C mRNA levels were significantly lower in NSFs compared to HDFs, RA-SFs and OA-SFs (Figure 3.19.A). Quantification of tenascin-C protein in the cell lysate fractions of RA-SFs, OA-SFs, NSFs and HDFs using the FnIII-B ELISA, revealed that HDFs express significantly more tenascin-C protein than RA-SFs, OA-SFs and NSFs, and that the tenascin-C protein levels were elevated in RA-SF relative to OA-SF cell lysate. NSFs expressed the least tenascin-C protein, though not significantly less than OA-SFs (Figure 3.19.B).

Confocal immunofluorescence revealed the cellular distribution of tenascin-C and fibronectin in permeabilised and non-permeabilised NSFs, RA-SFs and OA-SFs cultured for 48 hours. In non-permeabilised NSFs, tenascin-C was deposited in fibrillar structures that co-localise with fibronectin, and were widely distributed over the cell surface. In permeabilised NSFs slides, the organisation of fibronectin was punctate, and clustered around the edge of the cells in

small, dense foci of staining. Here, tenascin-C staining is barely observed, and was only detected as a faint shadow in the cytosol (Figure 3.19.C, F).

In non-permeabilised RA-SFs, tenascin-C and fibronectin co-localised in fibrillar extracellular structures, but there were also regions where tenascin-C immunostaining was solely detected. Some of these regions showed a dense fibrillar deposition of tenascin-C whereas others were less dense shaded areas where the staining covered the entire upper surface of the cells. In permeabilised cells, the fibronectin distribution remained unchanged compared to the non-permeabilised cells, but the tenascin-C staining comprised of small dense foci in the cytoplasm alongside the cell membrane. In RA-SFs, there was also a higher intensity of staining observed on the basal side of the cells; however this staining was relatively weak compared to the dense cytosolic foci (Figure 3.19.D,G).

OA-SFs exhibited less intense tenascin-C and fibronectin staining than NSF and RA-SFs, which in non-permeabilised OA-SFs was restricted to patches on the cell surface, with no fibrils visible. Fibronectin staining revealed short radiating fibres, with no tenascin-C co-localisation. In permeabilised cells, tenascin-C staining did not co-localise with fibronectin, and was present on the upper surface of the cells in a dense aggregate, with a faint green stain observed over the cell surface. Fibronectin distribution appeared to be dysregulated, punctate staining only present on the surface of the cell (Figure 3.19.E,H).

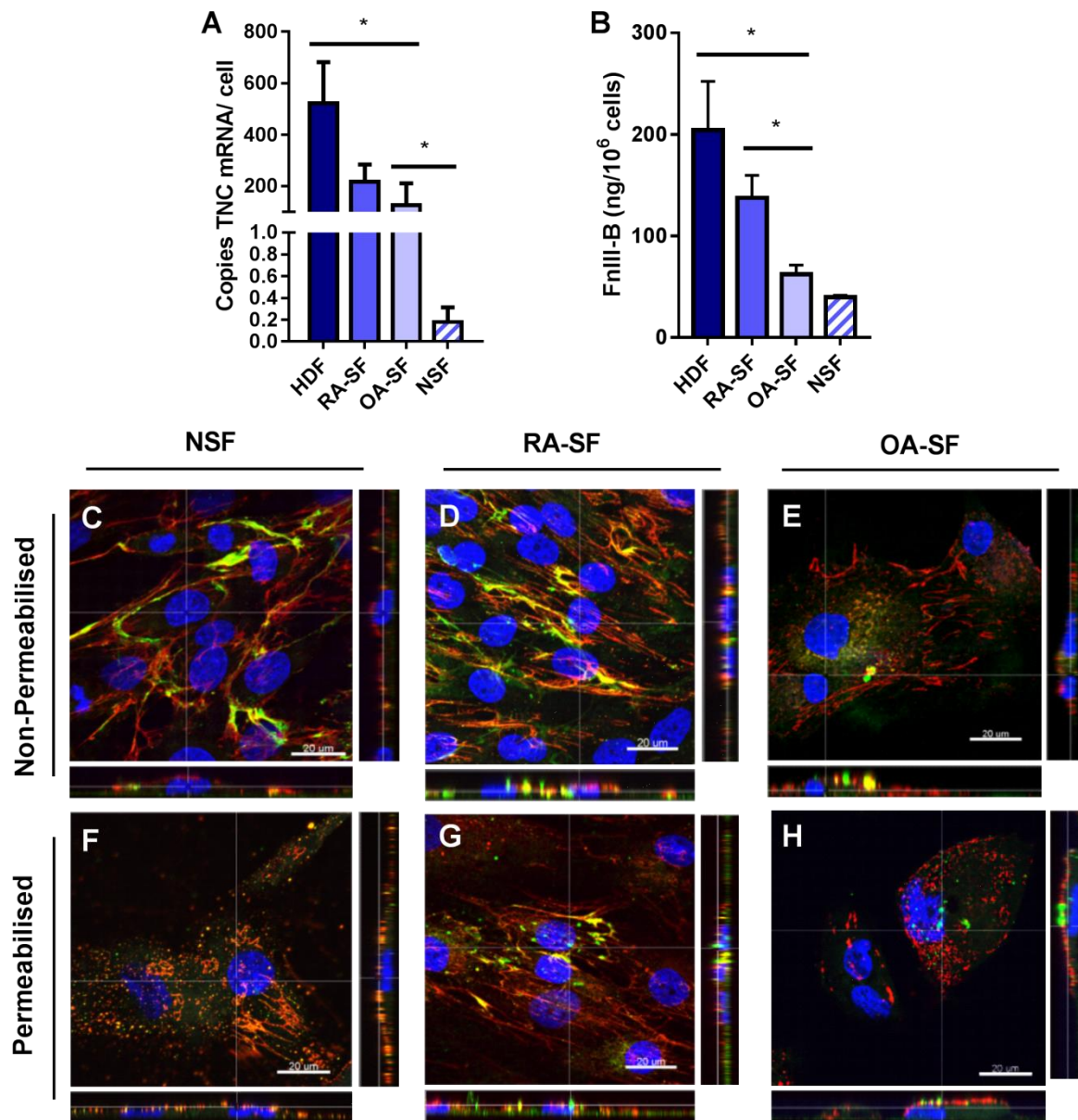


Figure 3.19. Comparing tenascin-C expression and cellular distribution in human NSFs, RA-SFs and OA-SFs. Primary human HDFs, NSFs, RA-SFs and OA-SFs were cultured for 48 hours, prior to quantifying tenascin-C mRNA by standard curve qPCR (A) and the quantification of tenascin-C protein in cell lysate using the FnIII-B ELISA kit (B); HDFs (n=4), NSFs (n=2), RA-SFs (n=3) and OA-SFs (n=3). Data are shown as mean + SEM. Un-paired t-tests were performed between cell types and statistical significance is displayed; *p<0.05. (C-H) Tenascin-C (green) and fibronectin (red) were immunolocalized in permeabilised and non-permeabilised NSF (n=2) (C,F), RA-SF (D,G) and OA-SF (E,H) (n=3) cell cultures rested for 48 hours. All slides were stained with DAPI (blue) alongside the secondary antibodies. NPCs were performed for each cell type. Images were visualised using the Olympus FV1200 IX83 Confocal System, with 60x objective and 2x zoom. Z-stack cross sections were compiled from n=45, 0.2µm step size scans.

Together these data show that tenascin-C expression is elevated in synovial fibroblasts during disease, and that the cellular organisation of tenascin-C in synovial fibroblasts differs according to cell type, with NSF and RA-SF appearing to deposit tenascin-C into fibrils, while in OA-SF, the distribution is punctate.

3.10 Summary

This chapter has shown that resting tenascin-C expression is cell-type specific, with U-87 MG and HDFs expressing high resting levels. This contrasts with monocytes, macrophages and MDDCs which express low levels, although here expression is greater than in T-cells; however neutrophils express no tenascin-C mRNA.

In U-87 MG cells, tenascin-C mRNA levels were always high; which contrasted firmly with observations in monocytes, macrophages and MDDCs, where, low tenascin-C mRNA levels were transiently upregulated following LPS stimulation, peaking around 4-8 hours following stimulation, and returning to baseline levels by 24 hours. Both CD4⁺ and CD8⁺ T-cells also exhibited large fold inductions in tenascin-C mRNA levels following stimulation with anti-CD3/CD28 Dynabeads®, however in contrast to monocytes, macrophages and MDDCs, the mRNA induction exhibited delayed and prolonged induction kinetics, with tenascin-C mRNA still elevated above baseline levels 7 days following stimulation. The peak levels of mRNA in activated CD4⁺ T-cells were comparable to that in activated M-CSF macrophages, but were lower than in resting U-87 MG cells. Neutrophils did not transcribe tenascin-C mRNA following stimulation with LPS or GM-CSF; contrasting with the observed induction of tenascin-C mRNA by LPS in monocytes, macrophages and MDDCs. In HDFs, the constitutively high resting tenascin-C mRNA levels were further induced by stimulation with IL-1 β , but not LPS; in line with observations in neutrophils. Furthermore, the transient IL-1 β induction kinetics were delayed relative to that seen in activated MDDCs, and had not returned to baseline levels by 120 hours after stimulation.

Resting U-87 MG cells predominantly accumulated tenascin-C protein in the cell lysate where it was mainly extracellular and punctate in organisation, but it was also abundantly detected in the supernatant. MDDCs however, showed a transient accumulation of tenascin-C in the cell lysate following LPS stimulation that was predominantly trafficked to the supernatant between 8-72 hours following stimulation. In contrast to HDFs, very little tenascin-C expressed by MDDCs remains cell associated with no tenascin-C fibrils or fibronectin co-localisation observed. In neutrophils, and T-cells, tenascin-C concentrations were too low to detect in the cell lysate or supernatant; this was despite the peak tenascin-C mRNA levels in T-cells being comparable to those in MDDCs at rest, where tenascin-C protein was detected.

NSFs expressed lower levels of tenascin-C mRNA, than detected in resting MDDCs; but expressed higher levels of protein. Tenascin-C expression was elevated in the diseased synovial fibroblasts relative to the NSFs, with the highest expression detected in RA-SFs; though the tenascin-C mRNA copies/cell were lower than detected in HDFs. Similarly to HDFs, tenascin-C expressed by RA-SFs and NSFs was organised into fibrils that co-localised with fibronectin; however in OA-SFs, less cell associated tenascin-C was detected, and a punctate expression pattern was observed that was more similar to that seen in U-87 MG cells.

Altogether, regulation of tenascin-C biosynthesis and cellular distribution is remarkably different in cells of different lineage and this has been systematically characterised here for the first time.

3.11 Discussion

This chapter details the first systematic characterisation of tenascin-C mRNA and protein biosynthesis in any cell type to date, and has served as a wide-ranging screen for tenascin-C expression in a range of immune cells and fibroblasts where very little was known about tenascin-C synthesis or cellular distribution. Methods for the quantification of tenascin-C mRNA, protein and tenascin-C visualisation by confocal immunofluorescence microscopy had not been previously established, and these protocols were optimised here. The methods described in this chapter were carefully selected with an emphasis on the absolute quantification of tenascin-C mRNA and protein in order to permit the direct comparison of tenascin-C synthesis data across cell types.

3.11.1 *Methods and Technical Justifications*

3.11.1.1 *qPCR protocols*

To quantify tenascin-C mRNA I adopted the standard curve qPCR method, as this gave the advantage of being able to quantify absolute levels of tenascin-C mRNA present in individual cells, and permitted direct comparison across different experiments and cell types. In order to do this, a TaqMan[®] qPCR assay was used, which amplified a short amplicon corresponding to a constitutively expressed exon spanning region of the FBG domain; allowing all tenascin-C mRNA transcripts to be detected. Samples were quantified against a purified tenascin-C plasmid (IRCFp5008F0112D; Source Bioscience), containing all constitutively expressed exons. Previous studies have shown that circularized DNA plasmids were the most robust qPCR standards for long term study purposes^{332, 333}; therefore for this study small concentrated aliquots were stored at -80°C to reduce freeze thaw damage, and provide consistent quality of the standard. In accordance with good qPCR practice guidelines, the same qPCR instrument was used to perform all of the analyses³³⁴. Limitations of the

technique may include differences in PCR amplification efficiency between plasmid DNA standards and sample cDNAs, resulting in inaccurate quantification of absolute mRNA copy numbers, but this would still allow relative comparisons between copy numbers across cell types.

In this study endogenous control genes were used to normalise the fold inductions in tenascin-C mRNA levels. In all cases, the absolute abundance of the endogenous control gene mRNA was quantified by standard curve qPCR using commercially available DNA plasmids; to assess the stability of expression. In this study, tenascin-C mRNA levels were normalised to one stably expressed control gene, but other studies have highlighted the improved accuracy of normalising gene expression to the geometric mean of multiple control genes³³⁵; however this was not possible in this study, as it was difficult to find even one stably expressed control gene for many of the cell types assessed. The relative abundance of control genes should closely resemble that for the target gene for optimal relative quantification. However, due to the low abundance of tenascin-C mRNA in monocytes, macrophages, MDDCs and T-cells, control gene levels were higher. These limitations were overcome by the 2-standard curve qPCR method for normalising gene expression, as normalisation was performed on absolute mRNA abundances, rather than relative Ct values.

3.11.1.2 ELISA protocols

In order to compare tenascin-C expression across cell types, tenascin-C was quantified using 2 commercially available ELISA kits. The first was the USCN ELISA, which had a standard curve with a small dynamic range, but detected a constitutively expressed tenascin-C epitope. The second kit was the FnIII-B ELISA which had a large dynamic range, but only detected tenascin-C variants containing the alternatively spliced FnIII-B domain.

Both ELISAs detected different quantities of tenascin-C in the same samples, with the USCN often detecting less than the FnIII-B ELISA. This is probably due to different antibody affinities for tenascin-C binding, and the inclusion of different tenascin-C standards in the two ELISA kits. Differences in quantification may also have occurred due to post translational modifications or proteolytic cleavage of the expressed tenascin-C. As a result of the kits quantifying tenascin-C against different standards, the absolute levels detected by the two ELISAs cannot be directly compared, but may be compared across cell types when using the same ELISA. Due to differences in the total volume of samples obtained from different cell types, tenascin-C quantification was instead normalised to the cell number as ng/ 10⁶ cells.

3.11.1.3 Confocal immunofluorescence microscopy protocols

Confocal immunofluorescence microscopy was performed as a method for localizing tenascin-C distribution in cell cultures, and as a way of complementing the findings of the ELISA; which quantified the amounts of tenascin-C present. Previously published protocols for immunostaining tenascin-C with the mouse anti-human BC-24 antibody in tissue sections were adapted for immunocytochemistry^{296, 327}. BC-24 binds to the constitutive EGF-L domains of tenascin-C and should permit visualisation of all tenascin-C isoforms. Tenascin-C co-localisation with fibronectin was assessed using the rabbit anti-human F3648 antibody, but differences in primary and secondary antibody binding efficiencies prevent quantification of the relative abundance of tenascin-C to fibronectin. Limitations of the assay included a potential bias that selected for cells which produced the most ECM and strongly adhered to the glass coverslips, therefore the cells present on the coverslips may not be representative of the total culture.

Overall, I selected three complementary assays which for the first time, characterised the transcription of tenascin-C mRNA, the expression of protein, and then localised tenascin-C in

cultures of resting and activated immune cells and fibroblasts; and in resting normal and diseased synovial fibroblasts.

The data in this chapter showed that in monocytes, macrophages, dendritic cells, T-cells, and HDFs, tenascin-C expression can be induced; but in a cell-type specific manner that differs in absolute magnitude, timescales, kinetics, and is also dependent on different stimuli. Such differences in the pattern of tenascin-C expression, suggests there are fundamental mechanistic differences in how tenascin-C expression is regulated by these cell types.

3.11.2 *Transcriptional regulation of tenascin-C*

Expression of tenascin-C is regulated in a stimulus specific manner in humans, mice, rats and chickens; the promoter elements of which are well conserved up to around 250bp upstream of the transcription start site, including a TATA box located 21 nucleotides downstream of the transcription start site⁷⁹. Regulation of the tenascin-C promoter is influenced by many transcription factors (reviewed in ⁶¹ and summarised in Table 3.1). The table illustrates how tenascin-C transcription may be induced or repressed in response to different subsets of stimuli including pathogen-associated molecular patterns (PAMPs), cytokines, reactive oxygen species, growth factors and mechanical stress. However, it is likely that not all of the transcription factors regulating tenascin-C expression are known; and our understanding of the details of this large *TNC* locus is only in its infancy. Here I discuss what is known about transcriptional control of tenascin-C in the context of the results generated in this chapter.

Table 3.1. Transcriptional regulators of tenascin-C expression

Stimulus	Transcription Factor/ Promoter	+ve/-ve regulation	Features of Study	Reference
PAMPs				
Lipopolysaccharide (LPS)	NF-κB	+ve	Identified 33 binding sites in <i>TNC</i> locus. In primary human monocyte derived dendritic cells tenascin-C expression was PI3K/Akt dependent	Goh et al. ²⁷³
Cytokines				
TNFα/ Sphingomyelinase	Postulated ATF-2/c-jun	+ve	JNK/SAPK-1 pathway activation increased tenascin-C expression in human epidermal keratinocytes.	Latijnhouwers et al. ¹⁷⁴
IL-4/ IFN-γ	Postulated STATs	+ve	Activate JAK-STAT pathway	Latijnhouwers et al. ¹⁷⁴
IL-13	unknown	+ve	Cultured human dermal fibroblasts, regulation was PI3K/Akt and/or PKC pathway dependent	Jinnin et al. ³³⁰
TNFα	NF-κB	+ve	TNFα driven tenascin-C expression was NF-κB p65 subunit (RelA) dependent in cultured human articular chondrocytes	Nakoshi et al. ³³⁶
Growth Factors				
TGFβ	Smad3/4, Ets1 Sp1, p300/ CREB-binding protein	+ve	Utilised WS-1 human dermal fibroblasts.	Jinnin et al. ³²⁹
PDGF	Sp1 Ets1/Ets2	+ve	Cultured human dermal fibroblasts, PI3K/Akt dependent	Jinnin et al. ³³⁷
	Fli1	-ve	Overexpression in human dermal fibroblasts inhibits effects of PDGF on tenascin-C expression	
Mechanical Strain				
Mechanical strain	NF-κB	+ve	Induced during mechanical strain via ROS in rat neonatal cardiac myocytes. Inhibited by anti-oxidants	Yamamoto et al. ²⁷⁵
Cyclic strain	MKL1	+ve	Binds CArG box (c-fos promoter)	Asparuhova et al. ³³⁸
Biomechanical stretch	NFAT5	+ve	Activates <i>TNC</i> expression following mechanical stress in vascular smooth muscle cells (VSMCs). May improve migratory activity in VSMCs	Scherer et al. ³³⁹
Other/ Unknown				
	Evx-1	+ve	Dependent on 89bp region containing TRE/AP-1 site in	Jones et al. ³⁴⁰

Stimulus	Transcription Factor/ Promoter	+ve/-ve regulation	Features of Study	Reference
			chicken tenascin-c promoter	
	Strain responsive element (SRE)		Chicken tenascin-C promoter	Chiquet-Ehrismann et al. ³⁴¹
	TATA box, Sp1, NF-1, C/EBP, AP-1, AP-2, <i>Krox-20</i> , <i>Pax</i>	+ve/ unknown	Sequence analysis of the promoter region identified multiple putative transcription factor binding sites	Gherzi et al. ³⁴²
	NF-1, TN control-element (TCE)	+ve	+ve regulation in mouse NIH-3T3 fibroblasts, C6 glioma and N2A neuroblastoma	Copertino et al. ³⁴³
	OCT	+ve	Required for +ve regulation by Brn2 in mouse N2A, inactive in C6 glioma	Copertino et al. ³⁴³
	Krox24/EGR-1 element	+ve/-ve	+ve regulation in mouse C6 glioma -ve regulation in mouse N2A neuroblastoma No effect in mouse NIH-3T3 fibroblasts	Copertino et al. ³⁴³
	OTX2	-ve	Homeodomain protein involved in anterior head formation. Represses tenascin-C expression in OTX2 transfected cells; U87-MG glioma cells, C6 rat glial tumour cells, O1 human primary glioblastoma, MRC-5 human fibroblasts, NIH-3T3 mouse fibroblasts and SEK-MEL-28 human melanoma cells	Gherzi et al. ³⁴⁴
	NF-κB/c-Jun	+ve	NF-κB and c-Jun synergistically trans-activate the tenascin-C promoter with c-Jun binding at a GCN4/AP-1 site in rat REF and RF3T3 fibroblast cell lines	Mettouchi et al. ³⁴⁵
Denatured type-I collagen	Unknown	+ve	Rat thoracic aorta (A10) VSMCs cultured on denatured type-I collagen express tenascin-C in ERK1/2 and β3 dependent. Promoter for transcription factor is in -43 to -165bp 5' of TSS	Jones et al. ³⁴⁶
	Fli1	+ve	Overexpression in human dermal fibroblasts results in +ve regulation. Modest activation observed with Ets1 and Ets2	Shirasaki et al. ³⁴⁷
Denatured type-I collagen	Prx1	+ve	Prx1/2 expression increases when A10 VSMCs cultured on denatured collagen substrate. Prx1 expression enhances <i>TNC</i> promoter 20-fold	Jones et al. ³⁴⁸
Focal Adhesion Kinase	Prx1	+ve	Mouse fibroblast cell-lines. FAK induces Prx1, promoting tenascin-C dependent migration	McKean et al. ³⁴⁹

Stimulus	Transcription Factor/ Promoter	+ve/-ve regulation	Features of Study	Reference
	GATA-6	-ve	Overexpression inhibited basal and decreased IL-4 /TGF β induced tenascin-C mRNA/protein levels in human foreskin fibroblasts	Ghatnekar and Trojanowska. ³⁵⁰
Notch2	RBPJk	+ve	Required for Notch-2 dependent transactivation of <i>TNC</i> promoter	Sivasankaran et al. ³⁵¹

3.11.3 *Cell-type specific basal expression of tenascin-C*

Basal levels of tenascin-C mRNA were highest in HDFs, where previously tenascin-C mRNA was detected by Northern blotting^{329, 330, 331}; although the reason for the high basal expression of tenascin-C in these cells is not yet known. Other studies have found that tenascin-C is expressed at high levels in intact human skin, but that it is restricted to the dermal-epidermal junctions, large blood vessels and eccrine glands, and was associated with epidermal proliferation^{352, 353}. The presence of tenascin-C in these locations, and in resting HDFs suggests that tenascin-C fulfils roles not limited to sites of inflammation, for example supporting cell adhesion, migration and proliferation. Furthermore, nothing is currently known about how tenascin-C expression is transcriptionally regulated in resting fibroblasts; however positive regulation was displayed by overexpression of transcription factors Fli1, Ets1 and Ets2 in HDFs, which all resulted in an increase in tenascin-C expression³⁴⁷. In contrast, tenascin-C transcriptional repression was achieved by GATA-6, which inhibited basal tenascin-C mRNA levels in human foreskin fibroblasts³⁵⁰.

One limitation of this study was that HDFs were isolated from non-palmar sites in patients with Dupuytren's contracture. Despite the fibroblasts being isolated from non-diseased locations and therefore being marked as normal, they were still isolated from diseased patients. It is not yet known if these fibroblasts bear a hallmark of the disease, or if they are epigenetically modified as a result of coming from a diseased patient.

Constitutively high levels of tenascin-C expression were detected in U-87 MG cells, and were in line with previous studies showing elevated tenascin-C mRNA and protein levels in cancers¹⁴⁹; particularly in glioma^{354, 355, 192, 165, 356}.

Tenascin-C mRNA levels were higher in macrophages and MDDCs than in monocytes from which they were differentiated *in vitro*, suggesting that the TNC promoter is regulated by transcription factors which are more active during differentiation, such as c-Jun/NF-kB or C/EBPs³⁵⁷. In contrast with the other myeloid cell types assayed, resting neutrophils did not express any tenascin-C. CD3+, CD4+ and CD8+ T-cells expressed lower basal levels of tenascin-C mRNA than monocytes macrophages and MDDCs.

The low basal levels of tenascin-C expression in resting myeloid and lymphoid immune cells indicates that tenascin-C expression is strictly repressed; perhaps because tenascin-C has a deleterious function on these resting cells; or that these cells rely on paracrine signalling by tenascin-C expressed by nearby cells to elicit cell responses.

Previous studies blocking resting protein synthesis with cyclohexamide in chick fibroblasts³⁵⁸ and HDFs³⁵⁹ showed an increase in the basal levels of tenascin-C mRNA, suggesting that here the tenascin-C gene is repressed by a protein with a high turnover rate. Furthermore, repression of tenascin-C expression was achieved by overexpressing microRNA-335³⁶⁰; revealing multiple mechanisms by which basal levels of tenascin-C expression can be negatively regulated. In the literature, low tenascin-C expression was previously reported in resting M-CSF macrophages³⁶¹, and MDDCs²⁷³ but no study has previously detected tenascin-C expression in resting T-lymphocytes; only that tenascin-C mRNA levels were lower in lymphocytes than in myeloid cell populations isolated from RA synovium²⁷³.

The notion that tenascin-C expression is tightly restricted in resting immune cells due to potential deleterious effects are supported by studies revealing that tenascin-C exhibits a

substantial range of effects on immune cells. Firstly, the Midwood group found that tenascin-C stimulates TLR4 mediated cytokine production⁴³, while other groups revealed that tenascin-C stimulates integrin mediated cytokine production²⁷⁷, inhibits myeloid cell migration^{49, 291}, stimulates myeloid cell migration³⁰², promotes hematopoiesis^{48, 362, 363}, stimulates erythroid cell differentiation³⁶⁴, stimulates Th2 and B-cell activation^{53, 294}, inhibits T-cell activation, promotes lymphocyte migration^{52, 293, 295}, and restricts T-cell actin polymerization and migration²⁹⁶. Therefore, it is feasible that inappropriate expression of tenascin-C by resting immune cells may dysregulate their function, leading to the initiation of inappropriate immune cell behaviours.

This thesis identified high resting tenascin-C expression in HDFs, which contrasted with the low basal levels of expression in immune cells and suggested fundamental cell-type specific differences in the way that tenascin-C expression is transcriptionally regulated across these resting cell types; albeit via currently unknown mechanisms. In addition, the data in this chapter has shown that tenascin-C is not only expressed during pathological states or during inflammation, but that it is also expressed in non-diseased resting cells.

3.11.4 *Cell type specific induction of tenascin-C biosynthesis*

Tenascin-C mRNA levels were rapidly and transiently increased in LPS stimulated monocytes, M-CSF, GM-CSF macrophages and MDDCs, in a manner consistent with previous studies findings in human²⁷³ and mouse³⁰¹ MDDCs. ELISA and immunofluorescence analysis confirmed that tenascin-C is predominantly secreted to the supernatant, and it does not associate to the cell surface. It was previously shown in other studies, that MDDC activation of the cell surface located TLR1/2, 2/6, 4, 5 by PAMPS PAM-3, LTA, LPS and flagellin respectively all promoted tenascin-C expression; but activation of the endosomally located TLR 3 and TLR8 by poly(I:C) and R848 which mimic viral

infection did not^{273, 365}; supporting the idea that tenascin-C expression by MDDCs is specifically associated with bacterial infection. In contrast, TLR4 activation of murine lung explants with LPS induced only minor increases on tenascin-C expression³⁶⁶.

In MDDCs, the blocking of LPS dependent TLR4 signalling by chemical inhibition, siRNA-mediated knockdown and overexpression of the endogenous NF- κ B inhibitor I κ B α revealed tenascin-C expression to be NF- κ B and PI3K/AKT dependent²⁷³. Furthermore, inhibition of the MAPK pathway with chemical inhibitors had a negligible effect on tenascin-C induction, indicating that here, signalling pathway is MAPK independent²⁷³. Tenascin-C mRNA induction kinetics in monocytes, macrophages and MDDCs appear to be highly cell-type and stimulus specific; as in other studies LPS stimulated THP-1 monocytes induced tenascin-C mRNA over 24 hours³⁶⁷, while in macrophages stimulated with oxidised-LDL, the tenascin-C levels peaked at 6 hours, and remained elevated for 48 hours²⁷⁴.

The kinetics of tenascin-C protein expression in activated MDDCs is slow relative to other TLR4 responsive genes that have previously been assessed, such as TNF α , IL-6 and IL-8; and raises the question as whether tenascin-C expression is secondary to expression of an early phase pro-inflammatory factor. Previous studies showed that TNF α is rapidly released in macrophages following trauma or exposure to LPS, and is an early stage mediator of tissue inflammation³⁶⁸. TNF α orchestrates a pro-inflammatory cytokine cascade, and is considered to be a master regulator of pro inflammatory cytokine production, that is further enhanced in macrophages primed with IFN γ ³⁶⁹. In macrophages, TNF α stimulation was shown to induce strong and rapid NF- κ B activation³⁷⁰, which contributed to the formation of an autocrine feed forward loop resulting in low level expression of IFN- β , which primed macrophages for enhanced expression of inflammatory response genes following subsequent challenge with inflammatory cytokines or microbial products³⁷¹.

Goh *et al*, showed TLR4 dependent tenascin-C expression to be NF-kB, and PI3K/Akt dependent, but tenascin-C mRNA synthesis was inhibited by treatment with cyclohexamide, showing that it required *de novo* protein synthesis²⁷³. This dependency on *de novo* protein synthesis suggested that tenascin-C could be a secondary response gene, allowing for the speculation that in MDDCs and macrophages, LPS activation of TLR4 induces rapid TNF α release, that primes the macrophage for further inflammatory stimulation and induced further *de novo* TNF α synthesis via activation of NF-kB, ERK, JNK and p38 MAPK signalling pathways. Therefore, NF-kB dependent tenascin-C expression could be induced following early TNF α release, which may explain the apparent time delay in tenascin-C mRNA and protein expression, compared to that reported for other TLR4 dependent cytokines such as TNF α , IL-6 and IL-8.

In HDFs, the data presented in this thesis does not support the notion that tenascin-C expression is TNF α dependent. Previous studies revealed that TNF α is secreted by synovial fibroblasts following LPS dependent activation of TLR4⁴³, and was reported to induce tenascin-C expression in human articular chondrocytes³³⁶, and human epidermal keratinocytes¹⁷⁴; in an NF-KB and ATF2/c-Jun dependent manner respectively. However, in this study HDF activation by LPS did not induce tenascin-C expression, suggesting that TLR4 dependent signalling alone is not sufficient to stimulate tenascin-C expression.

Here it was shown for the first time that human neutrophils do not express tenascin-C at rest, or following activation with the well characterised stimuli LPS³¹⁶ or GM-CSF³⁷². This suggested that in neutrophils, the tenascin-C gene promoter was not positively regulated downstream of TLR4/NF-kB²⁷³ or GM-CSF receptor (CD116)/JAK³⁷³ pathways, or was repressed by a negative regulator. Limitations of neutrophil *ex vivo* viability required a short assay time, which may have prevented mRNA detection at later time points.

It is possible that the cell-type specific nature of tenascin-C induction by LPS is the result of secondary signalling that occurs following TLR4 activation. Previous studies found that LPS dependent induction of tenascin-C by dendritic cells was dependent on de novo protein synthesis; as tenascin-C expression was reduced by cyclohexamide treatment. Furthermore, RNA polymerase II recruitment to the tenascin-C promoter was delayed, suggesting that tenascin-C was a secondary response gene which required protein synthesis and chromatin remodelling before transcription can proceed; meaning that tenascin-C expression is not a direct target of TLR4 signalling³⁷⁴. However, induction of tenascin-C expression by mechanical strain did not require de novo protein synthesis, suggesting that under those circumstances, tenascin-C was a primary response gene³⁷⁵. The regulation of histone deacetylase levels may provide an additional mechanism which LPS-dependent secondary response genes are transcribed; and could offer an explanation for the lack of LPS dependent tenascin-C induction observed in HDFs and neutrophils³⁷⁶.

This chapter described tenascin-C mRNA induction in activated CD4⁺ and CD8⁺ T-cells that was not translated into detectable levels of protein; indicating that the mRNA expression was too low to result in detectable tenascin-C expression, or that translation is blocked by a currently unknown mechanism. Despite this, the peak mRNA levels for CD4⁺ T-cells were higher than those in resting MDDCs, where tenascin-C protein was detected. Previous studies revealed that tenascin-C accumulated in lymphoid aggregates in inflamed synovium, but here the cells expressing tenascin-C were not determined³¹⁹; and this study would therefore suggest that the source of the tenascin-C was not lymphocytes, but perhaps the resident fibroblasts or traversing dendritic cells, which were shown to express tenascin-C.

Tenascin-C expression was induced in HDFs by IL-1 β , as previously described by McCachren³¹⁹; and selectively promoted the secretion of additional tenascin-C. However, tenascin-C expression was not induced by TLR4 dependent LPS stimulation, despite this

study also revealing that TLR4 activation caused a significant induction of IL-8 mRNA in HDFs; revealing that in fibroblasts monocytes, macrophages and dendritic cells, the transcriptional regulation of tenascin-C expression is downstream of distinct cell-type specific signalling pathways; supporting findings that tenascin-C expression was induced by signalling resulting from both sterile and non-sterile inflammation in fibroblasts and myeloid cell types respectively.

For reference, a table summarising the absolute resting and peak levels of tenascin-C mRNA present in cells is shown below (Table 3.2).

Cell Type	Basal copies TNC mRNA/cell	Peak copies TNC mRNA/cell	Conditions for peak stimulation
Glioma U87-MG	226.07	-	-
HDF	522.16	1206.74	48 hours +IL-1 β (10ng/ml)
M1 Macrophage	1.19	7.80	4 hours +LPS (10ng/ml)
M2 Macrophage	0.16	1.60	4 hours +LPS (10ng/ml)
MDDC	0.18	4.20	4 hours +LPS (10ng/ml)
Monocyte	0.037	0.46	4 hours +LPS (10ng/ml)
CD3+	0.0017	0.016	24 hours + anti-CD3/CD28 Dynabeads (1:1)
CD4+	0.0013	1.65	48 hours + anti-CD3/CD28 Dynabeads (1:1)
CD8+	0.00056	0.83	48 hours + anti-CD3/CD28 Dynabeads (1:1)
Neutrophil	0	0	No induction

Table 3.2 TNC mRNA copies/cell at basal and peak stimulated conditions in all of the cell types quantified. Tenascin-C mRNA copies/cell were quantified by standard curve qPCR in the cell types listed, following stimulation as described.

Induction of tenascin-C expression by fibroblasts was reported in other studies by IL-13³³⁰, PDGF³³⁷, IL-4³⁵⁰ and TGF β ^{329,350,272}, while mechanical stress was also shown to induce rapid tenascin-C mRNA synthesis that was NF- κ B/MAPK dependent³⁷⁷. The most well described inducer of tenascin-C expression in HDFs is TGF β ; here overexpression of the transcription factors Sp1, Ets1, Smad3, Smad4 led to a super-induction of tenascin-C expression by TGF β . In addition, the co-activating transcriptional regulator p300 was implicated in TGF β induced tenascin-C expression³²⁹. In chicken dermal fibroblasts, TGF β induced tenascin-C expression

was MAPK dependent, and was not inhibited by blockading Rho signalling; despite Rho blockade inhibiting tenascin-C expression in mechanically strained chick dermal fibroblasts³⁵⁸. Together with the data from this thesis, which described the induction of tenascin-C expression by IL-1 β ; these findings suggest that tenascin-C expression is induced in fibroblasts by cytokine and growth factor signalling, and that via these mechanisms tenascin-C expression may be further supported during the immune response, due to the elevated levels of cytokines and growth factors present during inflammation and during wound healing.

3.11.5 *Cell specific organisation and cellular distribution of tenascin-C*

Prior to this study, nothing was known about tenascin-C organisation and cellular distribution in immune cells, with only a few papers in the field displaying images of tenascin-C immunolocalisation and staining in tumour cell cultures^{296, 327, 378}, and in stem cell cultures³⁷⁹. Here I showed that tenascin-C organisation was cell type dependent and was modulated by inflammatory stimuli.

In U-87 G cells, tenascin-C was predominantly extracellular and punctate in organisation. Neither tenascin-C nor fibronectin were organised in fibrillar matrices, and their distribution closely resembled one another; indicating they may co-localise. Immunofluorescence analysis has previously been performed on glioma tissue sections using the BC-24 tenascin-C antibody only twice before; in one case tenascin-C staining was similarly punctate²⁹⁶, while in a U-87 MG mouse graft, the cells showed a more fibrillar staining pattern³²⁷. More recently, novel mAbs with constitutive and alternatively spliced tenascin-C FnIII epitopes were characterised in the laboratory of Andreas Faissner, and were used to stain U-87 MG, U-251 MG and U-373 MG glioma cell cultures, as well as tissue sections³⁵⁵. There, staining for tenascin-C was less punctate but showed densely staining foci underneath cells, neither of

which were observed in this study. These differences in tenascin-C organisation may result from the selective distribution of spliced FnIII binding epitopes in cell culture, or may be altered by surface coating substrates.

HDFs secreted high levels of tenascin-C into the soluble fraction and also retained it in cell associated fractions where it co-localised with fibronectin fibrils; perhaps supporting both autocrine and paracrine signalling. Furthermore tenascin-C staining was increased after stimulating with IL-1 β , perhaps as secreted tenascin-C was re-incorporated into the ECM. In contrast, MDDCs predominantly secreted tenascin-C to the soluble fraction. MDDCs do not express their own dense ECM, instead traversing the ECM synthesised by neighbouring cells. Faint fibronectin staining was observed around the MDDCs, but its distribution was disrupted by LPS treatment, indicating a change in cell surface receptor expression; or suggesting that secreted tenascin-C disrupts the fibronectin assembly, with previous studies showing that tenascin-C interferes with fibronectin fibril formation³⁸⁰. It is not yet known if the tenascin-C secreted by MDDCs is incorporated into the nearby ECM, or if it remains soluble and exhibits paracrine signalling properties.

Previous studies revealed that tenascin-C is incorporated into the ECM in a manner which is dependent upon the presence of heparan sulfate proteoglycans³⁸¹, fibronectin and $\alpha 5$, αv and $\beta 1$ integrins.³⁸² It may be that once incorporated into the ECM, tenascin-C stabilises fibronectin as tenascin-C knockout mice generally exhibit reduced fibronectin deposition^{55, 383}. A study by To *et al.*, found that all FnIII domains of tenascin-C bound to soluble fibronectin, but that the FnIII domains bound differentially to the N-terminal domain of fibrillar fibronectin preventing inter- and intra-chain interactions hence inhibiting fibronectin matrix assembly and fibrillogenesis³²⁰. These data show that in addition to the large hexabrachion structure providing ECM structural cues, individual tenascin-C fragments can also modify the ECM structure.

It is not clear how the functions of tenascin-C are affected by incorporation into a three-dimensional matrix, a process which is very likely to change the conformation of this molecule, perhaps exposing or concealing specific domains and binding sites. Soluble tenascin-C binds readily to fibronectin in glycerol gradients assays,³² where the extended conformation of both proteins appear to favour interaction.³⁸⁴ Conversely, fibronectin does not bind to tenascin-C that has been immobilized onto ELISA plates, but tenascin-C can bind to immobilized fibronectin^{32, 84, 385, 386}. In the structurally related FnIII domains of fibronectin, FnIII-1c was partially unfolded during increased myofibroblast tension³⁸⁷ and MMP-2 cleavage³⁸⁸, and triggered TLR2 and TLR4 dependent cytokine release in dermal and lung fibroblasts respectively^{387, 389}. As such the context and conformation of tenascin-C should be considered major players in defining ECM function.

Tenascin-C binds directly to other ECM molecules, and exhibits indirect effects on others. Tenascin-C binds to proteoglycan molecules that are themselves bound to fibronectin fibrils; evidenced by co-localisation of perlecan, fibronectin and tenascin-C. Perlecan is bound by FnIII3-5 of tenascin-C, and this is enhanced in the shorter ~190kDa isoform, as insertion of spliced FnIII domains after FnIII 5 attenuates perlecan binding³⁸¹; and may contribute to reduced cell adhesion in cells expressing larger tenascin-C isoforms such as tumours. Tenascin-C also binds to the lectican G3 domain, present in versican, aggrecan, brevican and neurocan³⁹⁰ where it is involved in cross linking aggrecan-hyaluronan complexes³⁹¹. Another factor in matrix formation is the ECM molecule periostin, which has been shown to bind to fibronectin and type I collagen, and also aid incorporation of tenascin-C into the ECM by acting as a bridge between tenascin-C and other ECM components³⁹². Tenascin-C binding has been reported with type I-IV and IX collagen but not to denatured collagen³⁹³. It was also shown that during wound healing, tenascin-C co-localised and directly bound with the ECM glycoprotein fibrillin-2; the effect of which reduced fibrillin-2 mediated cell adhesion³⁹⁴.

Fibrillin-2 also stores TGF β in the ECM, and in this way, expression or secretion of tenascin-C at sites of inflammation may modulate the uptake or release of TGF β ; a growth factor that promotes fibrosis during wound healing³⁹⁵.

The diverse range of ECM binding interactions described above for tenascin-C highlight the wide range of mechanisms by which tenascin-C exerts direct and indirect modulation of ECM assembly and signalling functions; both when the protein is intact, and when its structural integrity is compromised. Together with other findings, my data may support a model where fibroblasts synthesize a tenascin-C rich ECM that promotes local tenascin-C functions; while MDDCs do not, and may therefore not be affected by local signals.

3.11.6 *Tenascin-C expression is elevated in RA and OA synovial fibroblasts*

It is well reported that tenascin-C expression is elevated in disease. Here I compare tenascin-C expression in cultured NSFs, RA-SFs and OA-SFs, and reveal that tenascin-C mRNA and protein levels are elevated in synovial fibroblasts isolated from pathological joints compared to normal ones.

There is a clear difference in the expression of tenascin-C in fibroblasts located in different regions of the body. HDFs expressed constitutively high levels of tenascin-C, while NSFs expressed very little tenascin-C; although in this study only two NSF donors were assessed due to the low availability of this cell type, therefore the low donor numbers must be considered when drawing conclusions on the data.

Tenascin-C mRNA and protein was elevated in RA-SFs and OA-SFs relative to NSFs; and was consistent with previous findings where tenascin-C accumulated in RA synovium *in vivo*²⁷³, and that circulating tenascin-C levels were elevated in RA³⁰⁷. Furthermore, immunofluorescence microscopy revealed tenascin-C staining and co-localisation with

fibronectin in RA-SFs and NSFs, but with additional staining in RA-SFs that was not localised to fibronectin; indicating differences in the way tenascin-C is incorporated into the ECM. Such differences may result from ECM dysregulation as a result of pathology, or by alternative splicing of tenascin-C into disease specific isoforms that interact with different surface receptors or ECM components. OA-SFs displayed the lowest levels of tenascin-C staining, but expressed more cell associated FnIII-B containing protein than NSFs, this may suggest different regulation of splicing between these cell types, or that tenascin-C incorporation into the OA-SF ECM is inhibited compared to for NSFs.

Furthermore, in this study all fibroblast cell types were isolated and cultured up to passage 4, meaning that prior to experimentation, these cells had been cultured outside of their normal, or pathological inflammatory milieu for some time. It is therefore reasonable to conclude that negligible levels of soluble factors from the normal or diseased joints were still present in the cell cultures at the time of experimentation; therefore these cells cannot be considered truly normal or diseased, instead they were isolated from normal or diseased tissues. Nevertheless, even when removed from their normal or diseased environments, there were clear and long lasting differences in the alternative splicing patterns of tenascin-C; suggesting that epigenetic reprogramming of the synovial fibroblasts may occur during disease, which could result in the preferential expression of distinct tenascin-C splicing patterns.

Altogether, these data supported previous findings of elevated tenascin-C mRNA and protein levels in inflamed synovium³¹⁹, with higher expression in RA-SFs relative to OA-SFs, and an accumulation of tenascin-C in inflamed RA synovium *in vivo*²⁷³. Tenascin-C was previously shown to drive inflammatory joint disease in mice via interactions with TLR4⁴³, which was further evidenced by the finding that *TNC*^{-/-} mice are protected from erosive joint disease. Additionally, tenascin-C was recently found to drive persistent organ fibrosis in systemic sclerosis²⁷². Therefore, elevated tenascin-C expression by cells in a pathological state

supports a hypothesis that tenascin-C may drive inflammatory and fibrotic processes in human RA.

3.11.7 *Summary of tenascin-C expression*

For the first time, this study has compared and identified cell specific differences in tenascin-C expression at rest, and that tenascin-C expression is induced in a number of cell types following activation with different inflammatory stimuli. ELISA and Confocal immunofluorescence microscopy also revealed cell specific differences in the cellular distribution of tenascin-C, showing that tenascin-C can be predominantly cell associated or secreted. Finally, this study confirmed previous findings and showed elevated tenascin-C expression in synovial fibroblasts isolated from RA and OA patients, compared to healthy individuals. In the next chapter I extend these data to examine the alternative splicing pattern of tenascin-C in cells at rest and following activation, and in healthy and diseased cells, in order to determine whether different tenascin-C isoforms are expressed in a cell type specific manner, and ultimately if there may be cell type specific responses to individual tenascin-C isoforms.

Chapter 4 Characterising tenascin-C alternative splicing in inflammation and disease

4.1 Introduction

Alternative splicing allows a single gene to encode multiple distinct protein isoforms by permitting the exclusion of exons from the mature mRNA which greatly increases the size and diversity of the proteome. It is estimated that 95% of the ~21,000 protein coding genes in humans are alternatively spliced⁶⁴. As a result, >290,000 non-redundant peptide combinations can be produced⁶⁵. Tenascin-C was first observed to be alternatively spliced by Jones *et al* in 1988⁶², and the most recently discovered alternatively spliced FnIII repeat, AD2, was identified in 1997⁶⁷.

Alternative splicing of 9 of tenascin-C's 17 fibronectin type III-like domains (FnIII-A1, A2, A3, A4, B, AD2, AD1, C and D) generates enormous diversity in form; yielding 511 possible isoforms. Post-transcriptional modification of tenascin-C has been studied extensively in cancer and during development where disease and tissue specific isoforms are well characterised, and have been shown to exhibit distinct effects²⁴⁸. In cancer, the detection of specific alternatively spliced FnIII domains of tenascin-C has also been exploited for its diagnostic and therapeutic potential in detecting and predicting the disease stage in cancer^{181, 182, 183, 396, 186}; and for providing immunotherapeutic targets that are highly tumour specific in glioblastoma, lung cancer, breast cancer and Hodgkin's lymphoma (reviewed in ²⁴⁸).

However, nothing is known of how tenascin-C is alternatively spliced during inflammation or in inflammatory diseases. In vitro studies have revealed some functional evidence that alternatively spliced FnIII domains from tenascin-C, such as A1, A2 and D, have inhibitory effects on T-cell proliferation and transmigration^{155, 299}; but no characterisation of tenascin-C isoform expression in immune cells, or in fibroblasts exists.

This study will examine inflammation and disease specific tenascin-C isoforms made by a range of primary human immune cells and fibroblasts. Western blotting will be used to observe the size of tenascin-C protein isoforms expressed, and qPCR will be used to quantify the abundance of each alternatively spliced FnIII domain at the mRNA level.

These data comprise the first relative comparison in the sizes of tenascin-C isoforms expressed by immune cells and fibroblasts; and identify the presence of inflammation and disease specific tenascin-C alternative splicing patterns in resting and activated cells of the immune system, and in normal and diseased synovial fibroblasts.

4.2 Cell type specific expression of tenascin-C splice variants – strategy

The majority of studies published to date have used western blotting to characterize tenascin-C splicing. This method has previously revealed that human tenascin-C was expressed in isoforms of different sizes, and were visible as ‘smaller’ isoform that contains no alternatively spliced FnIII domains (~180-190kDa), and as ‘larger’ isoforms containing one or more spliced FnIII (from ~210 up to 330kDa)²⁴⁸.

These studies further showed that the splicing pattern in developing tissues changes over time, with a relative shift from the expression of larger isoforms in early mouse and chicken embryogenesis to smaller isoforms with increasing developmental age. Additionally, the little tenascin-C that is expressed in healthy adult tissues predominantly comprises the small variant, with larger isoforms re-expressed in the ECM of various tumours²⁴⁸.

4.3 Optimization of tenascin-C Western blot protocol

Western blotting of tenascin-C was performed using an antibody that binds within the constitutively expressed EGF-L repeats of the protein (mAb 1908), permitting visualisation of tenascin-C isoforms of all possible sizes, which are separated according to their molecular mass following SDS-PAGE under reducing conditions. Reducing SDS-PAGE gels were

prepared to 5% (w/v) polyacrylamide. Samples were run against the PageRuler™ Plus Prestained Protein Ladder with markers at 10, 15, 25, 35, 55, 70, 100, 130 and 250kDa to estimate protein size. Gels were run until the 55kDa protein ladder marker reached the bottom of the gel, to ensure that all possible tenascin-C bands could be visualised. Future gels were run until the 130kDa marker reached the bottom of the gel, to ensure individual tenascin-C bands were optimally resolved.

The sensitivity and specificity of the anti-EGF-L antibody was ascertained by Western blotting 3 different samples: i) tenascin-C purified from the conditioned media of tenascin-C overexpressing HEK cells in the Midwood lab, ii) commercially available tenascin-C purified from the conditioned media of the U-251 MG glioblastoma cell line and iii) U-87 MG cell culture supernatant, after 72 hours of culture. The purified HEK tenascin-C contained 2 visible bands; a higher well defined band at ~300ka, and a lower, smeary band between ~120-75 kDa. These bands were detected by Western blot when loaded between 1000ng and 50ng/well, but were not visible at 10ng without prolonged exposure to X-ray film. Tenascin-C from purified U-251 MG cells was detected in three bands, two well defined bands at ~300 and 260kDa, while the other was a smeary band between ~120-80kDa. A smeary band of this size was previously reported by the Midwood group, and corresponded to a degradation product that results from storing tenascin-C for long periods; the identity of the product was previously ascertained by staining with anti-EGF-L domain antibody, to reveal it is the N-terminal portion of tenascin-C. Supernatant from U-87 MG cells cultured for 72 hours, showed a single ~300kDa isoform running at the same size as the larger bands seen with HEK and U-251 MG purified tenascin-C (Figure 4.1.). Together these data show this mAb can detect purified tenascin-C, and that it also specifically detects tenascin-C amongst a mixture of cell synthesised proteins in conditioned media.

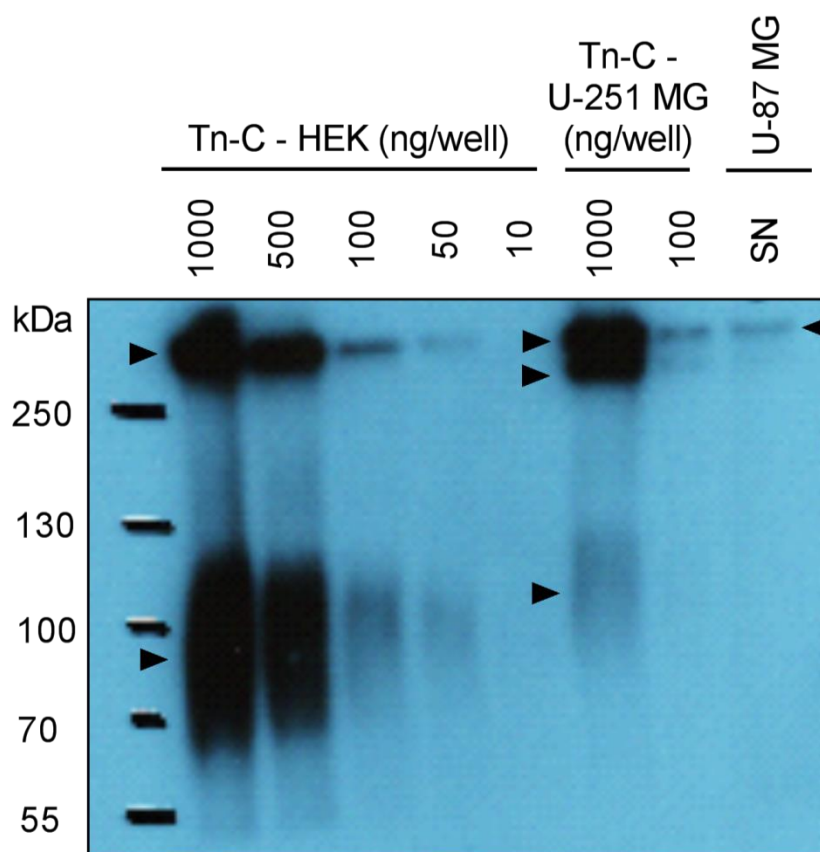


Figure 4.1. Validation of Western blotting for tenascin-C. Tenascin-C purified from HEK cells (Tn-C - HEK) (10, 50, 100, 500 and 1000ng per well), tenascin-C purified from U-251 MG cells (Tn-C – U-251 MG) (1000 and 100ng per well), and 20µl supernatant collected from U-87 MG cells cultured for 72 hours, were resolved by SDS-PAE in a 5% w/v polyacrylamide gel. Proteins were visualised after transfer to nitrocellulose and staining with mouse anti-human tenascin-C antibody mAb1908, followed by secondary staining with goat anti-mouse-HRP conjugated antibody, and exposure to ECL reagent. Samples were run against the PageRuler™ Plus Prestained Protein Ladder. The full un-cropped gel is displayed, and arrows display the location of protein bands. Gel was representative of 4 repeats.

4.4 U-87 MG express different cell associated and secreted tenascin-C isoforms

Previous studies have shown that cell associated tenascin-C synthesised by U-251 MG and U-87 MG glioma cells was a mix of isoforms of different sizes^{91, 165, 166}, but nothing was known of the sizes of secreted tenascin-C isoforms in glioma. Here I ran samples of cell lysate and supernatant from U-87 MG glioma cultured for 0, 4, 8, 24, 48 and 72 hours alongside a control containing 100ng/well of purified U-251 MG tenascin-C. The purified tenascin-C

served as a standard and was run on each gel, so that cell lysate and supernatant run on separate gels could be compared between different experimental repeats.

In the cell lysate, tenascin-C levels were greater than the 100ng/well purified tenascin-C standard at all time points after 0 hours, and cell associated tenascin-C accumulated steadily between 4 and 72 hours. Tenascin-C bands of two sizes were observed, estimated at ~300kDa and ~230kDa with the lower band being more defined than the higher molecular weight band; which comprised a doublet of two differently sized bands differing in size by ~10kDa. As a loading control, after Western blotting for tenascin-C, the membrane was stripped and re-probed with an anti-vinculin antibody (Figure 4.2.A). Tenascin-C secreted to the supernatant was less abundant than the 100ng/well purified tenascin-C standard, and was only detected at 48 and 72 hours following plating. A single ~300kDa sized tenascin-C band is detected (Figure 4.2.B).

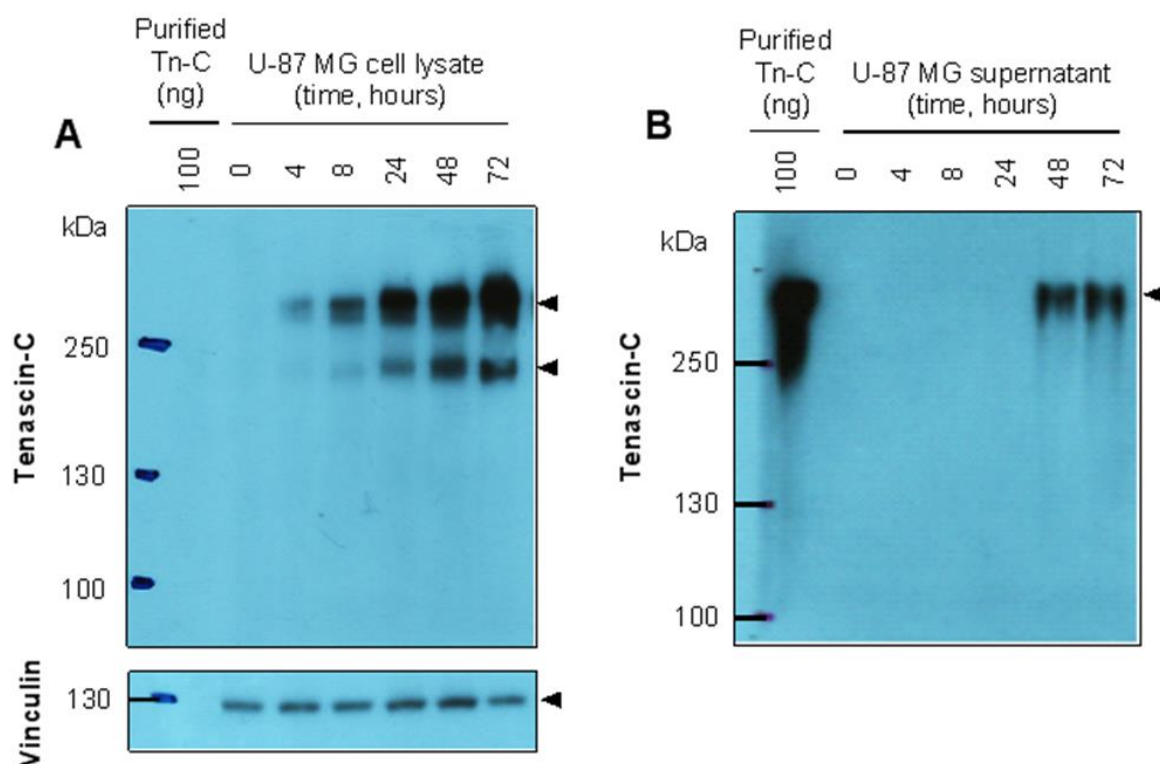


Figure 4.2. U-87 MG cells express different cell associated and secreted tenascin-C isoforms. U-87 MG cells were cultured for 0, 4, 8, 24, 48 and 72 hours, and tenascin-C present in the samples of cell lysate (A) and supernatant (B) were resolved by SDS-PAGE and Western blotting using mAb1908, both blots are un-cropped. 100ng of purified tenascin-C from U-251 MG cells was used as a positive control. Cell lysate blots were re-probed with the mouse anti-human vinculin mAb18058. Samples were run against the PageRuler™ Plus Prestained Protein Ladder. The gels showing cell lysate and supernatant were from the same experiment, and are representative of 3 independent experiments. Arrows display protein bands.

Together these data show that U-87 MG cells contain more tenascin-C in the cell lysate than the supernatant fractions; this was not in accordance with the findings of the USCN ELISA, but confirmed the results of the FnIII-B ELISA from chapter 3, which detected a greater concentration of tenascin-C normalised per 10^6 cells in the cell lysate relative to the supernatant. The cell lysate also contains 2-3 tenascin-C bands, while only one larger mass tenascin-C band is seen in the supernatant, meaning that the majority of tenascin-C is cell associated and a small amount of the larger isoform is secreted.

4.5 MDDCs express different cell associated and secreted tenascin-C isoforms

In chapter 3 I showed by qPCR, ELISA and IF that MDDCs express low basal levels of tenascin-C, which increase following LPS stimulation. Here I examined the size of tenascin-C isoforms in resting cells over time and any changes in the number and sizes of tenascin-C isoforms expressed by activated MDDCs.

For Western blotting samples from MDDCs, I included purified tenascin-C standards of 20 and 50 ng in each gel; this was less than for U-87 MG cells, due to the comparatively low abundance of tenascin-C in MDDC samples compared to U-87 MGs.

In these experiments, in order to attempt to more accurately estimate the sizes of tenascin-C isoforms which exceed the highest 250kDa molecular weight marker of the PageRuler™ Plus Prestained Protein Ladder, an additional ladder was included which contained higher molecular weight marker bands. This was the HiMark™ Prestained Standard with bands at 31, 41, 55, 71, 117, 171, 238, 268 and 460kDa. However, after running both MW standards on the same gel it was apparent that markers of the same mass from different ladders were migrating at different rates, which prevented the accurate quantification of tenascin-C isoform size. Therefore, for the purposes of this study all estimations of tenascin-C isoform size were estimated using the PageRuler™ Plus Prestained Protein Ladder, shown on subsequent Western blotting images in black. HiMark™ bands are shown in red for comparison.

In the cell lysate from unstimulated MDDCs cultured over time, no obvious tenascin-C bands were detected by Western blot in the 0, 4, 8, 24 and 48 hour samples. In contrast, treatment with 10ng/ml LPS induced an increase in the expression of 2 bands at ~300 and 280kDa tenascin-C that peaked at 8 hours, and were reduced at 24 and 48 hours following stimulation. At 8 hours following LPS stimulation, the ~280kDa band was most abundant,

but this was barely detected at the later 24 and 48 hour time points, when the ~300kDa isoform is most abundant. The peak tenascin-C MDDC band intensity was comparable to levels observed for the 50ng/well tenascin-C standard. The estimated mass sizes of the bands were lower in MDDCs than the upper ~300kDa band in tenascin-C standard.

Following tenascin-C staining, membranes were stripped and re-probed for β -actin to serve as a loading control. β -actin was selected as the loading control instead of vinculin, which was used for U-87 MG cell lysate samples, as it was more abundantly expressed than vinculin in MDDCs. This control confirmed equal loading of the wells (Figure 4.3.A).

In the supernatant, unstimulated MDDCs secreted low levels of tenascin-C with a poorly defined faint band at ~300kDa observed in cells at 24 and 48 hour time points, the levels of which were below the 20ng/well purified standard. Stimulation with LPS induced secretion of only one ~300kDa band at 48 hours. The larger band was more abundant than the 50ng/well purified standard. (Figure 4.3.B).

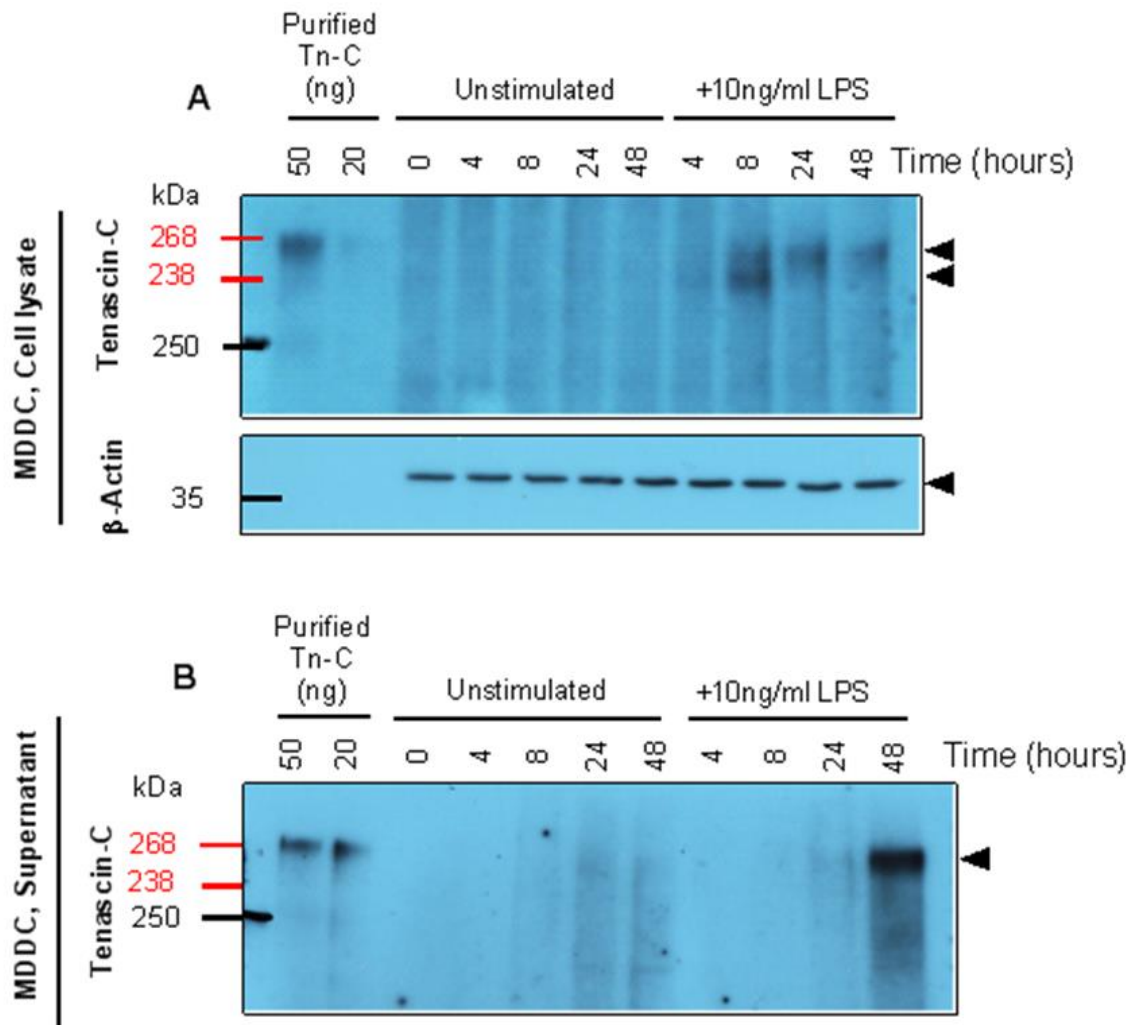


Figure 4.3. Activated MDDCs predominantly express large tenascin-C isoforms, and do not express small isoforms. MDDCs were plated and rested for 16 hours prior to stimulation with 10ng/ml LPS, for 0, 4, 8, 24 or 48 hours, or were left unstimulated. Tenascin-C present in samples of cell lysate (A) or supernatant (B) were resolved by SDS-PAGE and Western blotting using mAb1908. 50 and 20ng of purified human U-251 MG tenascin-C was loaded per gel as a positive control. Cell lysate blots were re-probed with the mouse anti-human β -actin mAb. Samples were run against the PageRuler™ Plus Prestained Protein Ladder (Black bars), and the HiMark™ pre-stained protein ladder (Red bars). The gels showing cell lysate and supernatant were from the same experiment, and are representative of 5 independent experiments. Arrows indicate the presence of protein bands.

These data show that LPS stimulated MDDCs predominantly secreted a single 300kDa tenascin-C band to the supernatant. Furthermore, a very faint band at 280kDa was transiently trafficked through the cell but was not retained; revealing differences in the way large tenascin-C bands are trafficked by MDDCs. The transient induction and kinetics of tenascin-C expression and secretion in MDDCs were supported by previous tenascin-C quantifications in LPS stimulated MDDCs using the FnIII-B ELISA, and observations with immunofluorescence microscopy.

4.6 Comparing cell associated and secreted tenascin-C isoforms in HDFs, NSFs, RA-SFs and OA-SFs

I previously showed that HDFs expressed more tenascin-C than MDDCs, and that tenascin-C levels were higher in RA-SFs and OA-SFs compared to NSFs. To determine whether tenascin-C isoforms of different sizes were expressed in these cell types I performed SDS-PAGE and Western blotting of cell lysate and supernatant fractions isolated from HDF, RA-SF, OA-SF and NSF after culture for 72 hours.

Samples of fibroblast cell lysate and supernatants from different donors were run against U-251 MG purified tenascin-C, which was loaded at 500 and 200ng/well. This was a higher amount than previously loaded, and reflected the greater abundance of tenascin-C in the fibroblast samples compared to MDDC and glioma samples.

In HDF cell lysate, tenascin-C bands of two sizes were observed that were both more abundant than the 500ng/well standard. The molecular masses were estimated at ~300 and ~250kDa, with the upper band being less defined than the lower band, and possibly comprising a doublet. Vinculin served as a loading control and was present in the samples at equal levels (Figure 4.4.A). Western blotting of synovial fibroblast cell lysate revealed lower overall tenascin-C levels in NSFs than in HDFs, RA-SFs and OA-SFs, with less intense

bands than the 200ng/well standard. Like HDFs, NSF cell lysate contained two bands of different sizes, but these were smaller than in HDFs, at ~280kDa and ~230kDa, expressing equal quantities of each. RA-SFs expressed two bands the same size as seen in NSFs, but here the larger band is predominant. OA-SFs also express isoforms the same size as in NSFs and RA-SFs, with the larger band also being predominant as in RA-SF cell lysate. Vinculin was present in the samples at equal levels (Figure 4.4.B). Phoretix densitometry analysis permitted relative comparison in pixel intensity of large and small tenascin-C bands, and found that HDFs expressed the greatest fold levels of large bands relative to small ones amongst all the fibroblast types; but that RA-SFs and OA-SFs also predominantly expressed large bands. These data contrast with NSFs, which expressed equal levels of large and small isoforms (Figure 4.4.C).

I next observed tenascin-C bands secreted to the supernatant from different fibroblast types. HDFs secreted isoforms of two sizes, the largest and predominant isoform is ~300kDa, and the smallest was ~250kDa; as was previously described in the cell lysate (Figure 4.4.D). NSFs and OA-SFs only secreted large isoforms at ~280kDa, but RA-SFs expressed both large and small variants at ~280kDa and ~230kDa (Figure 4.4.E). Comparative pixel densitometry analysis of the relative abundance of large and small bands in supernatant confirmed that HDFs, OA-SFs, RA-SFs and NSFs predominantly secrete larger tenascin-C isoforms relative to smaller isoforms. For OA-SFs where no small band was detected, the intensity of the large band was normalised against the background (Figure 4.4.F).

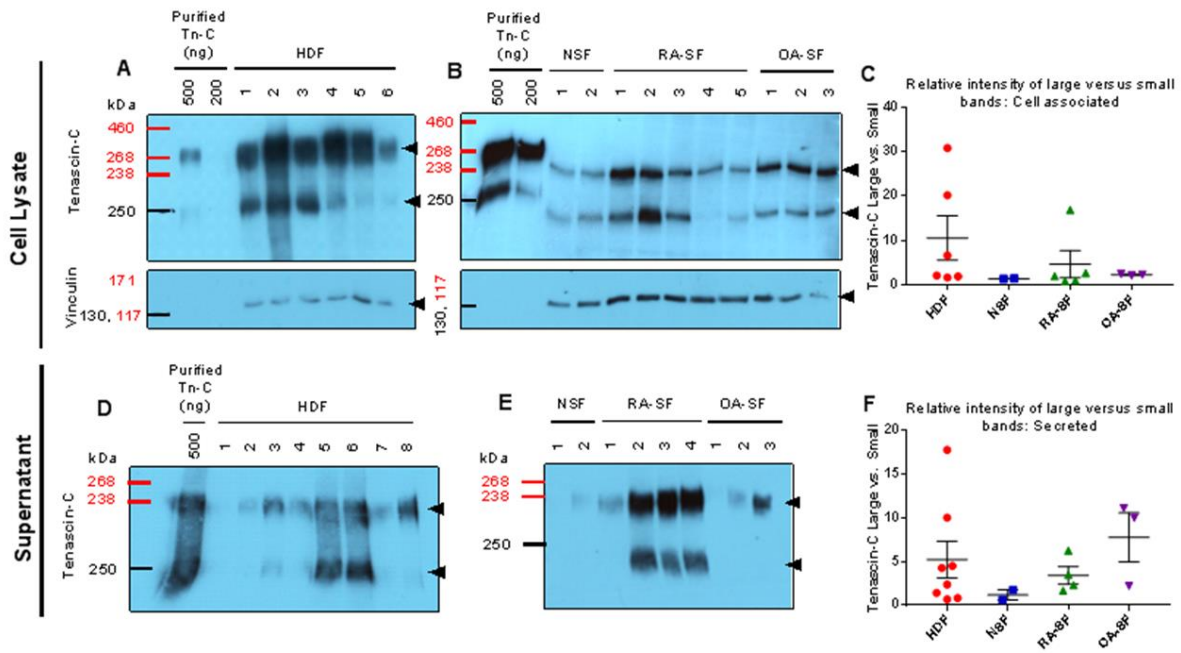


Figure 4.4. HDFs, RA-SFs and OA-SFs predominantly express large tenascin-C isoforms, whilst NSFs express equal ratios of large and small isoforms. Fibroblasts were cultured for 72 hours, and the sizes of tenascin-C isoforms present in the cell lysate of HDFs (n=6) (A), NSFs (n=2), RA-SFs (n=5) and OA-SFs (n=3) (B), were assessed by SDS-PAGE and Western blot using mAb1908. Membranes were stripped and re-probed for vinculin using mAb18058. Phoretix pixel densitometry showed the ratio of large to small tenascin-C bands present in the cell lysate (C). Tenascin-C isoforms present in the supernatant of HDFs (n=8) (D), NSF (n=2), RA-SFs (n=4), and OA-SFs (n=3) (E), were also assessed by SDS-PAGE and Western blotting. Phoretix analysis showed the ratio of large and small tenascin-C bands secreted in the supernatant (F). Samples were run against the PageRuler™ Plus Prestained Protein Ladder (Black bars), and HiMark™ ladder (Red bars). Purified U-251 MG tenascin-C standards were loaded to each gel, 500 and 200ng/well (A-B), 500ng/well (D-E). Data are shown as mean $\pm$ STDEV for n=2, and $\pm$ SEM for n $\geq$ 3.

These data show that overall tenascin-C expression is high in HDFs, and is elevated in RA-SFs and OA-SFs relative to NSFs; confirming previous findings when tenascin-C mRNA and protein were quantified and tenascin-C distribution was observed by immunofluorescence microscopy in chapter 3. Western blotting also identified differences in the relative ratios, sizes and number of tenascin-C bands seen in fibroblasts. Firstly, HDFs expressed tenascin-C bands that were ~20kDa larger than those seen in synovial fibroblasts. All NSFs, RA-SFs and OA-SFs expressed cell-associated bands of the same sizes, but the larger band was predominant in the diseased synovial fibroblasts. In fibroblasts, the large tenascin-C bands detected in the cell lysates were always present in the supernatant, but in HDFs and RA-SFs the smaller tenascin-C band was also detected.

In summary, HDFs express larger tenascin-C variants than synovial fibroblasts; and in the synovium RA-SFs express greater levels of large and small tenascin-C variants than NSFs and OA-SFs, meaning that while the tenascin-C variants expressed by synovial fibroblasts are the same size, there are disease specific differences in the ratios of large and small tenascin-C variants expressed.

4.7 Comparative analysis of tenascin-C isoforms amongst cell types

The aim of the sections above was to systematically examine the sizes of different tenascin-C isoforms expressed at rest and following stimulation, however due to the limitations of the protein ladders used in the Western blotting protocols, accurate comparisons of the absolute tenascin-C band sizes could not be made. To directly compare the relative sizes of tenascin-C bands across cell types, a single gel was loaded with samples of purified tenascin-C from U-251 MG supernatant, cell lysate from U-87 MG, HDF, NSF, RA-SF, OA-SFs cultured for 72 hours, and cell lysate and supernatant isolated from MDDCs stimulated with LPS for 8 and 48 hours respectively.

The samples were separated by SDS-PAGE for longer than previously described, in order to better resolve the small differences between the mass of tenascin-C bands. This comparison revealed that purified tenascin-C from U-251 MG supernatant contains isoforms of 2 sizes at ~300, 230kDa and that U-87 MG cell lysate contains isoforms of 4 sizes at ~300, 270, 240 and 230kDa. It also showed that HDF contains isoforms of 2 sizes that are also observed in U-87 MG, at ~300 and 240kDa. Furthermore, this gel confirmed that the two tenascin-C bands observed in NSFs, RA-SF and OA-SF cell lysate were smaller than those seen in HDFs. NSFs expressed bands of 2 sizes, at ~280 and 230kDa, RA-SFs expressed a similar mass lower isoform at 230kDa but a slightly larger ~285kDa large band, while OA-SFs expressed bands at ~280 and 225kDa. MDDCs only expressed large isoforms; the blot shows a very faint band in the 8 hour stimulated cell lysate at ~280kDa, and a very faint smeared band in the 48 hour stimulated supernatant at ~270-280kDa (Figure 4.5). Further Western blot images supporting this statement can be seen in Figure 4.3.

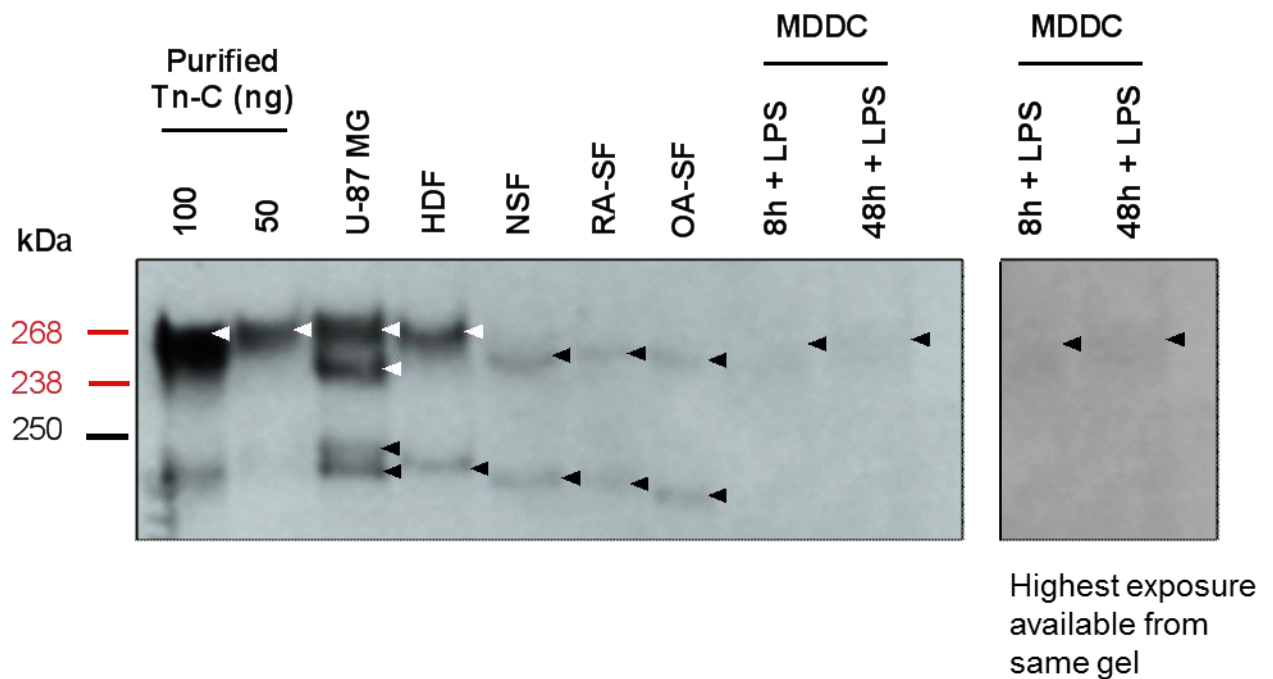


Figure 4.5. Comparing tenascin-C isoform sizes expressed by U-87 MG, HDF, NSF, RA-SF, OA-SF and activated MDDCs. The direct comparison of tenascin-C isoform sizes, expressed in the cell lysate of representative samples of resting U-87 MG (n=3), HDF (n=8), RA-SF (n=3), OA-SF (n=3) and NSFs (n=2) cultured for 72 hours, and from cell lysate and supernatant from MDDCs (n=5) stimulated with 10ng/ml LPS for 8 and 48 hours respectively, was resolved by SDS-PAGE and Western blot using mAb1908. Samples were run against PageRuler™ Plus Prestained Protein Ladder (Black bars), and HiMark™ pre-stained protein ladder (Red bars). Arrows display the presence of a protein band. The image on the right is an increased exposure of the same MDDC gel lanes shown in the image on the left.

To summarize, the combined Western blot revealed cell-type specific expression of different sized tenascin-C variants. U87-MG expressed the most variants of different sizes, with two large and two smaller variants; HDFs expressed variants of two sizes that were comparable to those expressed in U-87 MG cells. NSF, RA-SF and OA-SFs each expressed two tenascin-C variants, both of which were slightly smaller than those expressed in HDFs; while in activated MDDCs, very faint large tenascin-C bands were transiently detected after LPS stimulation. These variants had a higher mass than variants expressed in synovial fibroblasts, but smaller than those detected in HDFs and U-87 MG.

4.8 Summary of tenascin-C Western blotting

This study revealed cell-type specific tenascin-C protein isoform expression and cellular trafficking. In U-87 MG glioma, smaller tenascin-C bands (230-240kDa) were retained by the cells and were not secreted to the supernatant, while larger 300 and 270kDa isoforms were both cell associated and secreted. The transient induction and kinetics of tenascin-C expression and secretion in MDDCs were different from the steady accumulation of tenascin-C seen in U-87 MG cells; these observations were supported by previous findings in chapter 3. In MDDCs, no tenascin-C protein was detected at rest by Western blot, but following LPS stimulation, MDDCs predominantly secreted a single 300kDa isoform to the supernatant, and a slightly smaller 280kDa band that was transiently detected in the cell lysate, before being secreted to the supernatant.

HDFs expressed the highest levels of total tenascin-C; and constitutively expressed large 300kDa and small 250kDa isoforms to both the cell lysate and supernatant; which contrasted with U-87 MG and MDDCs which only secreted their larger isoforms. The HDFs, U-87 MG and MDDCs all shared common expression of a large 300kDa isoform, but the masses of the smaller isoforms were cell type specific. The HDF cell associated tenascin-C isoforms (300, 250kDa) were higher mass than those detected in synovial fibroblasts; NSFs (280, 230kDa), RA-SFs (285, 230kDa) and OA-SFs (280, 225kDa). RA-SFs expressed higher levels of tenascin-C protein than NSFs and OA-SFs, in accordance with previous results from chapter 3. RA-SFs also exhibited a tenascin-C isoform distribution pattern most like HDFs; where they expressed their high and low mass isoforms to both the cell lysate and supernatant. NSFs and OA-SFs only secreted large isoforms, but retained both large and small variants in the cell lysate; though here NSFs expressed large and small variants at roughly equal abundance, the OA-SFs predominantly expressed the larger variant.

4.9 Basal expression of alternatively spliced FnIII mRNA transcripts is cell type dependent

Western blotting is widely used for assessing tenascin-C alternative splicing, and here revealed the presence of tenascin-C variants with different molecular masses in different cell types. However, Western blotting protocols also have limitations for characterising tenascin-C alternative splicing; for example different tenascin-C isoforms of the same mass cannot be deduced as separate proteins and appear as the same band. Additionally, there are currently a lack of antibodies which specifically bind to the spliced domains FnIII-A1, A3, A4, AD2 and AD1, making it difficult to characterise the contents of the alternatively spliced isoforms. Therefore I decided to complement the Western blotting by generating a new method for assessing tenascin-C alternative splicing at the mRNA level, so that the presence of individual alternatively spliced FnIII may be quantified and compared in various cell types.

This method quantifies the absolute abundance of each alternatively spliced FnIII mRNAs using standard curve qPCR and custom primers compatible with SYBR-Green reagents and allows direct comparison of FnIII mRNA abundance across, and within cell types.

I assessed total tenascin-C mRNA levels using primers that anneal within the constitutively expressed domains FnIII 7-8, alongside primers specific for each alternatively spliced FnIII domain (Figure 4.6), in resting HDFs, GM-CSF macrophages, M-CSF macrophages, MDDCs, CD4⁺T-cells and CD8⁺ T-cells.

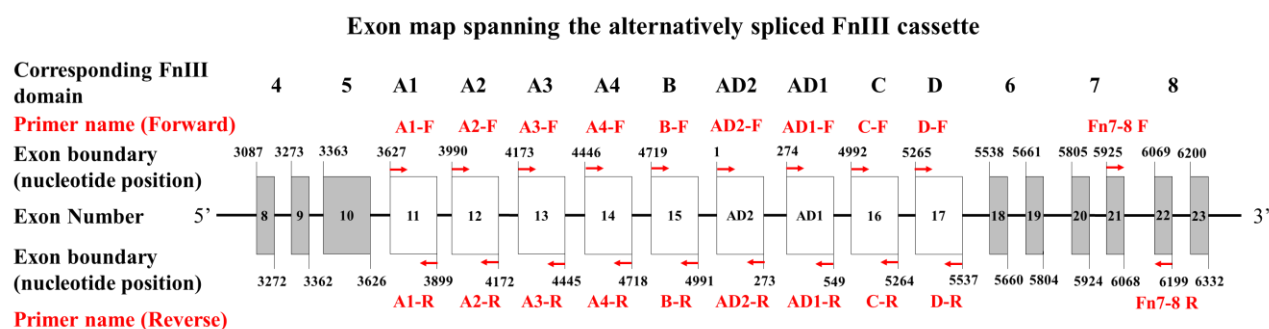


Figure 4.6. FnIII exon map of tenascin-C showing custom primers designed for qPCR analysis of splicing.

Custom DNA primers were designed to permit the quantification of alternatively spliced FnIII mRNA exons by standard curve qPCR. Primers were named eponymously with the FnIII domain to which the target exon corresponded, together with the forward (-F) and reverse (-R) suffixes. Targeted exons included those corresponding to the alternatively spliced FnIII domains A1, A2, A3, A4, B, AD2, AD1, C and D, and also to the constitutively expressed FnIII-7,8 which served as a control for total tenascin-C mRNA. The figure displays the exons corresponding to FnIII domains FnIII-4 (8-9), FnIII-5 (10), FnIII-A1 (11), FnIII-A2 (12), FnIII-A3 (13), FnIII-A4 (14), FnIII-B (15), FnIII-AD2 (AD2), FnIII-AD1 (AD1), FnIII-C (16), FnIII-D (17), FnIII-6 (18-19), FnIII-7 (20-21), FnIII-8 (22-23). The tenascin-C plasmid pBS KS II hTNC +AD2+AD1, was used to generate a standard curve for the quantification of FnIII mRNAs.

Basal levels of FnIII7-8 were highest in HDFs compared to the other cell types assessed. In HDFs FnIII7-8 containing mRNA was significantly more abundant than mRNA containing each of the alternatively spliced FnIII domains. Of the spliced domains, FnIII-B and D were most abundant and were significantly higher than FnIII-A1, AD2 and AD1; while FnIII-D was also found at significantly higher levels than FnIII-C (Figure 4.7.A).

In MDDCs, GM-CSF and M-CSF macrophages, basal levels of FnIII 7-8 containing mRNA were low relative to HDFs. Each spliced FnIII domain was expressed at the same level as FnIII 7-8; there were no significant differences between the abundance of FnIII7-8 and the other alternatively spliced FnIII domains in any myeloid cell type quantified. Total tenascin-C mRNA levels were slightly higher in GM-CSF macrophages than in the other myeloid cells assessed (Figure 4.7.B-D).

In CD4⁺ and CD8⁺ T-cells, levels of FnIII7-8 containing mRNA were low and comparable to those detected in MDDCs. Each spliced FnIII domain was expressed at the same level as FnIII 7-8. There was no significant difference in absolute levels of FnIII7-8 and any spliced FnIII domain, except for FnIII-A1 mRNA which was absent in both CD4⁺ and CD8⁺ T cells. CD4⁺ T-cells also exhibited lower FnIII-AD2 levels than CD8⁺ T-cells (Figure 4.7.E-F).

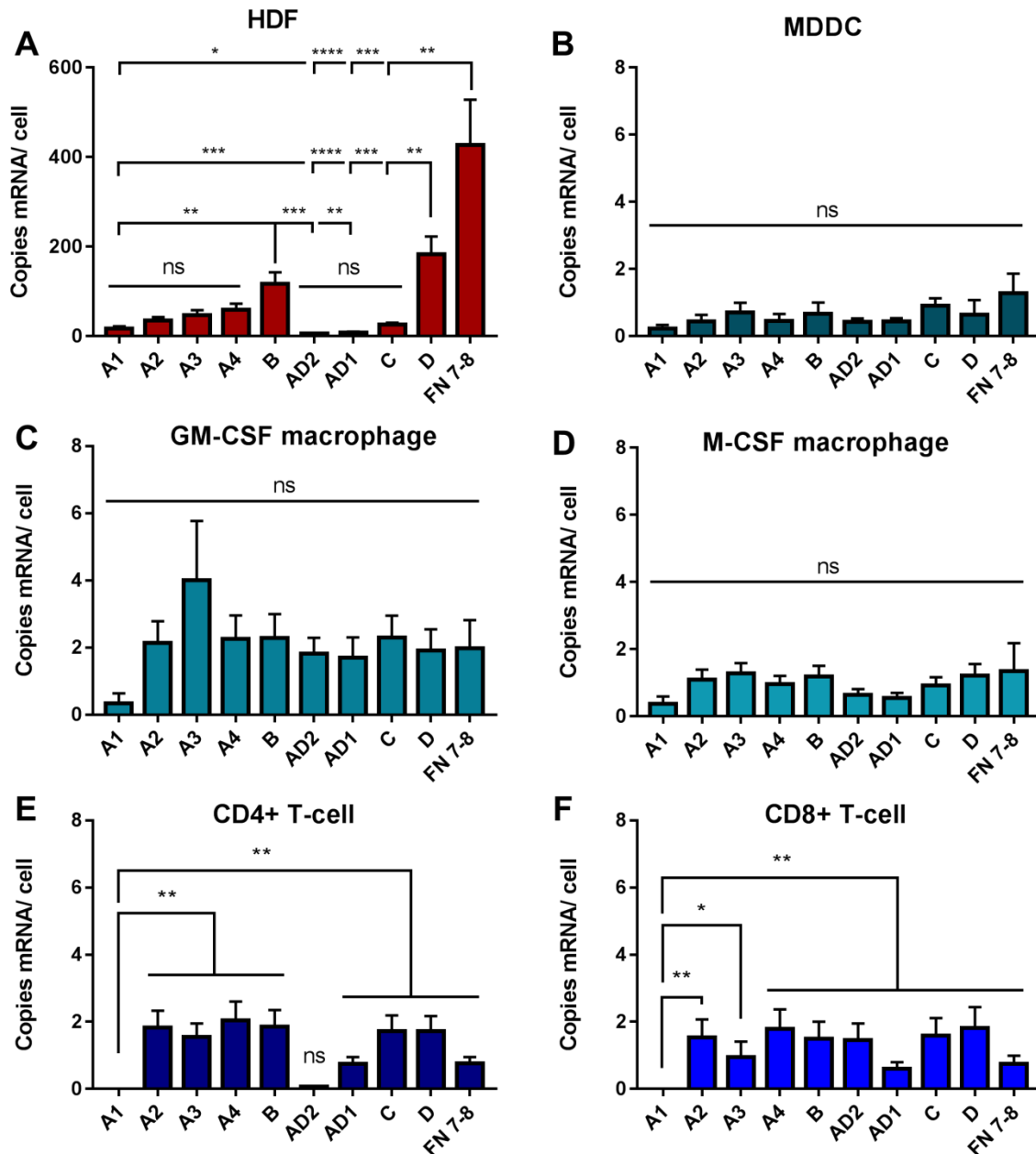


Figure 4.7. Absolute quantification of alternatively spliced tenascin-C FnIII mRNAs in resting HDFs, MDDCs, M-CSF, M-CSF macrophages and CD4+, CD8+ T-cells. The resting levels of alternatively spliced FnIII mRNAs corresponding to FnIII domains A1, A2, A3, A4, B, AD2, AD1, C, D, and the constitutively expressed FnIII 7-8, were quantified by standard curve qPCR. qPCR was performed on cDNA from HDFs rested for 48 hours (n=7) (A), in unstimulated MDDCs (n=6) (B), GM-CSF macrophages (n=4) (C), and M-CSF macrophages (n=4) (D) rested for 16 hours, and in CD4+ T-cells (n=5) (E) and CD8+ T-cells (n=5) (F) rested for 16 hours. Data are shown as mean + SEM. Un-paired t-tests were performed and statistical significance is displayed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and non-significant (ns).

These data confirm my findings of high basal expression of tenascin-C in HDFs, and low expression in immune cells previously described in chapter 3. This new splicing data

highlights that the alternative splicing of tenascin-C is different in fibroblasts, myeloid and lymphoid cells at rest, indicating that resting tenascin-C isoforms expressed by these cells contain a distinct repertoire of FnIII domains, with some domains more or less abundant.

4.10 All alternatively spliced FnIII exons are upregulated in activated MDDCs and macrophages except for FnIII-AD2 and AD1

In chapter 3 I showed that tenascin-C mRNA levels were transiently increased in myeloid cells in response to stimulation with 10ng/ml LPS, and that mRNA levels peaked 4 hours following stimulation. To determine if tenascin-C alternative splicing changes during activation, the absolute levels of induction of spliced FnIII mRNA were quantified by standard curve qPCR, and fold induction calculated by normalisation to endogenous control genes in MDDCs, GM-CSF and M-CSF macrophages that were left unstimulated, or that were stimulated for 4 h with LPS. The absolute quantification allows the direct comparison in the spliced FnIII mRNA abundances within a cell type, or across cell types; whereas the normalised fold induction best identifies whether the relative abundance of the spliced FnIII mRNAs changes in response to stimulation.

In MDDCs, the absolute resting levels of each spliced FnIII and FnIII 7-8 did not change over time, but following stimulation with LPS for 4 hours, the levels of FnIII7-8 were significantly increased. LPS stimulation also increased the levels of all spliced FnIII mRNAs except for FnIII-AD2 and –AD1; which were detected at significantly lower levels compared to all the other FnIII. In MDDCs, the peak level of FnIII7-8 was significantly greater than for all of the spliced FnIII mRNAs (See Appendix Figure 4.8.A). Normalisation of mRNA levels to GNB2L1 confirmed that LPS stimulation caused statistically significant inductions in all spliced FnIII and FnIII7-8 mRNAs, except for FnIII-AD2 and –AD1 (Figure 4.8.A).

Resting GM-CSF and M-CSF macrophages also exhibited no increase in absolute FnIII mRNA levels over time. LPS treatment increased the levels of FnIII7-8, to slightly higher levels in GM-CSF than in M-CSF macrophages. Following activation, the abundance of each spliced FnIII mRNAs in GM-CSF and M-CSF macrophages were increased to levels that were broadly similar across the two cell types; again with the exception of FnIII-AD2 and –AD1 which were present at lower levels as described for MDDCs. In GM-CSF macrophages, the peak levels of FnIII7-8 were significantly higher than for all FnIII except FnIII-A3; In M-CSF macrophages, the levels of FnIII-A3 and –B were not statistically different from FnIII7-8. Normalisation of the GM-CSF and M-CSF macrophage mRNA levels to HPRT1 revealed a similar induction pattern in response to LPS as seen in MDDCs (See Appendix Figure 4.8.B-C). Both macrophage cell types exhibited statistically significant inductions in FnIII7-8 and all spliced FnIII, except for AD2 and AD1, following LPS treatment (Figure 4.8.B-C).

To validate these findings, selected FnIII mRNAs were quantified in new MDDCs. The MDDCs were resting or stimulated with LPS for 4 hours, and mRNAs were quantified using a 10-point plasmid standard curve to assess the mRNA abundance by standard curve qPCR. For the validation, mRNAs corresponding to the following domains were selected; FnIII-A2, -C and FnIII7-8, because they were induced by LPS, and FnIII-AD2 and –AD1, because these were not induced by LPS treatment. This confirmed the LPS dependent increase in FnIII-A2, C and 7-8, and the lack of induction of FnIII AD2 and AD1. (See Appendix Figure 4.8.D-E).

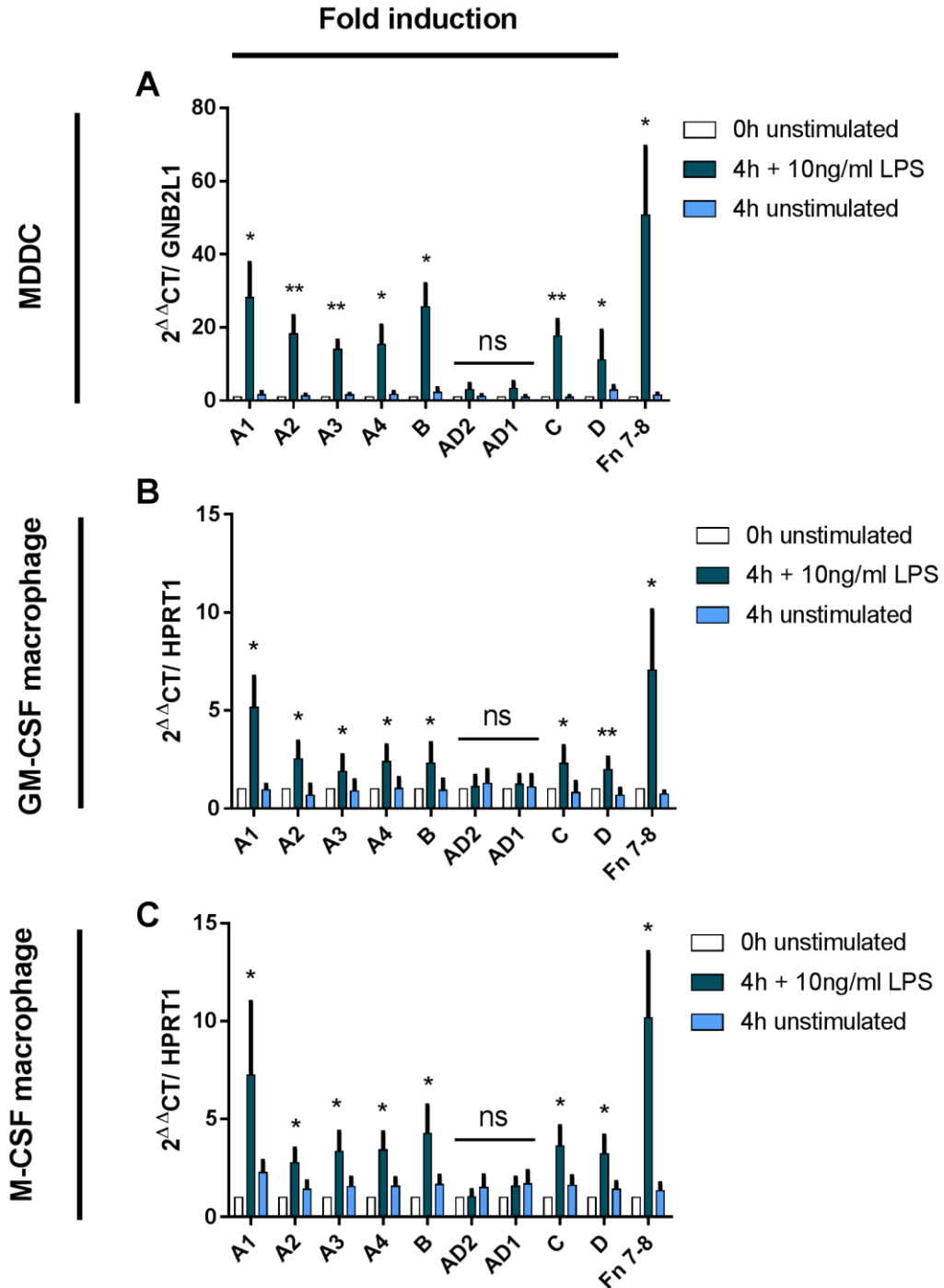


Figure 4.8. LPS stimulation induces transcription of all alternatively spliced FnIII mRNAs except AD2 and AD1 in MDDC, GM-CSF and M-CSF macrophages. Primary human MDDCs, GM-CSF and M-CSF macrophages were plated and rested for 16 hours and were then stimulated, or not-stimulated with 10ng/ml LPS for 0 and 4 hours. The relative fold inductions of alternatively spliced FnIII mRNAs and FnIII 7-8 in response to LPS stimulation were quantified by ΔCt qPCR in MDDCs (n=6) (A), GM-CSF macrophages (n=3) (B), and M-CSF macrophages (n=3) (C). Key, (white bars: 0h unstimulated/ dark blue bars: 4h + LPS/ light blue bars: 4h unstimulated). For ΔCt qPCR analysis, tenascin-C mRNA was normalised to GNB2L1 for MDDCs and to HPRT1 for GM-CSF and M-CSF macrophages. Data are shown as mean +SEM. 2-way ANOVA tests with multiple comparisons were performed within individual cell types (A-C). Statistical significance is displayed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and non-significant (ns).

Together, these data show that resting and activated myeloid cells make different tenascin-C splice variants, with activated myeloid cells upregulating all alternatively spliced FnIII apart from AD2 and AD1, leading to the expression of large tenascin-C isoforms that are low in FnIII-AD2 and AD1, and where FnIII-B,C may be expressed contiguously.

4.11 FnIII A1 mRNA is absent in CD4+ and CD8+ T-cells

In chapter 3 I showed that activation of CD4+ and CD8+ T-cells with anti-CD3/CD28 Dynabeads® resulted in a significant increase in tenascin-C mRNA levels which peaked 2 days following activation, and remained elevated for 7-10 days post stimulation. Here, I investigate the alternative splicing pattern of tenascin-C mRNA in these cells at both early and late activation time points.

Following stimulation of CD4+ T-cells for 2 days, FnIII 7-8 mRNA was elevated compared to unstimulated cells. The alternatively spliced FnIII were not induced. Levels of all spliced FnIII domains were comparable, with the exception of AD2 which was present at significantly lower levels than FnIII7-8, and A1, which was absent. 7 days following CD4+ T-cell culture, there was an increase in FnIII 7-8, -A3, -AD2, -AD1 and D, that was independent of cell activation, although FnIII-D levels were slightly increased following stimulation with the anti-CD3/CD28 Dynabeads®. Levels of FnIII-A2, A4, B, and C did not change. Levels of FnIII AD2 were the most abundant at this time point and A1 was still absent (Figure 4.9.A).

In CD8+ T-cells stimulated for 2 days, the absolute levels of FnIII7-8 mRNA increased significantly compared to unstimulated cells, but there was no increase in the spliced FnIII. The abundance of all spliced FnIII domains was comparable, apart from FnIII-AD1 where levels were low, and -A1, which was completely absent. After stimulation for 7 days, there was a stimulation independent rise in the levels of FnIII-A2, -AD2, -AD1, -C and -D. Levels

of A1, A3, A4, B did not change. At this time point, FnIII-C was the most abundant mRNA detected, with significantly higher levels than FnIII7-8, and A1 was still absent (Figure 4.9.B).

To confirm that FnIII-A1 was not expressed in these cells, the absolute abundance of FnIII-A1, FnIII7-8 and YWHAZ was validated by standard curve qPCR in different donor samples. The standard curve showing serial dilutions of tenascin-C plasmid quantified with FnIII-A1 primers, shows representative samples from stimulated CD4⁺ and CD8⁺ T-cell samples to be below the limit of detection for the qPCR assay, confirming the absence of FnIII A1 mRNA in resting or activated T-cells (See Appendix Figure 4.9.A). Standard curve analysis confirmed that FnIII A1 was absent, that FnIII7-8 levels were increased following stimulation, and that the control gene YWHAZ was stably expressed (Figure 4.9.C,D).

Together these data revealed that the tenascin-C splicing pattern does not change in activated T-cells, and that T-cells do not express FnIII domain A1.

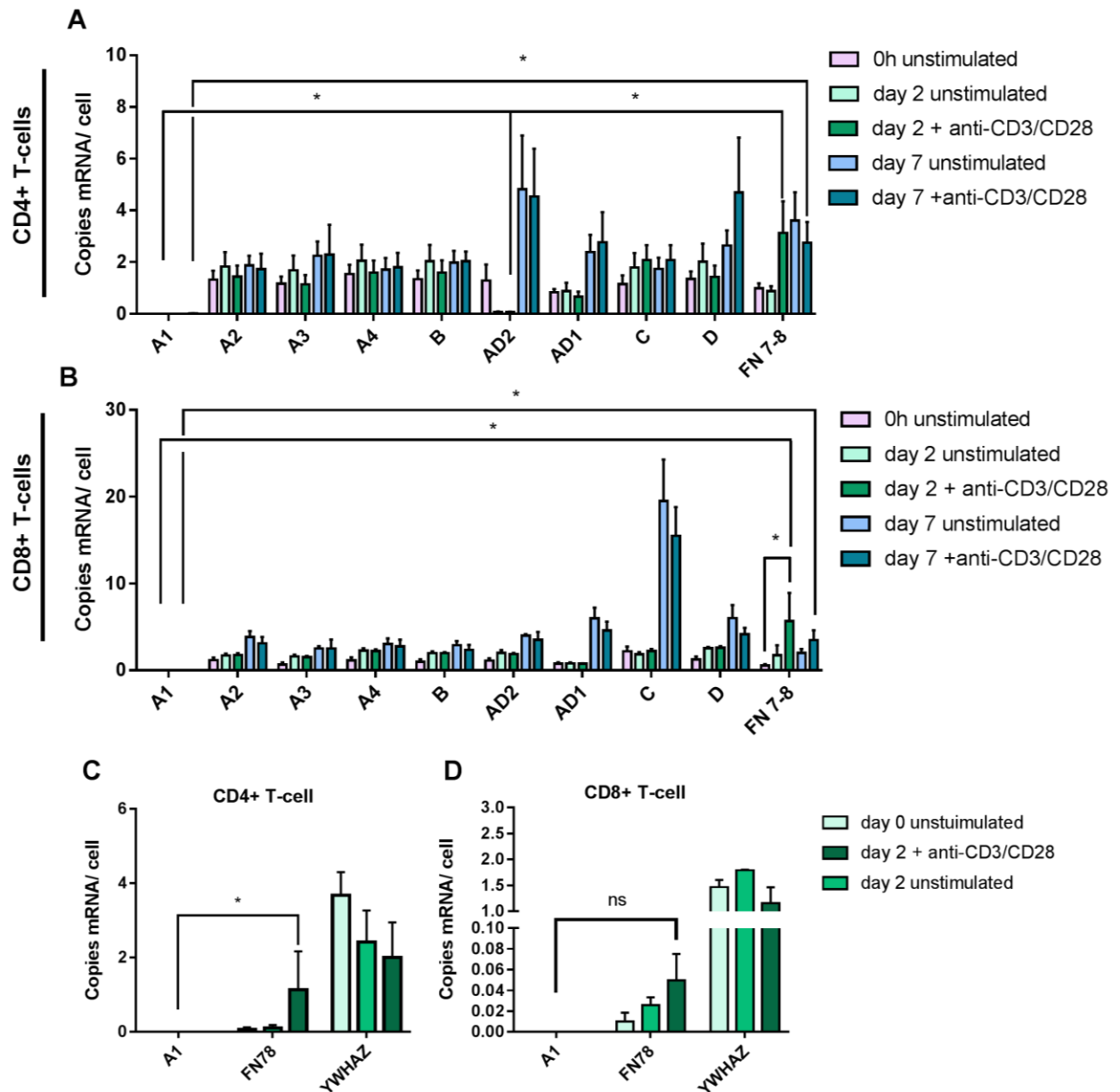


Figure 4.9 Alternatively spliced FnIII are not regulated in activated CD4+ or CD8+ T-cells, and FnIII A1 is absent. Primary human T-cells were harvested, purified and plated for 16 hours to rest. Following this, cells were stimulated with anti-CD3/CD28 dynabeads® at a 1:1 cell:bead ratio, and mRNA was extracted at 0h, 2 day and 7 day time points. The abundance of FnIII mRNA was quantified by standard curve qPCR in CD4+ T-cells (n=5) (A), and in CD8+ T-cells (n=5) (B). The absolute abundance of FnIII A1, FnIII7-8 and YWHAZ mRNAs were quantified by standard curve qPCR in CD4+ (n=5) (C) and CD8+ (n=5) T-cells (D). Data are shown as mean + SEM. 2-way ANOVA tests with multiple comparisons were performed within individual cell types (A,B) and un-paired t-tests were performed between stimulated and unstimulated cell types (C,D). Statistical significance is displayed as *p<0.05 and non-significant (ns).

4.12 IL-1 β does not impact tenascin-C alternative splicing in HDFs

In chapter 3 I showed that IL-1 β stimulation of HDFs induces tenascin-C mRNA and protein expression. However, nothing is known of whether activated HDFs express specific alternatively spliced tenascin-C isoforms. Therefore mRNA from HDFs stimulated with 10ng/ml IL-1 β for 0 and 4 hours was assessed, and the absolute level of alternatively spliced tenascin-C FnIII mRNA was quantified by standard curve qPCR, and the fold induction was calculated normalised to RPLP0.

Treatment with IL-1 β caused a small increase in FnIII7-8 levels after 4 hours, compared to cells that were not stimulated. It also resulted in a small increase in the levels of all alternatively spliced tenascin-C FnIII domains. The least abundant spliced FnIII domains were AD2 and AD1, and FnIII-B and -D were the most abundant spliced FnIII detected (Figure 4.10.A). Normalisation of the absolute mRNA levels to RPLP0, show that there is a small IL-1 β specific induction in all alternatively spliced FnIII and FnIII 7-8 mRNA, but this induction was only statistically significant for AD1 and FnIII 7-8 (Figure 4.10.B).

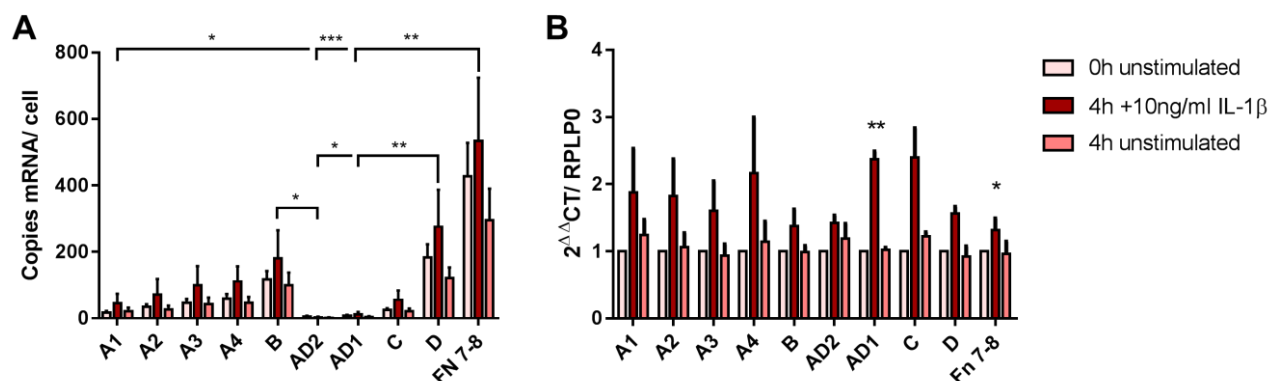


Figure 4.10. IL-1 β stimulation in HDFs does not significantly impact tenascin-C alternative splicing. The absolute abundance and the relative fold induction of alternatively spliced FnIII and FnIII 7-8 in response to stimulation with 10ng/ml IL-1 β for 0 and 4 hours, was quantified in primary human HDFs by A) standard curve qPCR (n=7), and B) $\Delta\Delta Ct$ qPCR normalized to endogenous control gene RPLP0 (n=7) respectively. Data are shown as mean $\pm$ SEM. 2-way ANOVA tests were performed and statistical significance is displayed as *p<0.05, **p<0.01, and ***p<0.001.

These data reveal that all alternatively spliced FnIII mRNAs are upregulated in activated HDFs, with each domain exhibiting a similar fold increase. This indicates that whilst global expression of tenascin-C by HDFs is increased by IL-1 β , the isoforms expressed have the same overall domain composition. As such, activated HDFs express higher levels of tenascin-C, and, as in resting cells, the tenascin-C isoforms present contain higher levels of FnIII domains B and D.

4.13 Alternatively spliced FnIII mRNAs are differentially regulated in disease

I previously showed significantly higher levels of tenascin-C expression in HDFs than in synovial fibroblasts, and elevated tenascin-C expression in RA-SFs and OA-SFs relative to NSFs. The effect of cell type on the alternative splicing of the tenascin-C mRNA transcript was assessed in resting HDF, RA-SF, OA-SF and NSF cells cultured for 72 hours.

Firstly FnIII 7-8 mRNA was quantified by standard curve qPCR, and revealed the same pattern of expression as the TaqMan assay previously described in chapter 3, with HDFs containing the most copies per cell, followed by RA-SFs and OA-SFs with the NSFs containing the least FnIII 7-8 (Figure 4.11). However, as expected for a different qPCR assay, the absolute numbers of mRNA copies/cell calculated is slightly different for FnIII 7-8 and for tenascin-C mRNA.

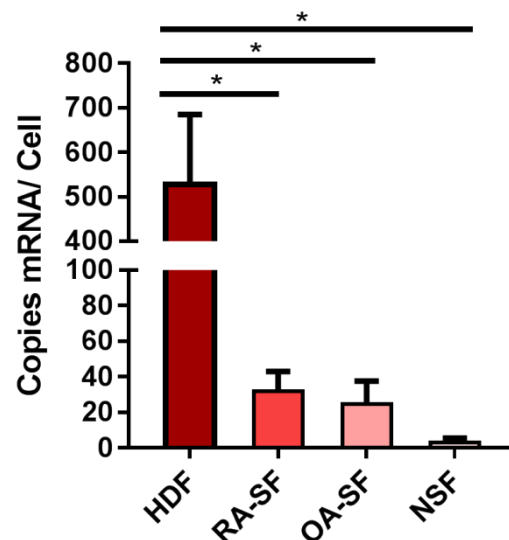


Figure 4.11. FnIII 7-8 mRNA abundance is elevated in HDFs, RA-SFs and OA-SFs relative to NSFs. The absolute abundance of FnIII 7-8 mRNA was quantified by standard curve qPCR. Cells were rested 72 hours prior to lysis, RNA extraction, and mRNA quantification in HDFs (n=7), RA-SFs (n=3), OA-SFs (n=3), and NSFs (n=2). Data are shown as mean $\pm$ STDEV for n=2, and as SEM for n $\geq$ 3. Un-paired t-tests were performed comparing FnIII7-8 copies/cell across cell types and statistical significance is displayed as *p<0.05.

I next examined levels of each alternatively spliced FnIII domain in these four types of fibroblasts compared to levels of FnIII7-8. In HDFs, FnIII7-8 mRNA levels were significantly higher than all other domains, of which FnIII B and D mRNAs were most abundant, and FnIII AD2 and AD1 the least abundant (Figure 4.12.A). In NSF, all FnIII domains were expressed at levels comparable to FnIII 7-8, except for FnIII A1; which was almost absent in NSF (Figure 4.12.B). RA-SFs, exhibited a pattern of FnIII expression similar to HDFs with FnIII 7-8 being the most abundant and with higher levels of FnIII-B and D, and low levels of FnIII AD2 and AD1. However the absolute levels of each domain were lower in RASFs than in HDFs (Figure 4.12.C). OA-SFs displayed a splicing pattern similar to NSF. Similarly to NSF, FnIII-A1 is almost undetected, and there is no difference in overall mRNA abundance between the FnIII 7-8 and any of the spliced FnIII mRNAs (Figure 4.12.D).

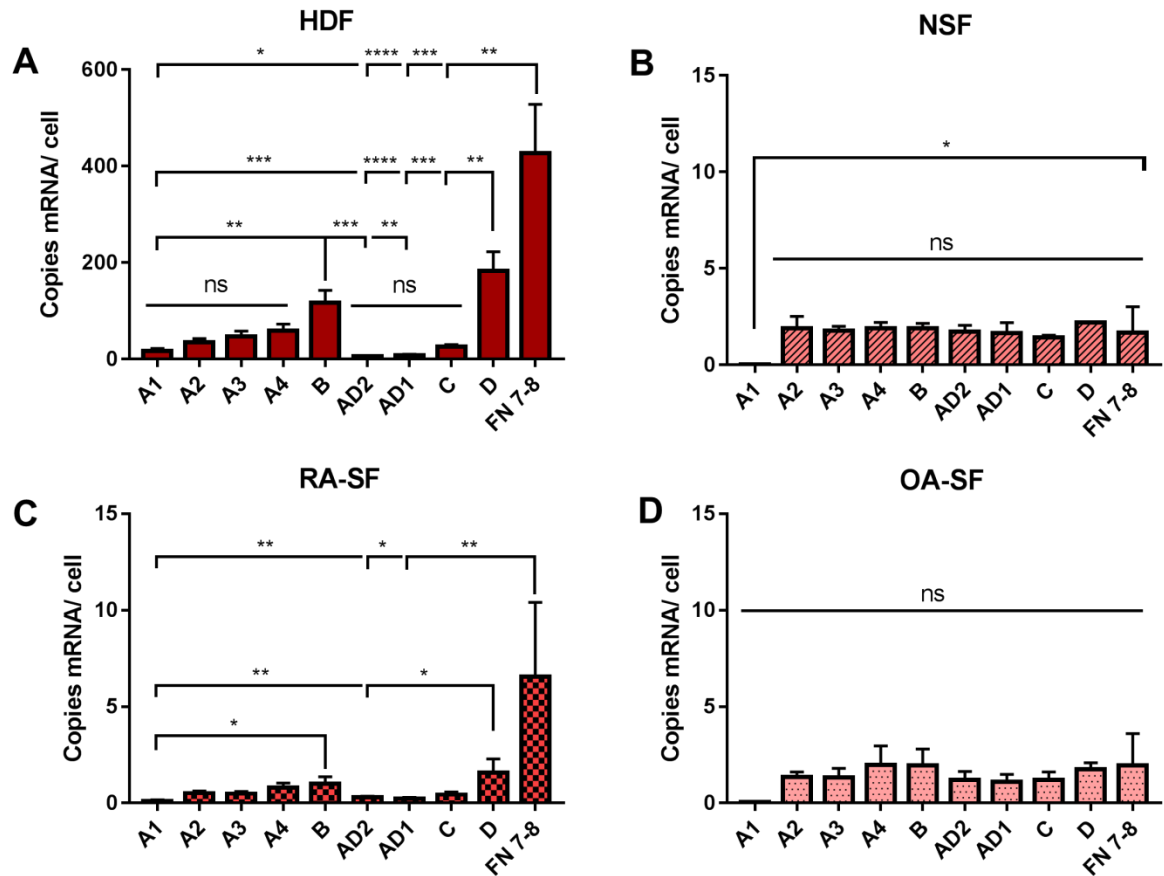


Figure 4.12. Absolute abundance of alternatively spliced FnIII mRNAs in HDFs, RA-SFs, OA-SFs and NSFs. Absolute levels of alternatively spliced FnIII and FnIII 7-8 mRNAs were quantified by standard curve qPCR in resting HDFs (n=7), RA-SFs (n=3), OA-SFs (n=3), and NSFs (n=2). Data are shown as mean $\pm$ STDEV for n=2, and as mean $\pm$ SEM for n $\geq$ 3. Un-paired t-tests were performed and statistical significance is displayed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and non-significant (ns).

4.14 Summary of tenascin-C alternative splicing characterisation

In resting cells, qPCR detected high basal levels of total tenascin-C mRNA in HDFs, where FnIII-B and D were over represented relative to the other FnIII, and AD2 and AD1 levels were low. In resting MDDCs, GM-CSF and M-CSF macrophages the total levels of tenascin-C mRNA were much lower than in HDFs, and contained roughly equal levels of each alternatively spliced FnIII mRNA compared to constitutively expressed FnIII 7-8 mRNA. In CD4⁺, CD8⁺ T-cells, total levels of tenascin-C mRNA were low, all spliced domains were detected at equally low levels as FnIII 7-8, except for FnIII A1, which was absent.

This study observed changes in tenascin-C splicing patterns in activated cells. HDFs stimulated with IL-1 β increased the abundance of total tenascin-C mRNA. Each spliced FnIII was also increased in stimulated HDFs to the same degree, meaning that higher levels of the same isoforms (rich in FnIII-B and D, and poor in AD2 and AD1) were observed in resting and activated cells (Figure 4.13.A)..

MDDCs, GM-CSF macrophages and M-CSF macrophages exhibited a clear increase in total tenascin-C mRNA abundance following LPS stimulation, and this was accompanied by an increase in abundance of all alternatively spliced FnIII mRNAs except for FnIII AD2 and AD1 which were selectively not upregulated (Figure 4.13.B-C).

CD4⁺ and CD8⁺ T-cells exhibited similar tenascin-C splicing behaviour in response to anti-CD3/CD28 stimulation over 2 and 7 days. Cell activation increased levels of total tenascin-C mRNA, but caused no overall change in the abundance of spliced FnIII at day 2. At day 7, stimulation slightly increased the overall levels of tenascin-C, but not the overall levels of the alternatively spliced FnIII. Interestingly, FnIII-A1 mRNA was never detected in CD4⁺ or CD8⁺ T-cells during any of the assay conditions in this study (Figure 4.13.D).

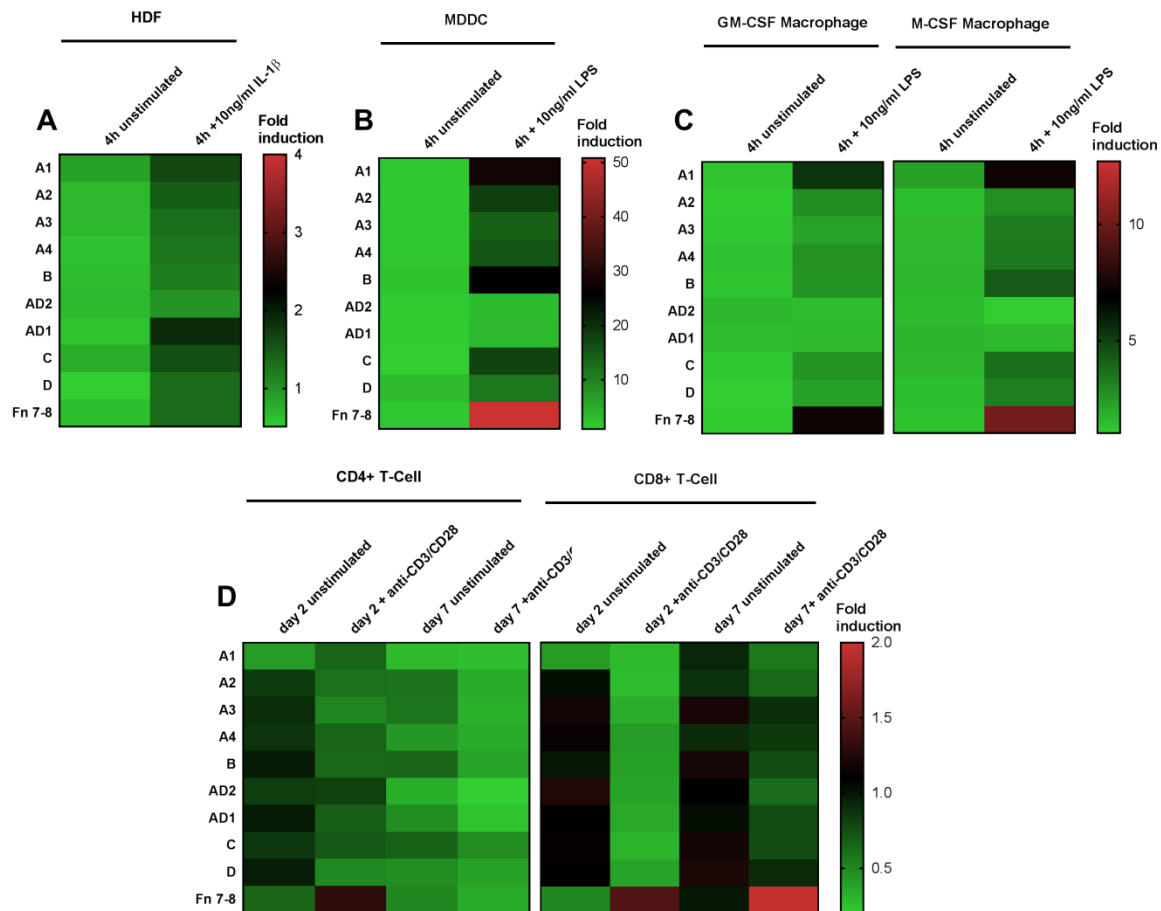


Figure 4.13. Heat maps displaying the fold induction of alternatively spliced FnIII mRNAs in activated HDFs, MDDCs, macrophages and T-cells. Heat maps show the fold induction of alternatively spliced FnIII and FnIII 7-8 mRNAs from basal conditions (0 hours, unstimulated) in A) HDFs stimulated $\pm 10\text{ng/ml}$ IL-1 β for 4 hours ($n=7$), B) MDDCs ($n=7$), C) GM-CSF and M-CSF macrophages rested 16h prior to stimulation with $\pm 10\text{ng/ml}$ LPS for 0 and 4 hours ($n=3$). D) CD4 $^{+}$ and CD8 $^{+}$ T-cells stimulated $\pm$ anti-CD3/CD28 dynabeads® for 0 hours, 2 days and 7 days ($n=4$). Heat maps display the mean fold induction relative to the 0h unstimulated time point, which has a value of 1 and is not included on the heat maps.

I also examined splicing in synovial fibroblasts. Expression of tenascin-C was in HDFs high, relative to the other fibroblasts, and as stated above, FnIII-B and D were abundantly transcribed, while FnIII-AD2 and AD1 levels were low. In contrast, NSF expressed low total levels of tenascin-C mRNA with an equal levels of spliced FnIII and FnIII 7-8 mRNA; although the transcripts completely lacked FnIII A1. RA-SFs displayed a similar splicing pattern to HDFs, but with lower absolute copies mRNA/cell; with relatively high levels of total tenascin-C mRNA, FnIII- B and D-, and with low levels of FnIII-AD2 and AD1. OA-

SFs exhibit the same splicing pattern as NSF, but with slightly lower abundance of spliced FnIII mRNAs (Figure 4.14).

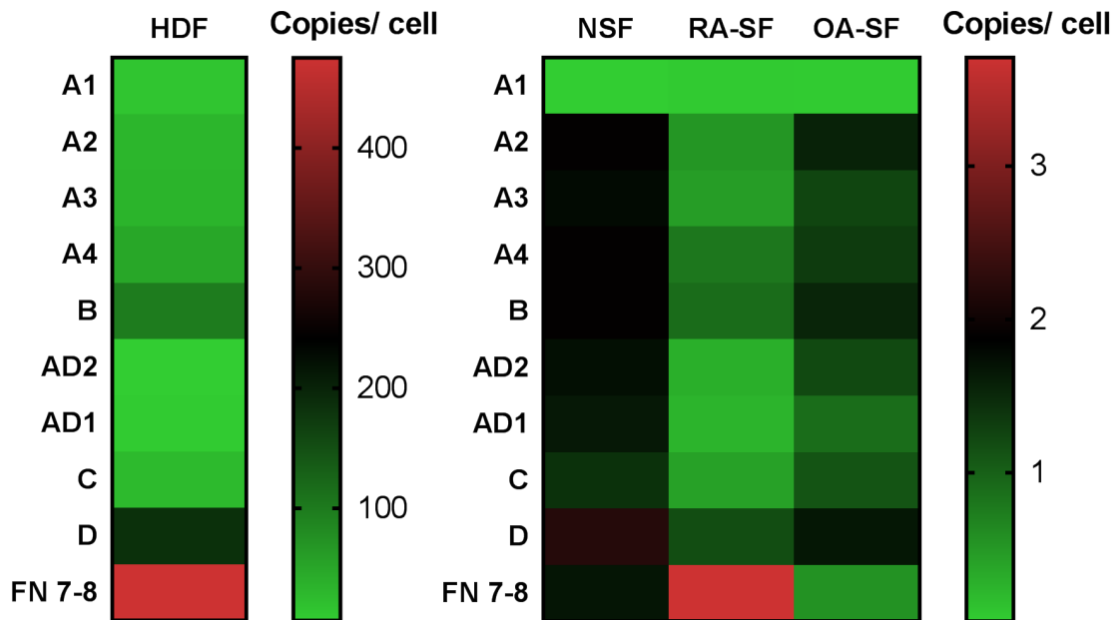


Figure 4.14. Heat maps illustrating the absolute abundance of alternatively spliced mRNAs in HDFs, RA-SFs, OA-SFs and NSFs. Heat maps represent absolute levels of alternatively spliced FnIII and FnIII 7-8 mRNAs in resting cells, quantified by 2-standard curve qPCR. Cell types are shown left to right, HDFs (n=7), NSFs (n=2), RA-SFs (n=3), and OA-SFs (n=3). Heat maps display mean copies/ cell.

4.15 Discussion

This chapter details the characterisation of tenascin-C alternative splicing in resting and activated MDDCs, GM-CSF macrophages, M-CSF macrophages, CD4⁺ T-cells, CD8⁺ T-cells, and HDFs, as well as in NSFs, RA-SFs and OA-SFs. Western blotting permitted the visualisation of differently sized tenascin-C protein isoforms that were expressed in cell lysate or supernatant fractions, but gave no information as to what alternatively spliced FnIII domains were present, or the total number of isoforms expressed. The second part of this study was dedicated to generating a novel method for characterising tenascin-C alternative splicing by conducting absolute quantification of individual spliced FnIII mRNAs, which gave greater sensitivity, permitted comparison of FnIII mRNA abundance across cell types, and comparison of different FnIII mRNAs within cell types. This study is the first time that standard curve qPCR has been utilised for the analysis of tenascin-C splicing, and the protocol generated here, has been published in *Methods in Cell Biology*, *in press*, see the end of thesis for attached document. Together these data reveal cell type specific tenascin-C splicing patterns in resting myeloid and lymphoid cells and fibroblasts, changes in splicing in activated cells of different lineages, and disease specific tenascin-C splicing.

4.15.1 *Western Blotting and estimation of tenascin-C isoform size*

Characterization of tenascin-C splicing by Western blot is widely reported in the literature, and typically entails the separation of tenascin-C isoforms by SDS-PAGE, and immunostaining with antibodies that bind to constitutively expressed tenascin-C domains, permitting the visualisation of all tenascin-C isoforms (reviewed in chapter 1 and ²⁴⁸). Several studies previously used antibodies that bind to individual alternatively spliced FnIII domains^{130, 155, 169, 173}, which enable identification of individual spliced FnIII domains present within tenascin-C protein bands. However, antibodies for FnIII-A3, AD2 or AD1 are not yet commercially available so a complete analysis of all domains cannot be undertaken this way. Additional limitations of this approach include the high structural homology of FnIII-A1 and -A4, which currently prevents the generation of antibodies that do not cross react with either domain. For this study I used an antibody that bound to a constitutively expressed epitope of tenascin-C as previously described²⁷³, so that I may observe all isoforms and compare the size and number of bands to those reported for other cell types.

I also investigated whether Western blotting would allow accurate estimation of the sizes of tenascin-C isoforms. Accurately doing so is dependent on identical treatment of samples prior to SDS-PAGE. Therefore, all samples are boiled in SDS to place a uniformly negative charge on the denatured proteins so only differences in mass impact the SDS-PAGE migration rate. The spliced FnIII domains A1-D have theoretical amino-acid chain masses of ~10kDa, meaning that inclusion of single additional spliced FnIII domains in the tenascin-C isoform will increase the mass in increments of ~10kDa; which may theoretically allow the estimation of the number of alternatively spliced FnIII domains present in a single tenascin-C band. This assumes that all of the mass added by spliced FnIII domains is attributable to non-post translationally modified protein, but tenascin-C is reported to be glycosylated.

NetOGlyc 4.0 sequence analysis revealed that 26 putative *N*-glycosylation sites are densely clustered within the alternatively spliced FnIII domains of tenascin-C, while 34 putative *O*-glycosylation sites are clustered within the constitutive tenascin-C domains^{248, 397} (Figure 4.15). Accurately quantifying the number of spliced FnIII domains present in tenascin-C isoforms is therefore not possible by Western blot unless the tenascin-C is deglycosylated, and the samples are separated by SDS-PAGE over a large migration distance, so the small ~10kDa differences in detected mass can be resolved. Even taking glycosylation into account, this method doesn't determine between isoforms of different sizes due to other post-transcriptional modifications, or which are proteolytically clipped.

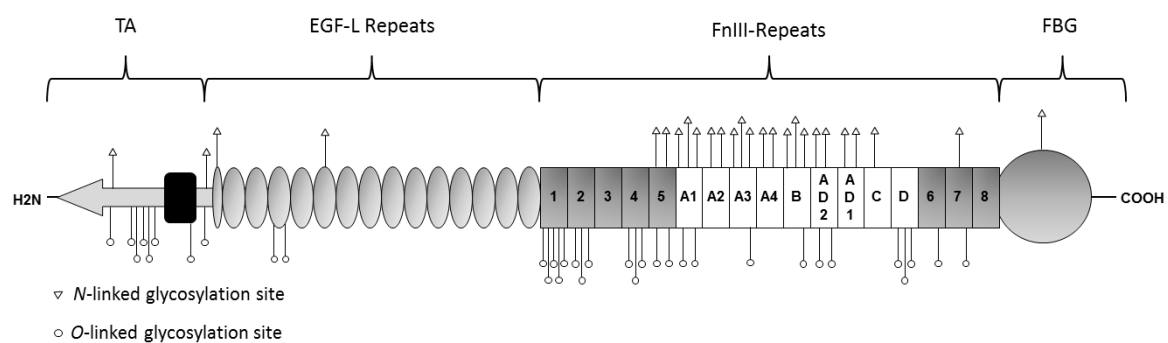


Figure 4.15 Predicted *N*- and *O*- glycosylation sites with tenascin-C. Tenascin-C possesses 26 putative *N*-linked glycosylation sites, and 34 putative *O*-linked glycosylation sites^{59, 63, 397, 398}; the locations of which are represented on the tenascin-C stick diagram by triangles and circles respectively.

To assess the extent of *N*-glycosylation of tenascin-C, U-87 MG cell lysate was incubated with *N*-glycosidase enzyme PNGase-F to cleave carbohydrate groups from the tenascin-C amino acid chain. Incubation with the enzyme reduced the mass by around ~20kDa, in line with previous observations^{75, 95, 110} (Figure 4.16); and *N*-glycosylation therefore accounted for at least 6.6% of the detected mass. Glycosylation of alternatively spliced FnIII domains can greatly impact cell function. The HNK-1 epitope, which may be found on FnIII-A1,A2,A4,B,C,D containing tenascin-C, and was previously shown to promote neural stem

cell proliferation by modulating the expression of the EGF-R^{126, 127}. Other studies have previously reported glycosylation on FnIII-B⁶³, and chicken FnIII-AD2 and AD1⁷⁶.

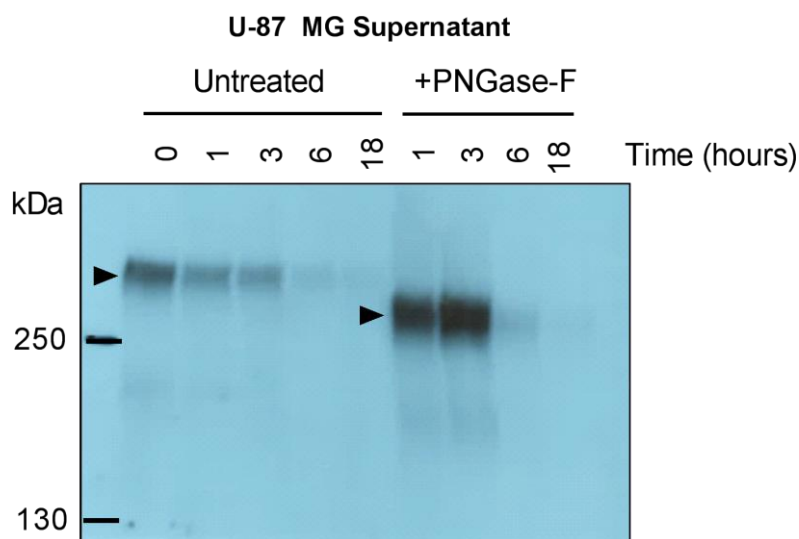


Figure 4.16. U-87 MG secreted tenascin-C is N-glycosylated. Supernatant from U-87 MG cells cultured for 72 hours was incubated in the absence, or presence of *N*-glycosidase enzyme PNGase-F for 0, 1, 3, 6 and 18 hours. Samples were then separated by SDS-PAGE and tenascin-C bands were visualised by Western blot using mAb1908. Samples were run alongside the PageRuler™ Plus protein ladder. The blot is representative of 3 independent experiments.

Another factor that precluded precise measurement of isoform size was the MW markers. This chapter used two protein ladders to estimate the molecular weight of tenascin-C isoforms. The first PageRuler™ Plus Prestained Protein Ladder was shown during this study to have an uppermost marker that ran at a lower molecular weight than the highest molecular weight tenascin-C isoforms. Therefore a second protein ladder, HiMark™ Prestained Standard (31-460kDa), which contained bands that exceeded mass of the tenascin-C isoforms, was used to attempt to more accurately estimate the higher molecular weight isoforms. However, when run alongside each other, the two protein ladders gave greatly different migration rates for protein markers that were reportedly the same sizes. Such an effect may result from differences in electrostatic charges present in the ladders, incomplete reduction of the ladders in SDS, or non-optimal SDS-PAGE conditions. This discrepancy,

supported my notion that accurate quantification of tenascin-C isoform size by Western blot is impractical, and that only the relative sizes of tenascin-C protein bands may be inferred. In this study, conclusions about the absolute sizes of tenascin-C isoforms were not made, but the sizes of tenascin-C bands were compared relative to the PageRulerTM Plus ladder.

Tenascin-C is also prone to proteolytic cleavage, which may result in an underestimation of the total mass due to cleavage of domains from the antibody binding epitope region, which may result in an underestimation of the total mass. Due to the possibility of the Western blot interpretation being confounded by these factors, I also developed a qPCR method to extend and complement this analysis. This new qPCR technique revealed the abundance of individual spliced FnIII mRNAs and identified domains that are over- or under-represented in resting and activated cells, and in fibroblasts from normal and diseased synovium.

4.15.2 *Absolute quantification of alternatively spliced FnIII mRNAs by standard curve qPCR*

I investigated a number of different ways to assess FnIII domain content at the mRNA level. Data shown in this chapter above uses a standard curve qPCR method to quantify the absolute abundance of individual spliced FnIII mRNAs; using primers that annealed within single exons. Samples were quantified against a tenascin-C plasmid standard that contains all of the spliced FnIII domains, and was at a known concentration. This method allowed the absolute levels of individual spliced FnIII domains to be directly compared across cell types, and of different FnIII within the same cell types. However, this method could not detect the FnIII domains present in an individual tenascin-C mRNA transcript, not which combinations of transcripts were expressed. This method could be improved in future by repeating the

protocol using exon spanning primers for adjacent FnIII, which would allow for the detection of FnIII pairs; to build a picture of the neighbouring FnIIIs that are transcribed.

Before starting the standard curve qPCR method I attempted a different published protocol which entails PCR using forward and reverse primers that anneal in the constitutively transcribed FnIII exons 8 and 18. In this way, the primers span the alternatively spliced FnIII A1-D cassette (exons 9-17), amplifying the entire contents of the spliced region. The amplicon size then infers the number of spliced FnIII mRNAs present in the transcript, and the contents of individual transcripts can be further identified by clone isolation, DNA sequencing or in-situ hybridization. This method has been successfully used to identify tenascin-C mRNA variants by Garwood *et al.*, in mouse oligodendrocyte precursor cells³⁹⁹. A similar nested PCR approach has previously been used by other groups to identify tenascin-C mRNA isoforms by nested PCR of the spliced cassette, followed by Northern blotting with cDNA probes, in lung adenocarcinoma¹⁵², and in breast cancer cells¹⁵¹; with an additional DNA sequencing step being performed on PCR products purified by agarose gel in human fetal membranes¹¹⁷.

I used the same primers in my study (T8F/T18R) as those published in^{117, 186, 188, 204}. Control primers to constitutively expressed FnIII 7-8 (T20F/23R) were generated to confirm the presence of tenascin-C mRNA in the samples; and primers to the control gene GAPDH (GAPDHf/GAPDHr) were used as a positive control for cDNA loading, and the PCR thermocycler conditions⁴⁰⁰. The predicted amplicon sizes of the T8F/T18R products are listed in Figure 4.17.A.

Using a plasmid containing full length tenascin-C as a positive control, I could amplify a product using primers T8F/T18R that corresponded to the full spliced FnIII cassette, at 2903bp. In cDNA from U-87 MG cells, these primers only amplified a single band of 443bp,

corresponding to tenascin-C without any spliced FnIII domains (Figure 4.17.B). A working example gel from the Garwood *et al.*, 2004 paper, is shown. Here, amplification of different mouse tenascin-C isoform mRNA sizes resulted in a clear 273bp ladder as the number of spliced FnIII in the amplified transcripts increased from 0-7³⁹⁹ (Figure 4.17.C). Subsequent analysis in U-87 MG time course samples, produced an amplicon ladder of product sizes that corresponded to tenascin-C isoforms containing 0, 1, 2, 3 and 5 spliced FnIII domains, but also yielded off target amplicons, not corresponding to tenascin-C isoforms (Figure 4.17.D). In resting and activated MDDCs, amplicons of sizes corresponding to variants with 0, 1, 2 and 7 FnIII were detected, many but non-specific bands were also observed (Figure 4.17.E). This method was also performed on cDNA from resting and activated HDFs, and from resting NSFs, RA-SFs and OA-SFs, where only the smallest predicted tenascin-C variant was detected, amongst a number of other non-specific bands (Figure 4.17.F).

I tried several ways to optimise the PCR reaction with these primers, but I could not improve the specificity of the reaction. This method was therefore unsuitable for my purpose of characterising tenascin-C mRNA splicing, due to its lack of specificity, and the experimental bias in the PCR that preferentially amplified small amplicons. I subsequently investigated a new method for characterising splicing at the mRNA level using standard curve qPCR.

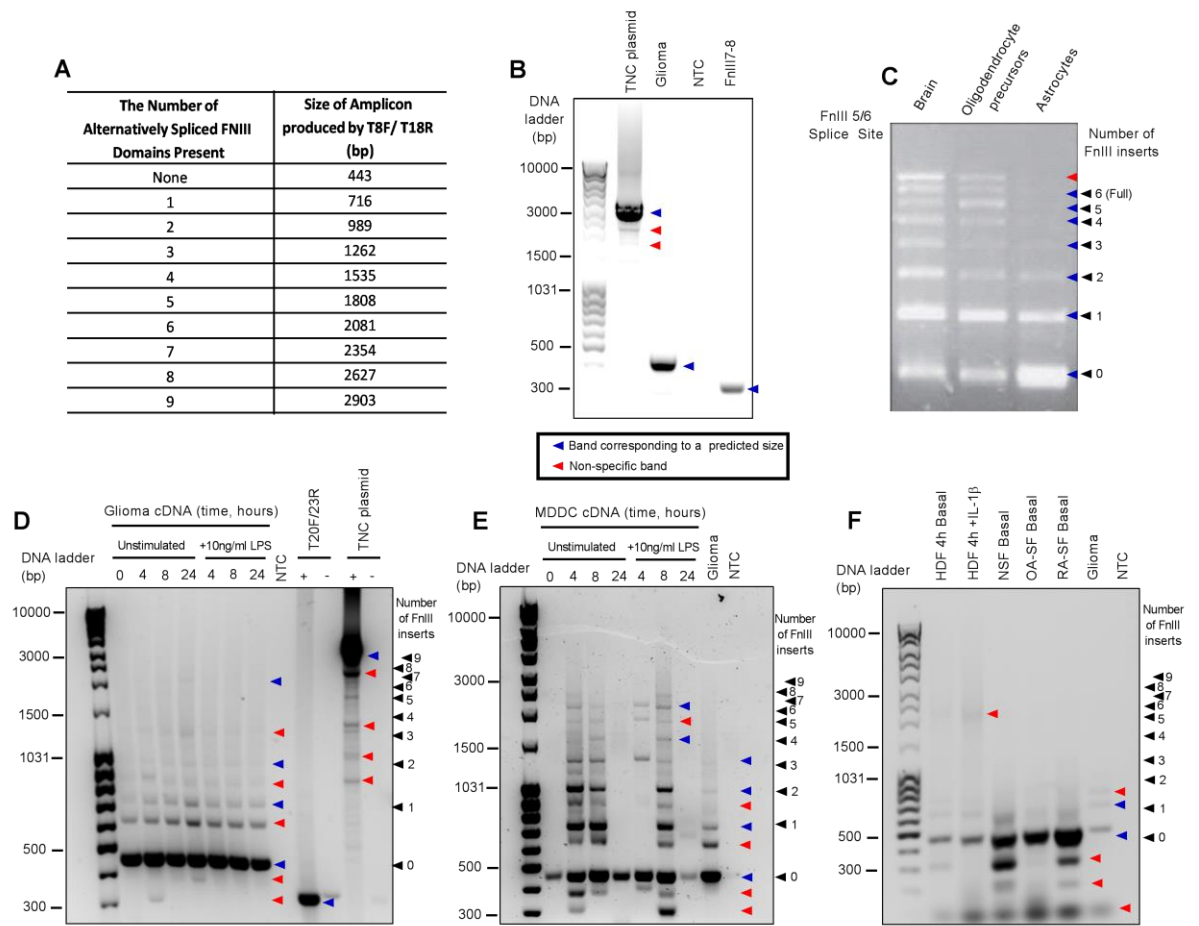


Figure 4.17. Method for determining the sizes of individual alternatively spliced tenascin-C mRNA transcripts using conventional PCR. The table shows the predicted sizes of amplicons resulting from the amplification of tenascin-C cDNA transcripts containing between 0 and 9 alternatively spliced FnIII exons (A). Gel shows a control experiment where the full FnIII cassette was amplified by T8F/T18R primers from a full length tenascin-C plasmid and U87-MG glioma rested for 24 hours, with negative no-template control and amplification of the FnIII 7-8 domains by T20F/T20R primers (B). Gel image is modified from Garwood *et al.*,³⁹⁹ and shows a working example of amplification of mouse tenascin-C cDNAs from using the T8F/T18R primer set (C). Gels show T8F/T18R primers run against purified TNC plasmid, and cDNA from resting and LPS stimulated U-87 MG at 0, 4, 8 and 24 hours, alongside T20F/23R primers amplifying FnIII 7-8 in the control plasmid (D). T8F/T18R amplified cDNA from resting and 10ng/ml LPS stimulated MDDCs (E). T8F/T18R amplicons from resting and 10ng/ml IL-1 β stimulated HDFs, were run alongside those for resting NSF, OA-SF, RA-SFs and U-87 MG glioma (F). PCR products were run on gels alongside the GeneRuler™ 1Kb DNA ladder. PCR was performed over 35-cycles, and amplicons were separated by gel electrophoresis in 2% w/v TopVision® agarose gels. Gel images are representative of 3 independent experiments. Specific PCR product bands are indicated by a blue arrow, and non-specific bands are indicated by a red arrow (B,C,D,E,F).

4.15.3 *Western blotting in U-87 MG cells reveal size specific isoform localisation*

Western blotting revealed that smaller tenascin-C bands were retained by U-87 MG cells and were not secreted to the supernatant, whereas the large tenascin-C bands were both retained, and secreted. This suggests that tenascin-C trafficking or localisation outside of the cell is dependent on the size or the content of the isoform; and may be regulated by alternative splicing, proteolysis, or other post translational modifications such as glycosylation. Little is known about how the alternatively spliced FnIII cassette may promote or hinder cell association of isoforms. The introduction of alternatively spliced FnIII domains with ligand binding sites such as $\alpha 7\beta 1$ seen in FnIII-D⁶⁴ may increase the likelihood of cell association, while the interruption of existing binding sites by incorporating additional alternatively spliced FnIII domains may occur; as was observed when the binding of contactin/F11 to tenascin-C domains FnIII-5,6 was interrupted by spliced domains^{33, 103}. Interestingly, both large and small tenascin-C variants also contain integrin binding sites in the constitutively expressed domains, but their presence here seems to have little impact on the localisation of isoforms; suggesting that this regulation is somewhat mediated by the protein size.

Alternatively, large and small tenascin-C variants may bind to different ECM components, which are then subsequently cleaved by proteases, resulting in the detachment of isoforms that exhibit a greater ability to adhere to proteolytically susceptible ECM components. It is not yet known if the physical size of the larger tenascin-C variants causes steric hindrance that results in the reduction of interactions between tenascin-C and its ECM ligands; as it could be expected here for larger tenascin-C variants to exhibit more hindrance, and therefore possible greater secretion to the supernatant.

Furthermore, small tenascin-C variants may be secreted to the supernatant, but may not be identified by the mAb1908, due to cleavage of the binding epitope, if it is clipped at the N-

terminus. Studies have previously reported proteolytic cleavage of constitutive and spliced domains by MMP-1, 2, 3, 7, leukocyte elastase, serine protease, ADAMTS-5 and meprin- β ^{331, 401, 402}, with MMP-7 digesting the N-terminal assembly domain and FnIII-A3-D within the alternatively spliced region^{33, 103}; and MMP-2 cleaving FnIII-A3 to expose a cryptic syndecan-4 binding site that promoted cell spreading^{39, 401}.

This Western blot protocol does not give any information as to the alternatively spliced FnIII content of the tenascin-C isoforms, and it is possible that the higher and lower molecular weight bands are mixtures of different isoforms; despite this, the large and small variants may contain some common spliced FnIII domains. More work on deducing the alternatively spliced FnIII content of tenascin-C isoforms expressed by U-87 MG cells is required to speculate on the mechanisms that may be selectively localising different sized tenascin-C isoforms to the cell.

4.15.4 *Tenascin-C expression in MDDCs and macrophages is modified by cell activation*

In MDDCs, Western blotting confirmed previous ELISA and immunofluorescence data from chapter 3, showing transient induction of tenascin-C in activated cells that was trafficked through the cell to be predominantly secreted. Western blotting further revealed that LPS treatment induces the expression of a large tenascin-C isoform that remained associated with the cell at low levels and was predominantly trafficked to the supernatant over time. Additionally, a second ~20kDa smaller isoform was transiently detected in the cell lysate very quickly after stimulation, before being promptly secreted; but perhaps at levels not detected in the supernatant by Western blot. This suggests a mechanism also exists in MDDCs that selectively traffics tenascin-C isoforms through the cell, in a manner that is isoform size dependent.

PCR confirmed that resting MDDCs express very low levels of tenascin-C isoforms, and revealed that each alternatively spliced FnIII is present at comparable levels, implying that these cells express large variants with each transcript expressing the maximum number of 9 spliced FnIII. In contrast, activated MDDCs express more tenascin-C, but with a different alternative splicing pattern to that seen in resting cells. This activation specific splicing pattern also produces long tenascin-C mRNA transcripts with 7 of the 9 FnIII domains (A1, A2, A3, A4, B, C and D), but very low levels of FnIII AD2 or AD1. The findings that MDDCs express an activation specific tenascin-C isoform are corroborated by the transient detection of a cell associated band ~20kDa smaller than the largest band in the cell lysate. It is not known if the splicing pattern changes after 4 hours following cell activation; but the absence of the slightly smaller isoform in later time points suggests so. Activated GM-CSF and M-CSF macrophages also induced tenascin-C variants lacking FnIII-AD2 and AD1, suggesting engagement of shared downstream transcription factors or alternative splicing machinery amongst these myeloid cells, although to date these have not been characterised.

In published reports, AD2 and AD1 have only ever been detected together in mRNA transcripts⁶⁷, suggesting that their expression is linked by a shared mechanism of induction. The presence of FnIII-AD2 and AD1 is not restricted to, but is elevated in breast cancer, and AD1 is associated with estrogen-receptor negative and grade III tumours¹⁸⁸. This raises the question as to whether the expression of FnIII-AD2 and AD1 domains is restricted in immune cells, but not other cell types such as cancers.

This absence of FnIII-AD2 and AD1 from the mRNA transcript in activated myeloid cells could result in FnIII-B and C becoming adjacent in the transcript; and this may have a number of functional consequences. In other studies, removal of FnIII has created a new function for the flanking FnIII domains that remain, for example, removal of all spliced FnIII conferred greater adhesion of contactin/F11 to domains FnIII-5,6^{33, 103}. Therefore, activated

MDDCs and macrophages may restrict the expression of FnIII-AD2 and AD1 to confer new functions encoded by FnIII-B, C. Specific functions have not been identified for FnIII-B, C when expressed in the same transcript, but independently both FnIII-B and C have been shown to promote neurite outgrowth, process extension and to direct neurite orientation respectively^{34, 141, 142}.

In addition to this, activated MDDCs and macrophages may restrict the expression of FnIII-AD2 and AD1 to inhibit deleterious functions conferred by these domains, or to relieve an inhibitory function preventing normal immune cell behaviours. Expression of AD1 is detected in many highly invasive tumours, and in breast cancer, both AD1/ AD2 and B,AD1,D are prevalent at the tumour invasion front and in cancers with higher risk of metastasis^{94, 186, 187, 188, 189}. This may suggest an adhesion or migration modulating role for FnIII-AD2, AD1 splicing. This idea will be further examined in chapter 5.

4.15.5 T-cells do not significantly modify tenascin-C splicing following activation, but FnIII A1 is always absent

In T-cells, I showed that stimulation with anti-CD3/CD28 increases tenascin-C mRNA levels, however the tenascin-C expression was so low it could not be detected by ELISA. Due to the low tenascin-C protein levels, tenascin-C protein splicing was not investigated in T-cell samples by Western blot, but was examined by PCR.

CD4⁺ and CD8⁺ T-cells expressed low basal levels of tenascin-C mRNA that contained comparable levels of all spliced FnIII except for FnIII-A1. These data indicate the presence of long tenascin-C mRNA transcripts at low abundance in resting T-cells. FnIII-A1 was not detected in CD4⁺ or CD8⁺ T-cells in resting or stimulated conditions, indicating that the

repression of FnIII-A1 may be shared across T-cell subsets. This may be due to a detrimental impact of this domain on T-cell function. For example, Puente-Navazo *et al.*, showed that recombinant, bacterially expressed FnIII-A1-A2 inhibited T-cell proliferation and cytokine expression in response to anti- CD3 and IL-2, or fibronectin treatment²⁹⁹. As FnIII-A1 is repressed in T-cells, and has previously been shown to impair T-cell function, it would indicate that tenascin-C is sometimes expressed by T-cells at functionally relevant levels, and that the repression of FnIII-A1 is an evolved response to prevent auto-inhibition of T-cell proliferation.

Alternatively, the high level of sequence and structural homology (>85% nucleotide sequence identity) between FnIII-A1 and A4, may make FnIII-A1 functionally redundant, as its roles may instead be fulfilled by another domain from the FnIII-A1-A4 cassette. However, in the Puente-Navazo *et al.*, study, recombinant FnIII proteins corresponding to all of the other alternatively spliced domains were assessed on their ability to inhibit T-cell polarisation; though only the FnIII-A1,A2 domains impaired function; making the functional redundancy argument unlikely.

Perhaps the inhibitory role of FnIII-A1,A2 on T-cells, is effected by another cell-type. FnIII-A1 mRNA was detected in MDDCs and macrophages, but was one of the lowest abundance FnIII mRNAs in HDFs, NSF, OA-SFs and RA-SFs. Therefore perhaps FnIII-A1 is selectively expressed by antigen presenting cells to act as a counterbalance for the usual T-cell activating role mediated by this cell type. The existence of such a mechanism is purely speculation, and there is currently no evidence to directly support this idea; but it highlights the extraordinarily complex mechanisms by which cell functions may be regulated by tenascin-C splicing in different situational contexts.

4.15.6 *Tenascin-C splicing is not modified by IL-1 β stimulation in HDFs*

HDFs constitutively express high levels of tenascin-C rich in FnIII-B and D, but low in FnIII-AD2 and AD1. HDF cell activation by IL-1 β induced the expression of all tenascin-C mRNA transcripts, in line with the induction kinetics previously observed using the TaqMan qPCR assay in chapter 3, and did not result in a shift towards transcription of cell activation specific isoforms at 4 hours following stimulation. The relatively high resting abundance of FnIII-B and D in HDFs, compared to FnIII-AD2 and AD1, increases the chances of FnIII-B and D being expressed contiguously. FnIII-B and D are reported to promote cell adhesion, migration, and proliferation, as-well as process extension outgrowth in fibroblasts, tumour cells and neurites^{34, 132, 141, 142, 144, 145, 155, 204}; while nothing is known about the direct role of FnIII-AD2 and AD1 in fibroblasts.

Western blot revealed that HDFs have an ability to secrete both large and small tenascin-C isoforms to the supernatant, unlike U-87 MG cells which retained the smaller isoforms. This may be due to the higher levels of tenascin-C expression by HDFs, which may saturate the local ECM with tenascin-C, resulting in the secretion of both large and small isoforms that cannot bind to ECM ligands. Additionally, it could be that the HDF expressed matrix permits greater tenascin-C incorporation into the ECM.

The cellular localisation of large and small variants may result from the expression of tenascin-C isoforms that are rich in FnIII-B and D; with both domains containing known ligand binding sites. FnIII-B and D promote neurite outgrowth in a manner dependent upon interactions with the contactin/F3 and $\alpha 7\beta 1$ integrin respectively^{34, 132}, the presence of these ligand binding sites may make the tenascin-C expressed by HDFs more ‘sticky’, even if the smaller tenascin-C variant only contains one of the two FnIII domains.

4.15.7 *Tenascin-C splicing by synovial fibroblasts is disease specific*

I showed that NSFs express low levels of tenascin-C, lacking in domain FnIII-A1, with equal levels of all spliced FnIII, with the exception of FnIII-A which was absent. Large and small isoforms are detected in the cell lysate at equal abundance, but only the large isoform is secreted to the supernatant; indicating that the small isoform is retained in a manner similar to that seen in U-87 MG cells, or is so low in abundance that it cannot be detected. Both NSF tenascin-C isoforms are slightly smaller than the variants detected in HDFs and U-87 MG cells, and this likely reflects the different alternative splicing patterns present in these cell types.

RA-SFs express more tenascin-C than NSFs, and the mRNA transcripts contained more FnIII-B and -D, and less FnIII-AD2 and AD1. The sizes of the tenascin-C bands expressed by RA-SFs are almost the same as in NSFs, except RA-SFs predominantly express the large band, and can secrete both to the supernatant like HDFs; which share a similar splicing pattern. The similarities in splicing patterns between HDFs and RA-SFs may be due to common evolved phenotypic traits, or the activation of a shared splicing factor.

The same pattern of tenascin-C expression and alternative splicing is seen for OA-SFs as for NSFs, with slightly different absolute levels. However, in OA-SFs, the cells predominantly express large tenascin-C bands rather than small ones; this concurs with the detection of larger tenascin-C mRNA variants by qPCR. Synovial fibroblasts predominantly express isoforms of the same sizes, with minor differences <10kDa in mass, perhaps accounted for by small post-translational modifications associated with the phenotypic differences of these cells.

The qPCR analysis indicated that HDFs and RA-SFs both predominantly express smaller tenascin-C isoforms rich in FnIII-B and D, while the NSFs and OA-SFs predominantly

expressed low levels of long tenascin-C isoforms with low levels of FnIII-A1. This pattern is not reflected in the Western blot analysis of the cell associated tenascin-C protein, where it is apparent that all of the fibroblasts express both large and small isoforms, with HDFs, RA-SFs and OA-SFs predominantly expressing larger isoforms. However, the qPCR analysis of tenascin-C splicing seems more in line with the pattern observed for secreted tenascin-C isoforms. Here, both HDFs and RA-SFs secrete large and small tenascin-C isoforms, while the NSF and OA-SFs only secrete large isoforms.

4.15.8 *Regulation of tenascin-C alternative splicing*

Typically the expression of larger tenascin-C variants containing more FnIII domains are associated with tumours, developing tissues or regions of pathology; while smaller tenascin-C variants are associated with non-pathological regions of cell condensation in adult tissues (reviewed in ²⁴⁸). Contrary to these previously described associations, this thesis has provided evidence that large tenascin-C variants are expressed by resting HDFs and dendritic cells, and by non-diseased NSFs; whereas smaller variants were up-regulated in activated dendritic cells and in RA-SFs, showing that large tenascin-C variants are not always associated with pathology or dynamic tissue remodelling.

These findings may also suggest that the expression of tenascin-C spliced FnIII domains is more widespread than is indicated in the literature; however with no other studies utilising the same research methods for the quantification of FnIII mRNAs, it is difficult to draw conclusions. In addition a pattern of tenascin-C splicing was observed that was broadly lineage specific, suggesting that cell type specific mechanisms modulate splicing.

The mechanisms by which the complex process of tenascin-C splicing is controlled are beginning to emerge. Regulation of tenascin-C expression is mediated by growth factors and cytokines in a situation-specific response. During embryogenesis in humans and mice,

tenascin-C expression is up-regulated by TGF β .^{92, 403} TGF β -1 provides the most significant up-regulation of tenascin-C although expression is also responsive to TGF β -2, -3 and -5.⁴⁰⁴ TGF β -1 is also observed to induce preferential expression of the small tenascin-C isoform lacking FnIII A1-D in endometrial adenocarcinoma and NIH-3T3 cell lines.³²⁴ *In vivo*, tenascin-C expression closely correlates with expression of TGF β in the heart, bone marrow megakaryocytes, breast cancer, ovarian cancer and inflamed joints.^{405, 406} FGF is also a potent inducer of tenascin-C expression, with the expression pattern of FGF2 in the mouse CNS corresponding with that of tenascin-C.⁹² In addition to FGF up-regulating tenascin-C expression on the whole, FGF1 and FGF2 has been shown to preferentially induce the expression of large tenascin-C splice variants in the lateral ventricles of the rat brain, and the NIH-3T3 fibroblast cell line respectively.^{92, 324, 407}

The expression of tenascin-C is also influenced by the presence of pro-inflammatory cytokines, which can either up-regulate the expression of all tenascin-C isoforms, or induce the preferential expression of long or short isoforms. This effect has been investigated in keratinocytes, where IL-4 treatment induced the expression of short and long mRNA transcripts equally, with a relative abundance of 1:1.5. In contrast, the potent immune-activator tumour necrosis factor-alpha (TNF α) preferentially induces the expression of short tenascin-C transcripts, while IFN γ favours the expression of long transcripts³³¹; displaying the responsiveness of the tenascin-C splicing machinery to inflammatory signals.

Another important regulator of tenascin-C splicing is intracellular pH (pHi). In cell cultures of lung and dermal fibroblast cell-lines, a physiological pH ~7.0 results in the expression of small tenascin-C splice isoforms lacking the alternatively spliced FnIII domains A1-D. However when cells are exposed to more basic pH ~7.30-7.50 as is the case in artificial culture experiments, fetal tissues and aggressive tumours, tenascin-C expression switches to the longer-isoform with one or more alternatively spliced FnIII repeats included in the

transcript.^{170, 214, 408} pH regulation of tenascin-C splicing may therefore contribute to the diverse range of tenascin-C splice variants which are found in human fetal membranes.¹¹⁷ Similarly, malignantly transformed fibroblasts principally express large tenascin-C isoforms regardless of the extracellular pH *in vitro* (pHo); interestingly, this is because these cells maintain a basic pHi throughout a wide range of pHo.¹⁷⁰ Bumke et al.⁴⁰⁹, confirmed that the large isoform is preferentially expressed at basic pHo, but that serum starvation in fibroblasts reduces expression of the large tenascin-C isoform; independent of culture media pH.

Little is known regarding the specific mechanisms by which splicing is induced. However, overexpression of the RNA binding protein and splicing factor Sam68 in neural stem cells, resulted in the preferential expression of larger tenascin-C isoforms¹²⁵. Additionally, induction of the splicing factor protein SRSF6 in transgenic mice resulted in expression of full-length tenascin-C isoforms, which were absent in normal un-induced skin. The presence of the full-length tenascin-C isoform accompanied hyperplasia of keratinocytes, although this effect and the abundance of full-length tenascin-C could be reverted following removal of SRSF6 induction.²⁰² SRSF6 mediated alternative splicing regulation of tenascin-C results in the excessive proliferation of keratinocytes in response to tissue injury, and therefore SRSF6 can modulate tissue homeostasis *in vivo* by promoting the expression of long tenascin-C isoforms in preference to shorter ones. These previous studies highlight the complexity with which tenascin-C alternative splicing is regulated at rest and during inflammation; and gives some context to the mechanisms by which cell-type specific splicing regulation of tenascin-C is likely to occur.

4.15.9 *Summary*

The relative abundance of large and small tenascin-C isoforms visualised by Western blot in the cell lysates and supernatants of U-87 MG, MDDC, GM-CSF, M-CSF macrophage, HDF, NSF, RA-SF and OA-SF, and the details of FnIII domain abundances detected by qPCR are summarized in Table 4.1.

For the first time this study has directly compared the relative sizes of tenascin-C protein isoforms expressed by immune cells and fibroblasts following cell activation and in disease and FnIII domain abundance. It has shown cell type specific expression of tenascin-C isoforms basally. HDFs overexpress FnIII-B and D, domains which are implicated in processes associated with cell plasticity; and T-cells do not express FnIII-A1 mRNA, a domain shown previously to inhibit T-cell activation. This study has also revealed cell stimulation specific regulation of the tenascin-C mRNA isoform repertoire transcribed by MDDCs, GM-CSF and M-CSF macrophages, which upregulated all FnIII mRNAs except for AD2 and AD1. In diseased fibroblasts, similarities were observed in tenascin-C splicing patterns between HDFs and RA-SFs, and between NSFs and OA-SFs. In chapter 5 I go on to examine the functional relevance of alternatively spliced FnIII domains that are differentially regulated during inflammation and disease.

Summary of tenascin-C splicing data in chapter 4		
Cell Types	qPCR (mRNA)	Western blot (protein)
U-251 MG	N/A	CL: 300 , 260kDa large band was predominant
U-87 MG	All FnIII mRNAs were detected in the qPCR controls	CL: 300 , 270, 240, 230kDa large band was predominant. SN: 300kDa
HDF	Resting and activated overrepresented FnIII B, D, underrepresented FnIII AD2, AD1	CL: 300 , 250kDa large band was predominant SN:300kDa
MDDC	Resting: FnIII A1-D present Activated: FnIII-A1-D, low level FnIII-AD2,AD1	Activated: CL: 300 , 280kDa SN: 300kDa (All bands were very faint)
M-CSF Macrophage	Resting: FnIII A1-D present Activated: FnIII-A1-D, low level FnIII-AD2,AD1	N/A
GM-CSF Macrophage	Resting: FnIII A1-D present Activated: FnIII-A1-D, low level FnIII-AD2,AD1	N/A
CD4+ T-cell	Resting FnIII A2-D present Low level FnIII-A1	N/A
CD8+ T-cell	Resting FnIII A2-D present Low level FnIII-A1	N/A

Summary of tenascin-C splicing data in chapter 4		
Cell Types	qPCR (mRNA)	Western blot (protein)
NSF	All FnIII present, except FnIII-A1	CL:280, 230kDa equal abundance SN: 280kDa
RA-SF	Overrepresented FnIII B, D, Underrepresented FnIII AD2, AD1	CL: 285 , 230kDa large band predominant SN: 285, 230kDa
OA-SF	All FnIII present, except FnIII-A1	CL:280, 225kDa large band predominant SN: 280kDa

Table 4.1. Table summarising Western blot and qPCR data on tenascin-C alternative splicing in the cell types assessed. This table displays a summary of the observations made about tenascin-C alternative splicing, as detected by Western blot and qPCR methods in the following cell types; in purified tenascin-C from U-251 MG cells, from U-87 MG, HDFs, MDDC, GM-CSF macrophage, M-CSF macrophage, CD4+ T-cell, CD8+ T-cell, NSF, RA-SF and OA-SF. Protein samples were obtained from the cell lysate (CL), and the supernatants (SN) of resting and activated cells, as indicated in the table. The predominantly expressed protein band is written in **bold**. Sizes of tenascin-C bands were estimated using the PageRuler™ plus protein ladder.

Chapter 5 Assessing the function of inflammation- and disease-specific alternatively spliced FnIII domains

5.1 Introduction

The functional implications of alternative splicing of tenascin-C have been best examined in tumour cells and developing cells during embryogenesis. Previous studies that characterised the associations and functional consequences of alternatively spliced FnIII domains, highlighted several mechanisms by which the inclusion or omission of FnIII domains could exert functional effects in cells; and this study served to investigate whether such mechanisms are also relevant in the context of tenascin-C splicing during inflammation.

Alternative splicing of tenascin-C exerts influence over the protein's function by either including or omitting alternatively spliced FnIII domains. For example, studies have shown that the introduction of a specific spliced FnIII domain into the tenascin-C transcript may confer a new ligand binding site, which may have both stimulatory and inhibitory consequences. Such examples include the expression of FnIII-D, which introduced a new integrin binding site, and promoted $\alpha 7\beta 1$ dependent rat hippocampal neurite outgrowth^{34, 145}; or the inclusion of FnIII-A1 and A2, which inhibited rat retinal neurite outgrowth¹⁴⁴. The inclusion of specific alternatively spliced FnIII domains can also increase the susceptibility of tenascin-C to proteolytic cleavage; here MMP-2 degrades both small and large tenascin-C variants within a constitutively expressed region, while MMP-3 and -7, both digested alternatively spliced FnIII domains from the larger tenascin-C isoform¹⁴⁶. Furthermore, It was found that MMP-2 processing of FnIII-A2 may expose a cryptic binding site that permitted interaction with heparan sulfated side-chains of syndecan-4 to promote $\beta 1$ integrin clustering and cell spreading^{39, 146}. Also, perhaps counter-intuitively, the selective omission of FnIII domains from tenascin-C causes a conformational change that can confer a gain in function

by bringing two non-contiguous FnIII domains together, to form a new ligand binding site. For example, it was previously shown that a ~190kDa tenascin-C variant containing no alternatively spliced domains bound to contactin/F11 via FnIII 5-6, but that this binding was disrupted by the presence of alternatively spliced FnIII domains in larger isoforms that are inserted between domains FnIII-5 and -6; making larger tenascin-C isoforms containing spliced FnIII domains anti-adhesive for embryonic chick neurons¹⁰³.

Tenascin-C alternative splicing may also permit additional function-modulating post translational modifications to occur, such as glycosylation. Human tenascin-C possesses 26 potential *N*-glycosylation sites, of which 21 are in the FnIII domains^{63, 398}. Analysis of the human tenascin-C cDNA sequence revealed a cluster of 10 potential *N*-linked glycosylation sites within the alternatively spliced FNIII repeats A1-A4, three further putative sites were identified within FnIII B,^{59, 63} and four more sites were identified in the FnIII AD1 and AD2 domains³⁹⁸. Tenascin-C glycosylation may protect the protein from proteolytic degradation. The abundance of putative *N*-glycosylation sites within the alternatively spliced FNIII A1-D closely associate with the FnIII domains that are susceptible to protease degradation by MMP-1, -2, -3 and -7;^{117, 146} and may therefore exist to improve the half-life of tenascin-C.

Spliced FnIII domains have also been implicated in the progression of a number of different cancers; for example, overexpression of FnIII-B,AD1,D enhanced cell growth and invasion of breast cancer cell lines¹⁸⁹, while a recombinant full length FnIII-A1-D, bound cell surface annexin II, and suppressed gemcitabine mediated cytotoxicity in pancreatic cancer²⁰⁹ (reviewed in ²⁴⁸). In cancer, alternatively spliced FnIII domains are thought to be so specifically expressed, yet absent in normal healthy tissues, that therapeutic antibodies are being developed targeting FnIII-A1 (F16) for the treatment of lung cancer, breast cancer and Hodgkin's lymphoma^{214, 215}; and FnIII-C,D domains (81C6) for the treatment of glioblastoma multiforme respectively^{213, 214, 211, 410}. The identification of inflammation specific alternative

splicing patterns for tenascin-C may be of interest for future therapeutic development for the treatment of inflammatory diseases.

In chapter 4, I identified differences in the alternative splicing pattern of tenascin-C between resting and activated MDDCs, GM-CSF and M-CSF macrophages, and between diseased, and non-diseased fibroblasts. To date, the functional relevance of tenascin-C splicing during inflammation, and in inflammatory disease, has been subject to limited investigation. In this chapter I clone, express and purify recombinant alternatively spliced FnIII protein domains from tenascin-C, and assess their impact on a number of cellular functions including cytokine expression, cell adhesion, cell morphology and cell migration. Together these data reveal that alternatively sliced FnIII domains of tenascin-C can modify the behaviours of human myeloid cells and dermal fibroblasts.

5.2 Cloning, expression and purification of recombinant proteins

I found in chapter 4 that LPS activated MDDCs and macrophages induced the expression of all alternatively spliced FnIII, except for AD2 and AD1. From this data it was inferred that activated myeloid cells express different tenascin-C variants when activated, compared to when resting, and that the omission of FnIII-AD2 and -AD1, increased the chances of FnIII-B and -C being expressed contiguously inside the same tenascin-C molecules. In HDFs, and RA-SFs, FnIII-B and -D domains were overrepresented, while FnIII-AD2 and -AD1 were underrepresented compared to in NSFs and OA-SFs, indicating that FnIII-B, -C, and -D have more chance of being expressed contiguously; whereas in NSFs and OA-SFs, FnIII-AD2, AD1 are more likely to be included in the same transcripts. From this data I inferred that that in HDFs and RA-SFs, FnIII-B and -D domains comprise a greater proportion of the spliced FnIII domains present, and may be more frequently expressed together in the same transcripts than in NSFs and OA-SFs; which appear to have similar splicing patterns (Figure 5.1).

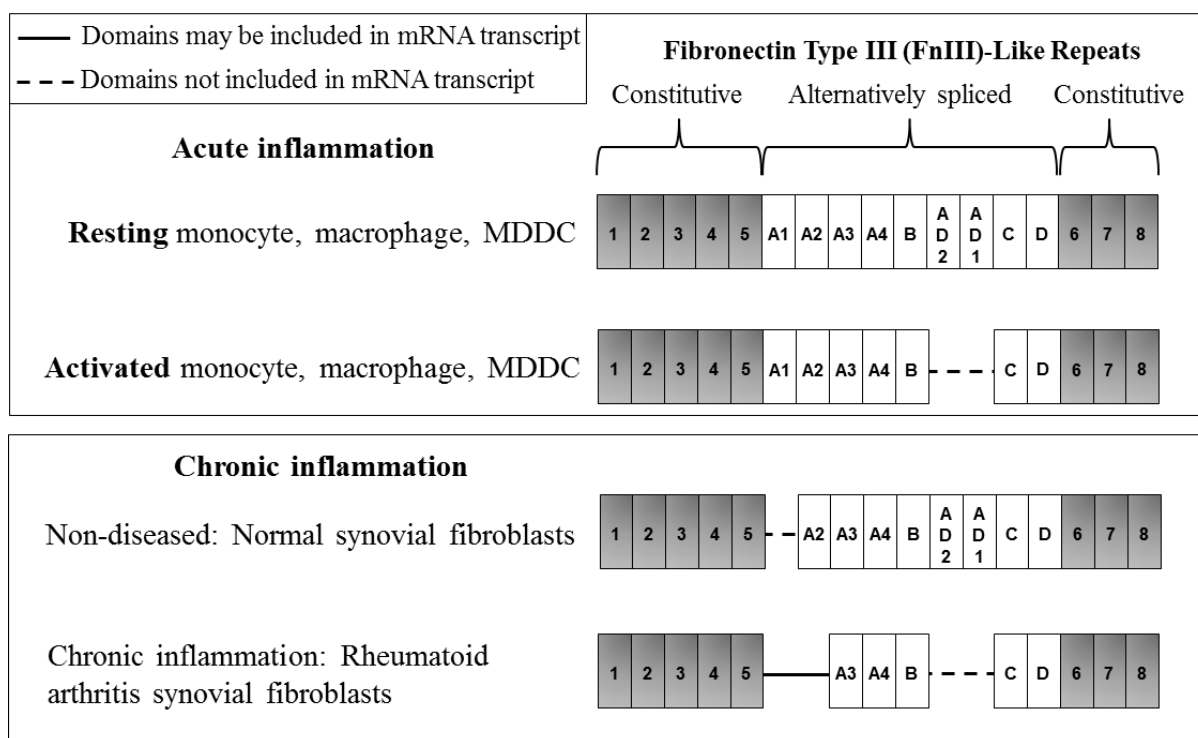


Figure 5.1. Tenascin-C isoforms expressed by resting and activated myeloid and fibroblast cells.. The illustrations represent the full FnIII cassette (FnIII-1-8), and show the alternative splicing pattern of tenascin-C mRNA transcripts expressed by monocytes, macrophages and MDDCs at rest and following stimulation with +10ng/ml LPS. Alternatively spliced tenascin-C isoforms expressed by NSF and RA-SFs are also summarized.

The omission of FnIII-AD2, AD1 domains from the tenascin-C transcripts following myeloid cell activation; and their underrepresentation in RA-SFs raised questions as to whether AD2 and AD1 inhibit dendritic cell or RA-SF functions during inflammation. Conversely, I was also interested in testing whether the removal of FnIII-AD2,AD1 from the full transcripts permitted additional functions due to the contiguous expression of FnIII-B,C,D domains; and if including FnIII-AD2,AD1 between domains FnIII-B and C, prevented a function during inflammatory processes. In order to these questions being raised, recombinant proteins containing the alternatively spliced domains FnIII AD2 and AD1 (AD2AD1), FnIII B,C and D (BCD) and FnIII B,AD2,AD1,C and D (BAD2AD1CD) were selected for synthesis; and were cloned and expressed in *E.coli* (Figure 5.2).

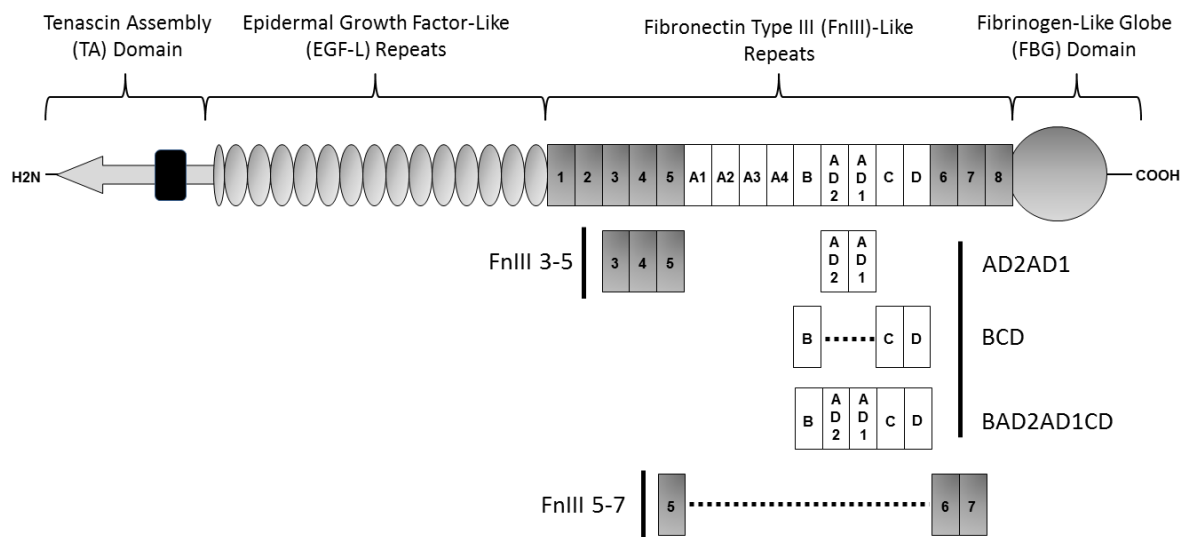


Figure 5.2. Recombinant FnIII protein domains selected for functional analysis. Schematic representation of full length tenascin-C containing the N-terminal tenascin assembly domain (TA), followed by 14.5 epidermal growth factor-like repeats (EGF-L), and up to 17 fibronectin type III-like repeats (FnIII), of which 9 (white boxes) are alternatively spliced, capped with a fibrinogen-like globe domain (FBG-C). Recombinant proteins used for this study include FnIII 3-5, AD2AD1, BCD, BAD2AD1CD and FnIII 5-7. Boxes separated by a dotted line represent contiguity.

I chose to examine the impact of these protein domains on two cellular functions. Firstly, I wanted to know if alternatively spliced FnIII domains impact cytokine expression. Full length tenascin-C has been shown to induce cytokine expression in various cell types^{27, 43, 257, 273}. Previously the Midwood group identified that full length tenascin-C and the recombinantly expressed FBG domain induce expression of IL-6, IL-8 and TNF α in monocytes via interactions with the TLR4. In the same study, recombinant FnIII proteins corresponding to the constitutively expressed regions of tenascin-C including the TA domain, EGF-like repeats, FnIII 1-3, FnIII 1-5, FnIII 3-5, FnIII 5-7, FnIII 6-8 did not induce cytokine expression, but the activity of the alternatively spliced FnIII domains was not investigated⁴³. The constitutively expressed regions of fibronectin do not induce cytokine expression, but inclusion of the alternatively spliced extra domain-A (EDA) repeat did induce TLR4 dependent cytokine synthesis⁴¹¹; raising the question as to whether the structurally analogous

alternatively spliced FnIII domains in tenascin-C, can also induce TLR4 dependent cytokine synthesis.

The second aim was to determine whether these recombinant domains impact cell adhesion. Fibroblast cell adhesion to fibronectin and tenascin-C is well documented. Broadly, tenascin-C is considered to be an anti-adhesive protein, as it disrupts cell adhesion to fibronectin; but its constitutive FnIII domains contain RGD sequences that permit $\alpha 8 \beta 1$ integrin binding in FnIII-3⁴¹², and $\alpha \nu \beta 3$ integrin to bind in the FnIII-3 and FBG regions^{278, 279, 413}. It is possible that the alternatively spliced FnIII domains also regulate adhesion processes, as studies have shown spliced FnIII domains to cause a loss in focal adhesions, and increase the migration of arterial endothelial cells via binding to annexin II^{40, 140}; while the FnIII-A2 modulated fibronectin fibrillogenesis via a cryptic binding site to syndecan-4^{39, 380}. To date, the effects of alternatively spliced FnIII domains on myeloid cell adhesion have not been studied; and together, early findings of adhesion modulating properties of alternatively spliced FnIII in other cell types, raises the question of whether these domains impact fibroblast and immune cell adhesion during inflammation.

For each functional assay different controls were used. To assess the induction of cytokine expression, immune cells were stimulated with LPS, recombinant FBG domain from tenascin-C (FBG-C) which served as positive controls; and FnIII 5-7, which served as a negative control. FBG-C and FnIII 5-7 were cloned by the Midwood lab as previously described⁴³; FBG-C was expressed and purified by Dr. Lorena Zuliani-Alvarez, and FnIII 5-7 was expressed and purified by myself as detailed below.

As controls for adhesion assays, I used full length fibronectin, tenascin-C, FnIII 5-7 and FnIII 3-5. Fibronectin served as a well characterised positive control for fibroblast adhesion^{82, 414}. Purified tenascin-C was included in the study as a negative control for fibroblast cell

adhesion^{82, 415}, and permitted the direct comparison between the activity of the recombinant spliced FnIII proteins and the full length tenascin-C. FnIII 5-7 protein corresponded to the constitutively expressed FnIII 5,6,7 domains of tenascin-C, and did not previously display adhesive properties in fibroblasts, so served as a negative control (Figure 5.3). Finally, FnIII 3-5 was expected to support cell adhesion due to the presence of an RGD motif in FnIII-3⁴¹⁶, and therefore served as a positive control for the adhesion assays. These proteins were already available to the Midwood lab; fibronectin was purified from human plasma as previously described in ³⁸⁰, tenascin-C was purified from overexpressing HEK cells, and FnIII 3-5 from *E.coli* as previously described in⁴³.

His-tagged AD2AD1, BCD and BAD2AD1CD protein domains were cloned and expressed in BL21 (DE3) *E.coli* using protocols described for the constitutive FnIII of tenascin-C⁴³. FnIII 5-7 was purified from the insoluble fraction of the bacterial culture as was previously described⁴³, whilst AD2AD1 was purified from the insoluble fraction and was refolded (Figure 5.4). The other FnIII BCD (Figure 5.5), and BAD2AD1CD (Figure 5.6) proteins were purified from the soluble fraction of the bacterial culture. Enrichment of each protein by Ni-NTA chromatography was confirmed by SDS-PAGE and coomassie staining, purity was assessed by silver stain, and protein identity was confirmed by Western blotting with an anti-his tag antibody. The expected molecular masses of the recombinant proteins were as follows; FnIII 5-7 28.7 kDa; AD2AD1 19.94kDa; BCD 33.0kDa; BAD2AD1CD 56.0kDa. The circular dichroism (CD) spectra were assessed for each protein fraction to confirm the presence of α -helix or β -sheet secondary protein structure that could be abolished by denaturation. The level of contaminating endotoxin in all purified fractions was below 10pg/ml, with the exception of BAD2AD1CD where only fraction 2 was under this threshold, and therefore suitable for use in cellular assays.

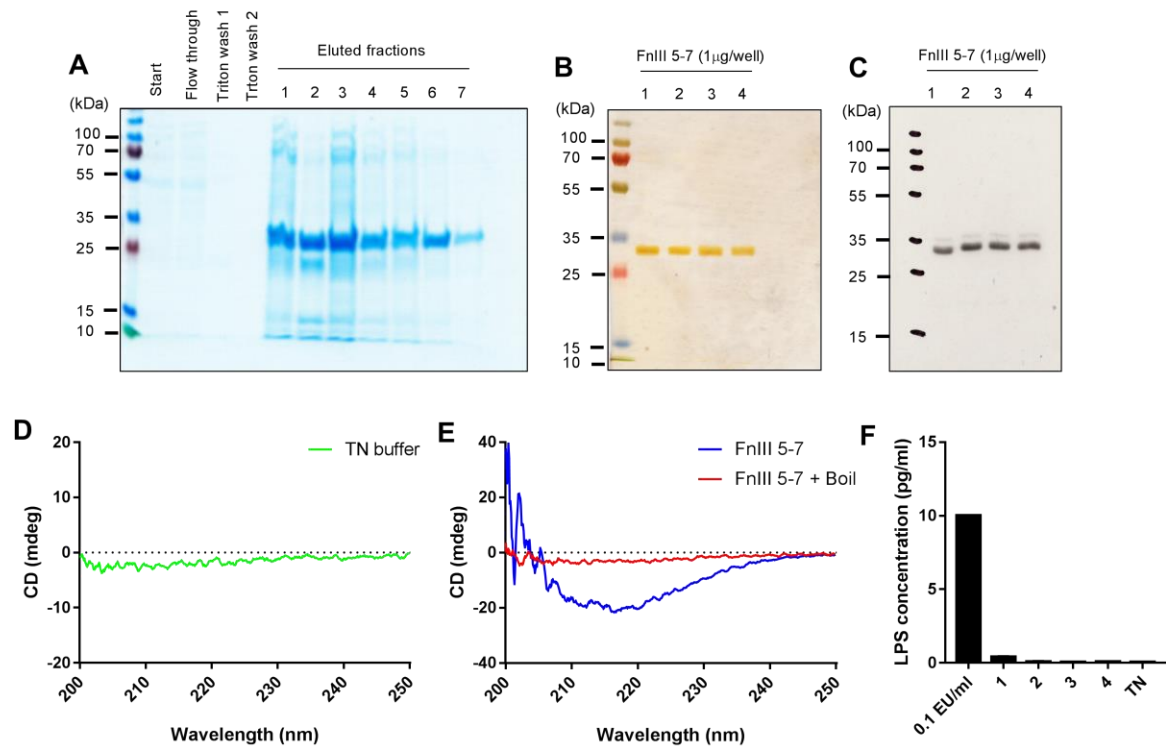


Figure 5.3. Expression and purification of FnIII 5-7. FnIII 5-7 was previously cloned in the Midwood lab with an N-terminal 6xHis tag. I induced expression in BL21 (DE3) *E.coli*, and purified FnIII 5-7 from the soluble bacterial fraction using Ni-NTA chromatography. (A) Coomassie blue stained SDS-PAGE gel showing 30 μ l of the starting material loaded onto the Ni-NTA column (Start), 30 μ l flow through, 30 μ l of the two triton washes and 30 μ l of the 7 eluted fractions (1-7). (B, C) 1 μ g of fractions 1-4 were loaded per well and separated via SDS-PAGE before detection by silver stain (B) or Western blot with mouse anti-tetra His mAb (C). (D, E) Circular-dichroism analysis of buffer blank (TN buffer) (D), or 0.2mg/ml samples of FnIII 5-7 fractions before (blue) and after (red) boiling; this graph is representative of each fraction (E). Endotoxin concentrations measured in endotoxin units per ml (EU/ml) were calculated in 1 μ M FnIII 5-7 fractions 1-4 (1-4) and in the TN buffer (TN) diluent; the graph shows mean + SEM (F).

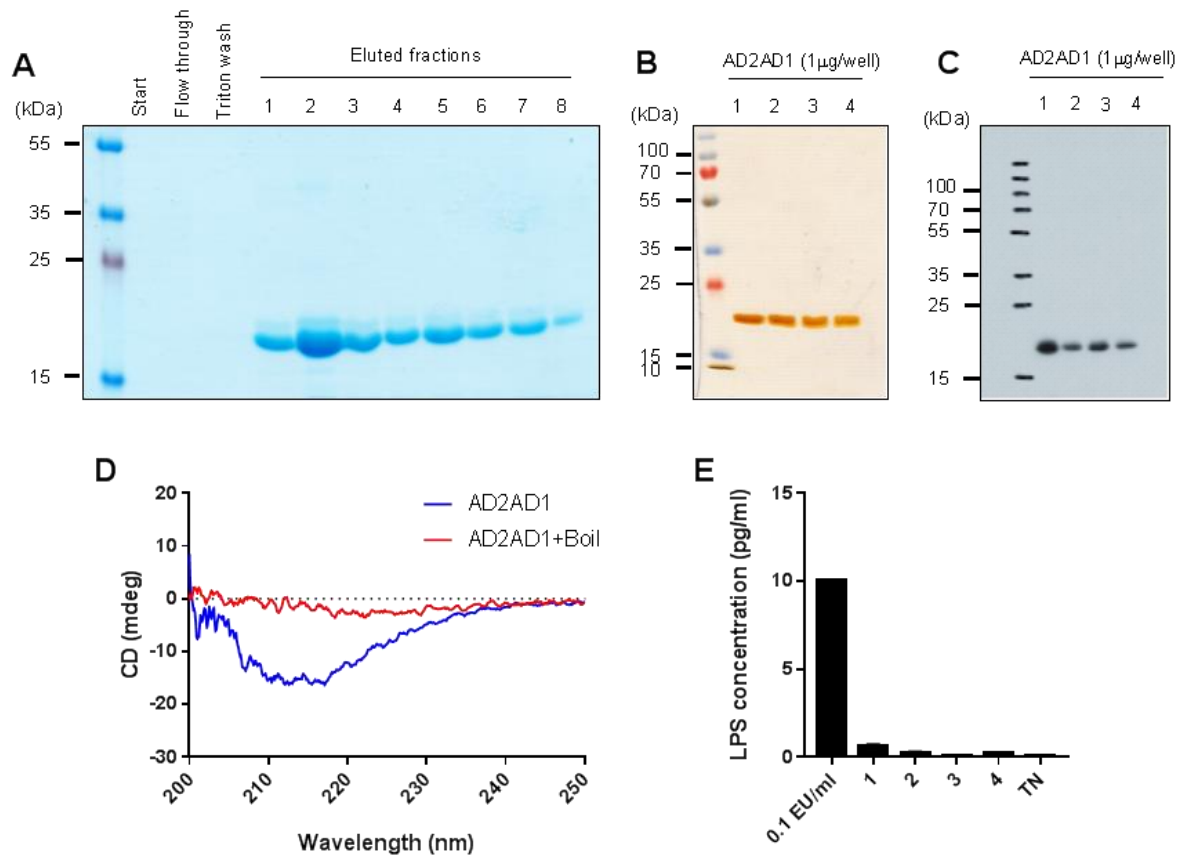


Figure 5.4. Expression and purification of AD2AD1. I cloned AD2AD1 with an N-terminal 6xHis tag, which was then expressed in BL21 (DE3) *E.coli*, and purified from the insoluble fraction using two rounds of Ni-NTA chromatography. (A) Coomassie blue stained SDS-PAGE gel showing 30 μ l of the starting material loaded onto the Ni-NTA column (Start), 30 μ l flow through, 30 μ l of the triton wash and 30 μ l of the 8 eluted fractions (1-8). (B, C) 1 μ g of fractions 1-4 were loaded per well and separated by SDS-PAGE before detection by silver stain (B), or Western blot with mouse anti-tetra His mAb (C). Circular-dichroism analysis of 0.2mg/ml samples of AD2AD1 fractions before (blue) and after (red) boiling; the graph shown is representative of each fraction (D). Endotoxin concentrations measured in endotoxin units per ml (EU/ml) were calculated in 1 μ M AD2AD1 fractions 1-4 (1-4) and in the TN buffer (TN) diluent; the graph shows mean + SEM (E).

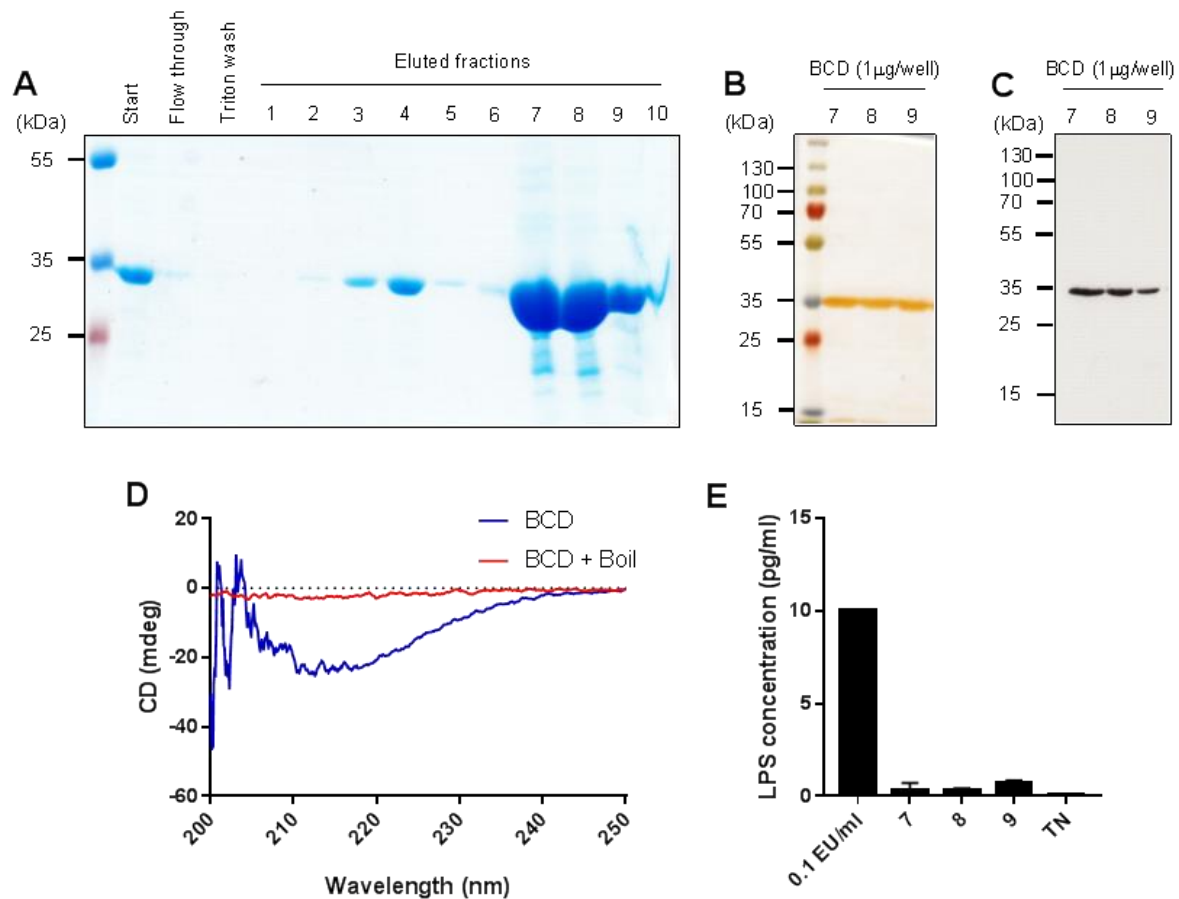


Figure 5.5. Expression and purification of BCD. I cloned BCD with an N-terminal 6xHis tag, which was then expressed in BL21 (DE3) *E.coli*, and purified from the soluble fraction using Ni-NTA chromatography. (A) Coomassie blue stained SDS-PAGE gel showing 30 μ l of the starting material loaded onto the Ni-NTA column (Start), 30 μ l flow through, 30 μ l of the triton wash and 30 μ l of the 10 eluted fractions (1-10). (B, C) 1 μ g of fractions 7-9 (7, 8, 9) were loaded per well and separated by SDS-PAGE before detection by silver stain (B), or Western blot with mouse anti-tetra His mAb (C). Circular-dichroism analysis of 0.2mg/ml samples of BCD fractions before (blue) and after (red) boiling; the graph shown is representative of each fraction (D). Endotoxin concentrations measured in endotoxin units per ml (EU/ml) were calculated in 1 μ M BCD fractions 7-9 (7, 8, 9) and in the TN buffer (TN) diluent; the graph shows mean + SEM (E).

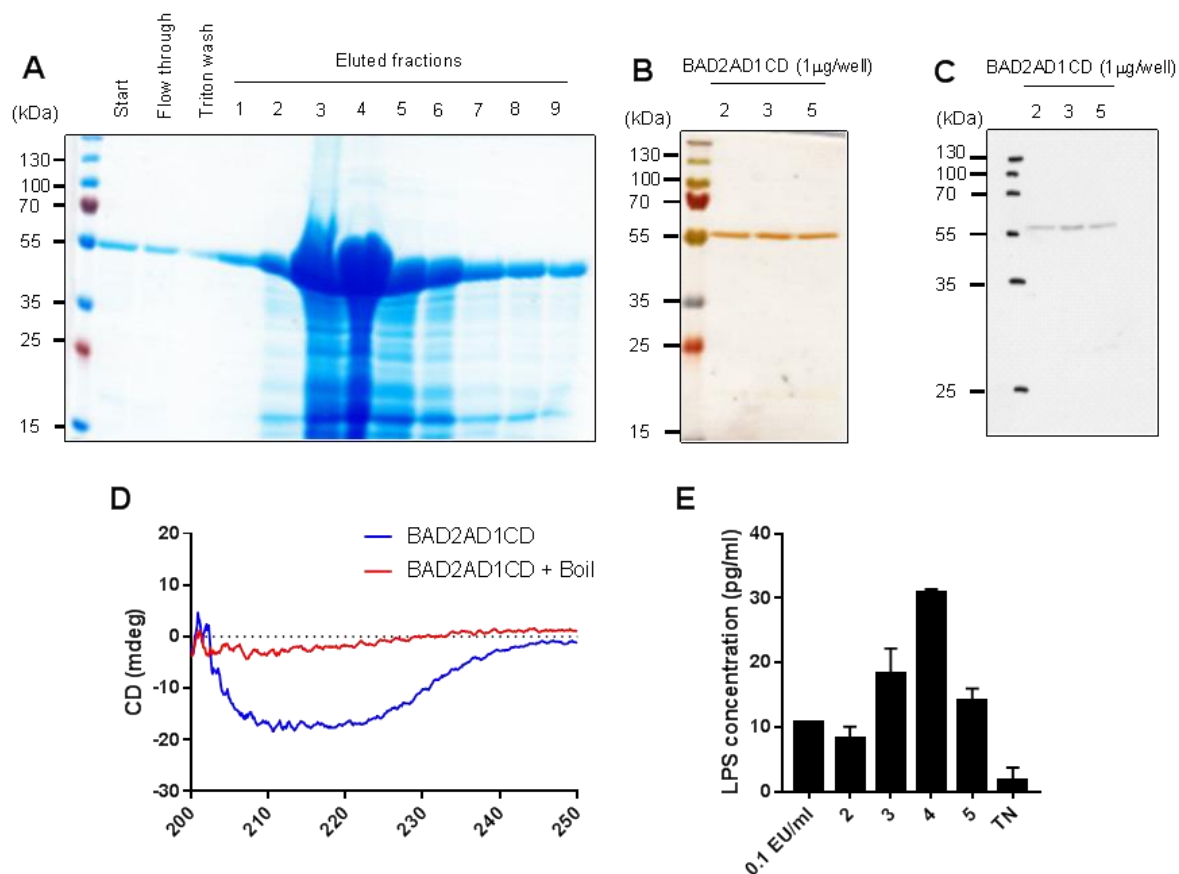


Figure 5.6. Expression and purification of BAD2AD1CD. I cloned BAD2AD1CD with an N-terminal 6xHis tag, which was then expressed in BL21 (DE3) *E.coli*, and purified from the soluble fraction using Ni-NTA chromatography. (A) Coomassie blue stained SDS-PAGE gel showing 30 μ l of the starting material loaded onto the Ni-NTA column (Start), 30 μ l flow through, 30 μ l of the triton wash and 30 μ l of the 10 eluted fractions (1-9). (B, C) 1 μ g of fractions 2, 3 and 5 (2, 3, 5) were loaded per well and separated by SDS-PAGE before detection by silver stain (B), or Western blot with mouse anti-tetra His mAb (C). Circular-dichroism analysis of 0.2mg/ml samples of BAD2AD1CD fractions before (blue) and after (red) boiling; the graph shown is representative of each fraction (D). Endotoxin concentrations measured in endotoxin units per ml (EU/ml) were calculated in 1 μ M BAD2AD1CD fractions 2, 3 and 5 (2, 3, 5) and in the TN buffer (TN) diluent; the graph shows mean + SEM (E).

5.3 Tenascin-C fragments induce cytokine expression in macrophages

To determine whether the alternatively spliced FnIII domains of tenascin-C may induce TLR4 dependent cytokine synthesis, the effect of treating primary human M-CSF macrophages with exogenous alternatively spliced FnIII domains on TNF α , IL-6 and IL-8 cytokine expression, was quantified by ELISA.

M-CSF macrophages were stimulated with 1ng/ml LPS or 1 μ M purified FBG-C as positive controls, and 1 μ M FnIII 5-7 as a negative control, for 24h before assessing TNF α , IL-6 and IL-8 synthesis by ELISA as described in ⁴³. Cells were also stimulated with 1, 0.5, 0.25, 0.1 and 0.01 μ M AD2AD1, BCD or BAD2AD1CD proteins. As further controls, 1 μ M of each protein ligand was subjected to denaturation by boiling or to digestion with proteinase K (PK), and to pre-incubation with PMB to bind contaminating endotoxins. The activity of protein ligands is ablated by boiling and PK digestion, but not following treatment with PMB; while the opposite is observed with LPS⁴³. PMB is an antibiotic for gram negative bacteria that binds the negatively charged portion of the LPS molecule; hence inactivating the endotoxin. TLR4 dependency was assessed by pre-incubation of cells with TAK242, a small molecule TLR4 antagonist, using DMSO as a vehicle control for this inhibitor. In this section, only the results from the positive and negative controls, and for the BCD domain are shown. Data from the full experiment including the AD2AD1 and BAD2AD1CD stimuli can be seen in Appendix Figure 5.7.

Unstimulated macrophages produced negligible TNF α levels. As expected, TNF α was significantly induced by treatment with LPS and was not reduced by boiling or DMSO treatment, but was ablated by PMB and TAK242 pre-incubation. FBG-C treatment also induced TNF α expression which was not reduced by DMSO, and was lower following PMB treatment; but this induction was ablated by boiling, PK and TAK242 treatment. As expected, FnIII 5-7 was not active, and similarly AD2AD1 treatment resulted in very low levels of

TNF α release. BCD and BAD2AD1CD both induced significant levels of dose dependent TNF α expression that was not reduced by PMB or DMSO treatment, but was ablated by boiling, PK and TAK242 treatment (Figure 5.7.A; Appendix Figure 5.7.A).

IL-6 levels were negligible in resting macrophages. LPS significantly induced IL-6 secretion that was not reduced following boiling or DMSO treatment; and were reduced but not completely ablated by PMB or TAK242 treatment. FBG-C also induced IL-6 levels, which were reduced by PMB and DMSO treatment but remained elevated above the levels expected when ablated by boiling, PK and TAK242 treatment. As expected FnIII 5-7 was not active, and AD2AD1 and BAD2AD1CD treatment did not induce IL-6 levels. BCD induced statistically significant, dose dependent secretion of IL-6 that was not reduced by PMB or DMSO treatment, but was reduced by boiling, and ablated by PK and TAK242 treatment (Figure 5.7.B; Appendix Figure 5.7.B).

IL-8 was also negligibly expressed in resting macrophages, and LPS treatment significantly induced IL-8 levels that were slightly reduced by boiling and DMSO treatment, but was further reduced by PMB and TAK242 treatment. FBG-C stimulation induced IL-8 levels, that were not reduced by PMB or DMSO, but was ablated by boiling, PK and TAK242 treatment. Here FnIII 5-7 did induce IL-8 secretion in a manner that was not reduced by boiling, PMB or PK but was ablated by treatment with TAK242 and DMSO. AD2AD1 treatment significantly induced secretion of IL-8, which was not ablated by any of the controls, contrary to what was expected; and BCD induced significant dose dependent secretion of IL-8, that wasn't reduced by PMB and DMSO treatment, but was reduced by boiling, PK and TAK242 treatment (Figure 5.7.C; Appendix Figure 5.7.C).

The absolute levels of cytokines expressed following induction were lowest for TNF α , and were the highest for IL-8; this reflects the perceived accuracy of the ELISAs, as the lower

detected cytokine levels were observed by the ELISAs where samples were the least diluted. Equally high levels of induction were seen for LPS, FBG-C and BCD, but with lower levels of induction for BAD2AD1CD. In the IL-6 ELISA, the largest induction was observed with 1 μ M FBG-C, while 1ng/ml LPS and 1 μ M BCD produced lower overall levels that were comparable to each other. In the IL-8 ELISA the largest induction was by 1 μ M BCD, followed by AD2AD1; here the levels of induction caused by LPS, FBG-C and FnIII 5-7 were comparable.

Cell viability, as assessed by MTT assay was not negatively affected following any treatment, although pre-incubation of the cells with PMB slightly increased viability relative to the other treatments (Figure 5.7.D; Appendix Figure 5.7.D).

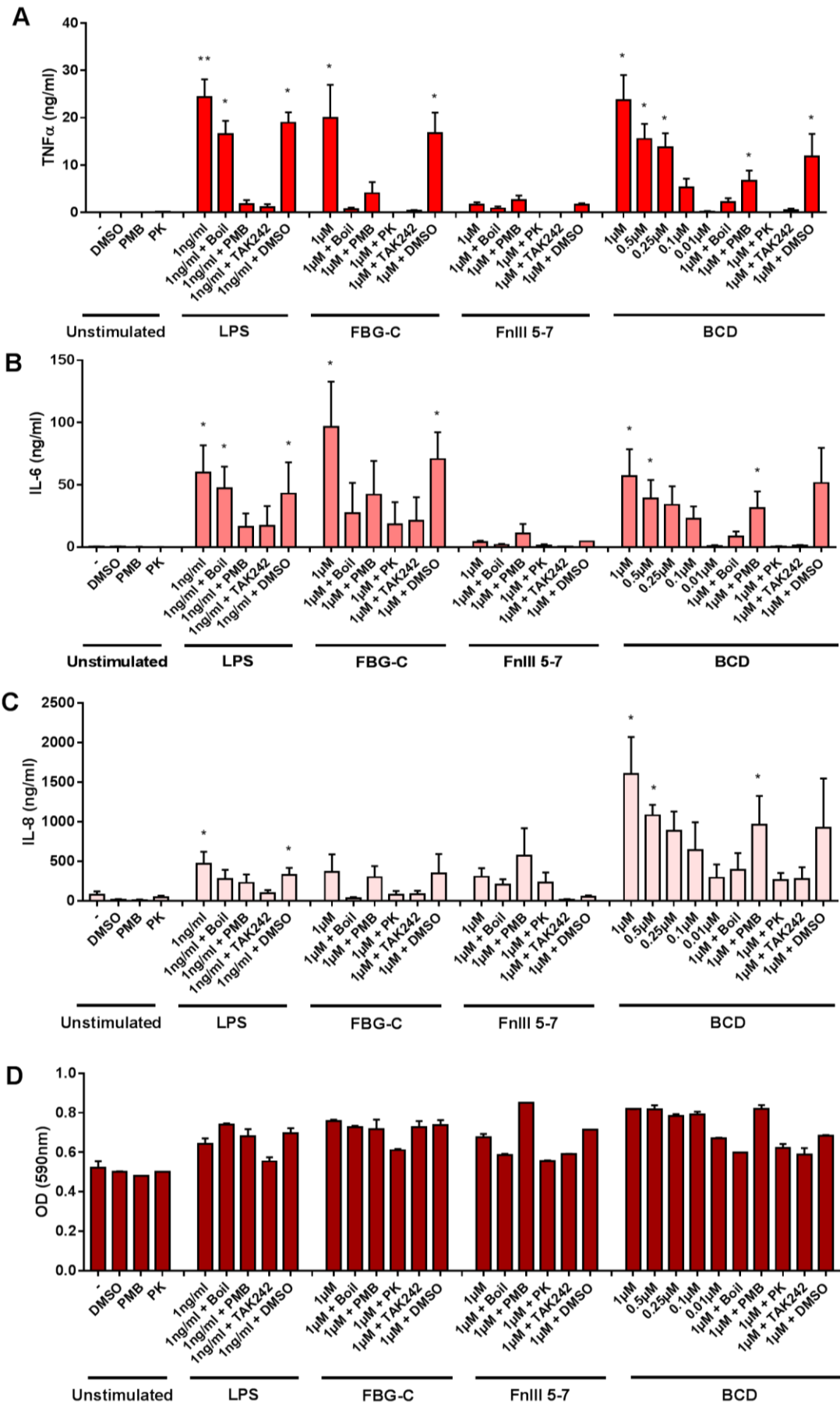


Fig 5.7. The legend is on the following page.

Figure 5.7. Cytokine expression is induced in M-CSF macrophages by recombinant FnIII BCD fragments. M-CSF macrophages were plated and rested for 16 hours prior to stimulation with 1ng/ml LPS, 1µm FBG-C or 5-7, or 1, 0.5, 0.25, 0.1 and 0.01µM BCD and AD2AD1 or BAD2AD1CD (See Appendix Figure 5.7) for 24 hours. Controls included boiling of the stimulus (+Boil; 90°C for 30mins), 30 minute pre-incubation with polymyxin-B (+ PMB) or 6 hour pre-incubation with TAK242 or DMSO. Cytokine release was quantified by ELISA for TNFα (A), IL-6 (B) and IL-8 (C); and cell viability was assessed by MTT (D). Data are shown as mean + SEM from n=4 independent experiments. One way Anova with multiple comparisons was performed and statistical significance is displayed as *p<0.05, ** p<0.01.

Overall, it may be concluded that BCD induced expression of TNFα and IL-6 in a manner that appears to be TLR4 dependent. BCD also induced expression of IL-8, but here the TLR4 dependency of this induction could not be established. BAD2AD1CD appeared to selectively induce TNFα expression in a TLR4 dependent manner, but not IL-6 or IL-8. Also, AD2AD1 was not active in inducing TNFα or IL-6 expression, but did cause an increase in IL-8 levels; however due to the failure of the negative controls detected by this ELISA, it is unclear whether this is the case. In summary, the recombinant BCD and BAD2AD1CD do exhibit the ability to selectively induce different cytokine expression in a TLR4 dependent manner, as has been previously described for the fibronectin EDA domain; but AD2AD1, much like the constitutive FnIII domains does not induce cytokine expression in a TLR4 dependent manner.

5.4 Modulation of cell adhesion and morphology in HDFs by alternatively spliced tenascin-C fragments

In order to assess the effect of alternatively spliced FnIII domains on fibroblast cell adhesion, HDF adhesion assays were performed on coatings of recombinant FnIII proteins AD2AD1, BCD, BAD2AD1CD, FnIII 3-5, FnIII 5-7 and full length proteins fibronectin and tenascin-C.

Firstly, the protein coating protocols and concentrations for the future cell-based assays were optimised to ensure equal protein coating, so that the effects of cell adhesion between different proteins at the same concentration could be directly compared. Most published cell

adhesion data coats plates with proteins measured in mg/ml, but I reasoned that this was not good for comparing adhesion to domains of different molecular masses. To assess equal protein coating, tissue culture plates were coated with either 5µg/ml or 0.2µM of tenascin-C, FnIII 3-5, FnIII 5-7, AD2AD1, BCD, BAD2AD1 or a BSA blank. The His-tag of bound proteins was detected using an HRP conjugated mouse anti-tetra His antibody. Differences in the absorbance (450nm) following coating with 5µg/ml of each protein or protein domain were observed when compared to coating with 0.2µM (Figure 5.8.A). Coating with 0.2µM resulted in equal protein binding, whilst coating with 5µg/ml resulted in an overrepresentation of smaller proteins like AD2AD1 (19.94kDa), and an underrepresentation of larger proteins like BAD2AD1CD (56kDa) (Figure 5.8.B).

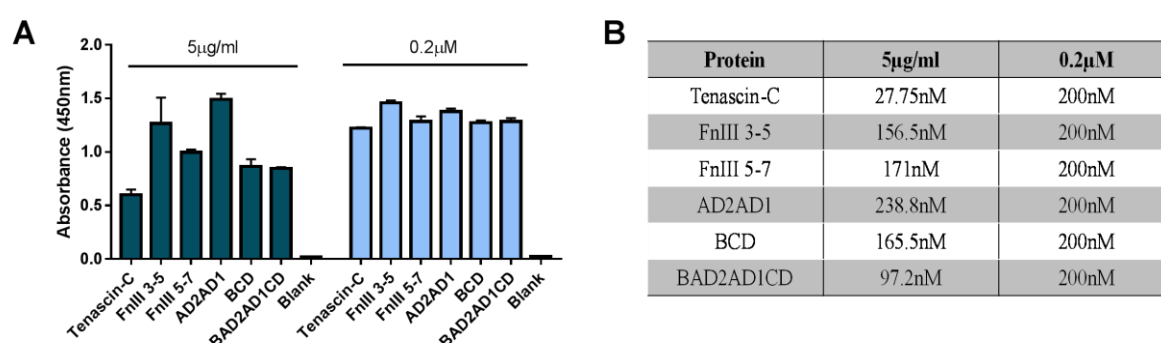


Figure 5.8. Protein coating controls. Tissue culture plates were coated with either 5 µg/ml or 0.2 µM of tenascin-C, FnIII 3-5, FnIII 5-7, AD2AD1, BCD and BAD2AD1CD, or were incubated with TN buffer (blank) for 16h at 4°C. Following blocking with 5% BSA and washing, bound protein was detected with an HRP conjugated anti-tetra His mAb, and absorbance was read following incubation with TMB buffer for 20 mins, at OD 450nm. The mean OD from 3 replicates + SEM are shown in (A), and the discrepancy in nM concentrations resulting from coating with 5µg/ml protein (B).

Next, I determined the degree of adhesion of HDFs to serially diluted concentrations of tenascin-C, fibronectin, FnIII 3-5, FnIII 5-7, AD2AD1, BCD and BAD2AD1CD. This allowed for the quantification of dose dependent HDF cell adhesion to these substrates, and permitted calculation of EC₅₀ and B_{max} values, describing the relative potency of the coating substrates, and the receptor density for the coating in HDFs respectively (Figure 5.9.A).

HDFs exhibited high dose dependent levels of adhesion to fibronectin, as was expected⁸², with a low EC_{50} and comparatively high B_{max} values relative to the other coatings (Figure 5.9.B). Also as expected, tenascin-C was a poor substrate and promoted little fibroblast adhesion (Figure 5.9.C); whereas the FnIII 3-5 protein promoted high adhesion levels comparable to fibronectin, consistent with published data; due to the RGD sequence and integrin binding site located within the FnIII-3 domain^{412, 278, 279, 413}. FnIII 3-5 also showed comparable EC_{50} and B_{max} values to fibronectin coating (Figure 5.9.D).

The FnIII 5-7 coating promoted dose dependent adhesion that was higher than for tenascin-C but less than for fibronectin; with a higher EC_{50} and a lower B_{max} value than for fibronectin and FnIII 3-5 (Figure 5.9.E). All of the alternatively spliced FnIII proteins promoted HDF adhesion. AD2AD1 coating permitted dose dependent levels of adhesion comparable to FnIII 5-7, but with an EC_{50} comparable to fibronectin and FnIII 3-5, and a B_{max} value similar to FnIII 5-7 (Figure 5.9.F). BCD promoted similar levels of adhesion as AD2AD1, exhibiting similar EC_{50} and B_{max} values (Figure 5.9.G); while adhesion to BAD2AD1CD appeared slightly greater than for FnIII 5-7, AD2AD1 and BCD, with EC_{50} levels similar to for FnIII 5-7, and a B_{max} value comparable to fibronectin (Figure 5.9.H).

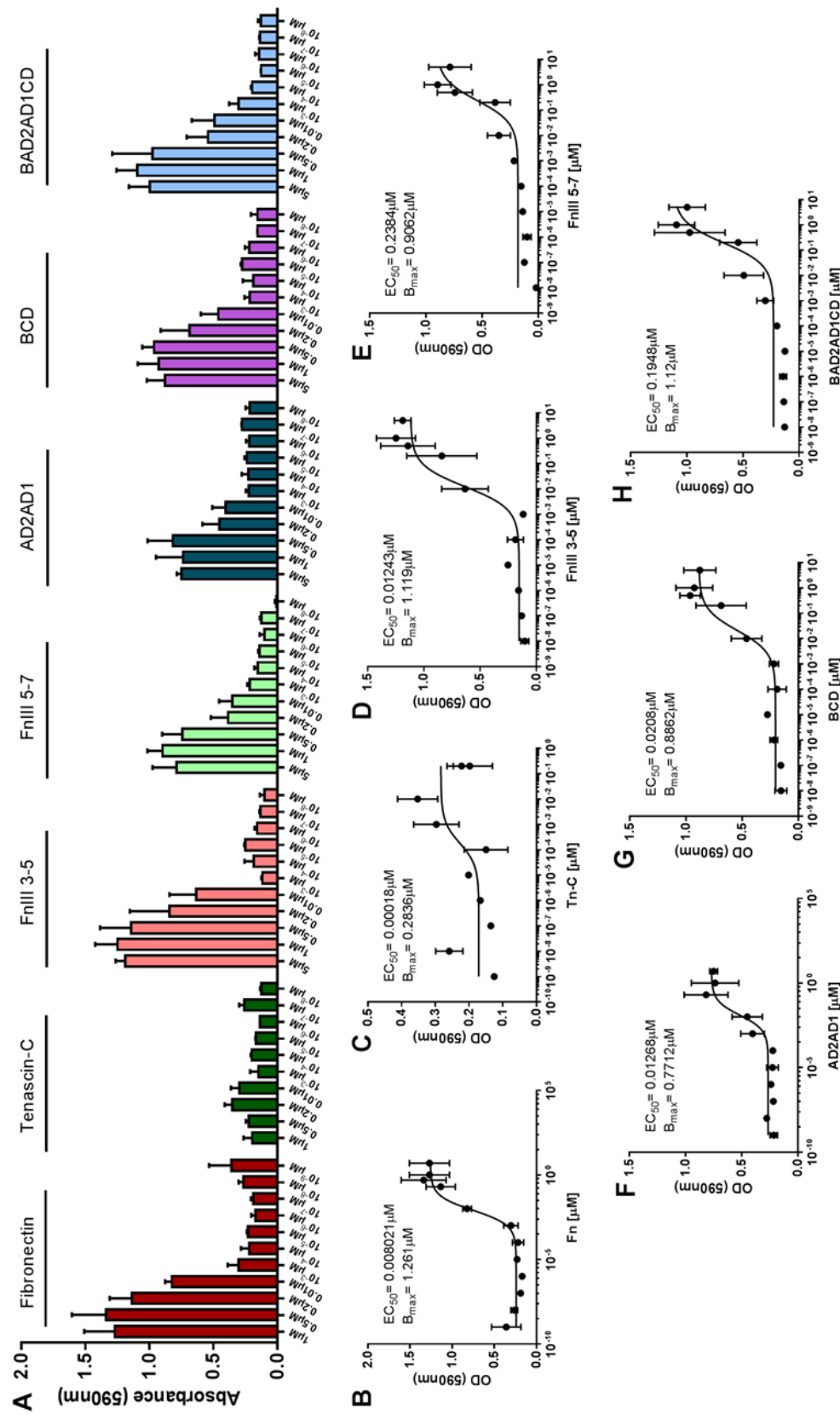


Figure 5.9. HDf cells adhere to recombinant FnIII 5-7 and to alternatively spliced FnIII proteins but not tenascin-C. 96-well plates were coated with the stated concentrations of each protein for 16h at 4°C. Wells were washed and blocked with 10% BSA prior to addition of HDf cells at 4×10^4 cells/well for 60 minutes at 37°C. Cells were fixed and stained with 0.1% crystal violet before being solubilised in 10% acetic acid. Absorbance at 450nm was measured using the Fluostar plate reader (A). EC_{50} and B_{max} values were calculated for each substrate: fibronectin (B), tenascin-C (C), FnIII 3-5 (D), AD2AD1 (F), FnIII 5-7 (E), BAD2AD1CD (H) and BCD (G). Data are shown as mean $\pm$ SEM, with background levels of cell adhesion to uncoated; with the background absorbance from 10% BSA blocked wells subtracted.

In summary, these data show that the positive and negative controls for the HDF adhesion assay worked as expected, and that FnIII 5-7, and the alternatively spliced FnIII proteins AD2AD1, BCD and BAD2AD1CD promoted greater HDF adhesion than tenascin-C; but not to levels as high as for fibronectin or FnIII 3-5.

Differences in the cellular morphology caused by different protein coatings were next observed in cells plated on 0.01 μ M fibronectin, tenascin-C and FnIII coatings; the concentration at which the greatest differences in the total levels of adhesion were observed. Here, the levels of HDF adhesion to fibronectin and FnIII 3-5 were significantly greater than to tenascin-C; where the least adhesion was detected. The FnIII 5-7, AD2AD1 and BCD coatings supported higher levels of adhesion than tenascin-C, but this was not significant, with only the BAD2AD1CD coating promoting significantly more cell adhesion than tenascin-C (Figure 5.10.A).

Morphologically, the HDFs appeared more angular, displaying small cellular processes when cultured on fibronectin, FnIII 3-5, BCD and BAD2AD1CD, but were more rounded when adhered to tenascin-C, FnIII 5-7, AD2AD1 and the BSA control (Figure 5.10.B-I).

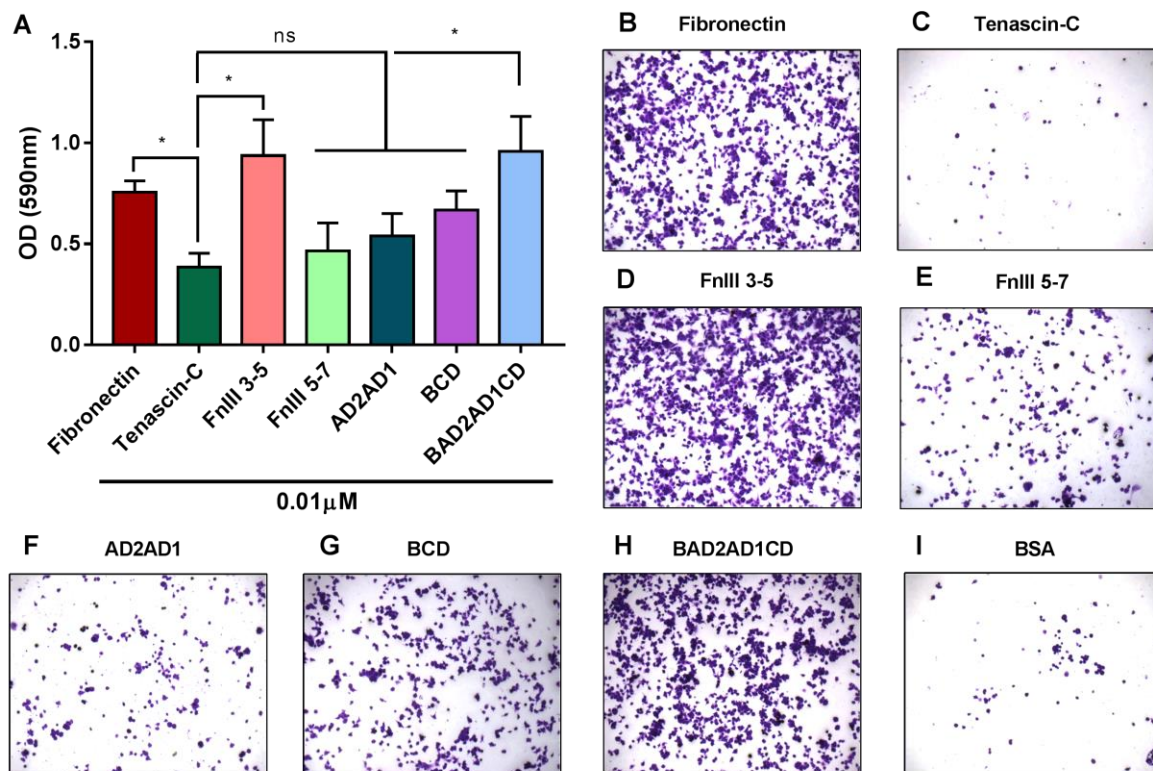


Figure 5.10. FnIII 3-5 and BAD2AD1CD promote greater HDF adhesion than full-length tenascin-C. 96-well plates were coated with the 0.01 μ M of each protein as indicated, for 16h at 4°C, or were left uncoated (BSA). Wells were washed and blocked with 10% BSA prior to addition of HDFs at 4×10^4 cells/well for 60 minutes at 37°C. Cells were fixed and stained with 0.1% crystal violet before being solubilised in 10% acetic acid. Absorbance at 450nm was measured using the FluoStar plate reader (A), and images were taken with the Nikon Eclipse Ts2r light microscope at 40x objective (B-I). Data are shown as mean + SEM, from 4 separate experiments (n=4) with the BSA blank subtracted. One way Anova with multiple comparisons was performed, *p<0.05, non-significant (ns). Images are representative of 4 independent experiments.

To examine cell morphology in more detail, I cultured HDFs on glass coverslips coated with 0.01 μ M fibronectin, tenascin-C and recombinant FnIII proteins, and stained the cells for vinculin and actin; so that the conformation of the cell cytoskeleton and potential focal adhesions that would suggest integrin involvement in adhesion could be observed by confocal immunofluorescence microscopy.

Fibroblast adhesion to fibronectin is integrin dependent^{417, 418}, and therefore I would expect to observe discrete regions of co-localisation between vinculin and actin around the edges of the cell, that would indicate the presence of focal adhesion formation. From the staining, actin

filaments were clearly visible, while vinculin faintly stained the cell interior and did not strongly co-localise with actin, indicating that focal adhesions were either not present, or were not being observed by this assay. However, cells cultured on fibronectin appeared morphologically larger and better spread than for any of the other coatings (Figure 5.11).

HDFs plated on tenascin-C, FnIII 5-7 and BCD exhibited similar vinculin and actin distributions to each other, and were more rounded than those on fibronectin. Actin was abundantly stained around the edges, and in some small foci in the centre of the cells; while vinculin appeared to faintly stain the centre of the cells with little co-localisation with actin. Cells plated on FnIII 3-5 and AD2AD1 were also small and rounded, with strong co-localisation of actin and vinculin around the edges of the cells, with some small projections staining positively for actin or vinculin. HDFs plated on BAD2AD1CD were also rounded, with few cell processes as described for AD2AD1. Vinculin staining is distributed around the cytosol, and actin is visible in the centre of the cell in punctate foci. Compared to all proteins tested, the levels of actin staining in the BSA control were barely detected, while vinculin was detected around the edge of the cell outline (Figure 5.11).

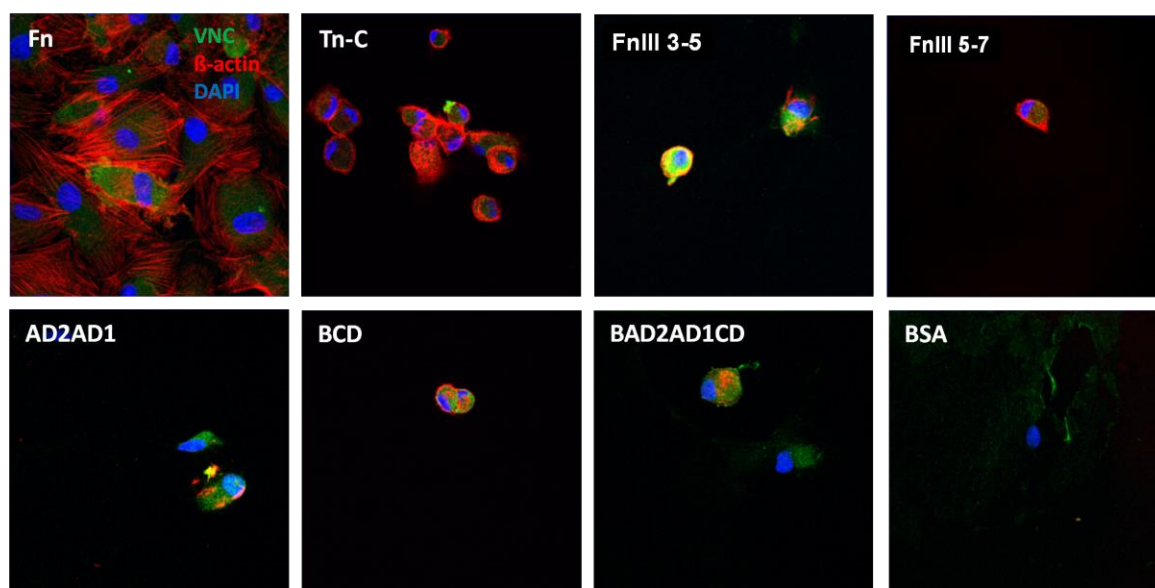


Figure 5.11. Assessing morphology and the distribution β -actin and vinculin in HDFs cultured on fibronectin, tenascin-C and tenascin-C fragments. Glass coverslips were coated with 0.01 μ M fibronectin, tenascin-C, FnIII 3-5, FnIII 5-7, AD2AD1, BCD, BAD2AD1CD or 10% BSA for 16h at 4°C. Coverslips were washed and blocked with 10% BSA prior to addition of HDFs at 4×10^4 cells/well for 60 minutes at 37°C. HDFs were then fixed, permeabilised in 0.02% Triton X-100, and stained for β -actin using 1:200 AlexaFluor 568nm labelled Phalloidin (Red) and 1:200 mouse anti-human vinculin primary antibody (Green). All slides were stained with DAPI diluted 1:250 (Blue), alongside the goat anti-mouse AlexaFluor 488nm secondary antibody at 1:1000. Representative images 3 separate experiments are shown. Images were visualized with the Olympus FV1200 IX83 Confocal System, with a 60x objective and 2x zoom.

In summary, these data show that HDF adhesion is greatest to fibronectin, FnIII 3-5 and is low in tenascin-C as was previously described. Furthermore, recombinant proteins corresponding to constitutive FnIII domains, FnIII 5-7, and alternatively spliced FnIII domains promoted slightly greater cell adhesion than tenascin-C, though culture on BAD2AD1CD promoted significantly higher levels of HDF adhesion than AD2AD1 or BCD individually. For the confocal immunofluorescence microscopy, the fibronectin coating promoted higher levels of HDF adhesion and cell spreading than the other coatings, but did not contain observable focal adhesions. Despite promoting significantly greater adhesion in the cell adhesion assay, FnIII 3-5 and BAD2AD1CD did not exhibit higher levels of adhesion than tenascin-C, FnIII 5-7 or AD2AD1 on the glass coverslips following

immunofluorescence staining. However, a clear co-localisation of vinculin and actin was observed when HDFs were cultured on FnIII 3-5 and AD2AD1 compared to the other protein coatings.

5.5 Modulation of cell adhesion and morphology in MDDCs by alternatively spliced tenascin-C fragments

Activated MDDCs transiently express tenascin-C containing all of the alternatively spliced FnIII domains except for AD2 and AD1. In this section, I examine the functional consequences of omitting the FnIII-AD2 and AD1 domains from the FnIII-B,C,D region, by assessing MDDC adhesion and morphology after plating on AD2AD1, BCD and BAD2AD1CD recombinant proteins, and comparing the adhesion to tenascin-C.

I first determined the degree of MDDC adhesion to serial dilutions of purified fibronectin, tenascin-C, FnIII 3-5, FnIII 5-7, AD2AD1, BCD and BAD2AD1CD; from which EC_{50} and B_{max} values were calculated.

MDDC adhesion to fibronectin was dose dependent, and higher than the baseline for uncoated wells (Figure 5.12.A,B); however the overall levels of adhesion were lower than detected for tenascin-C. This result was surprising, but not unexpected, as direct MDDC adhesion to tenascin-C had not previously been investigated. The EC_{50} and B_{max} values for tenascin-C were higher than observed for fibronectin (Figure 5.12.C). In MDDCs, the alternatively spliced FnIII proteins promoted less cell adhesion than full length tenascin-C (Figure 5.12.A). FnIII 3-5 coating resulted in lower levels of adhesion than fibronectin and tenascin-C, which were not dose dependent (Figure 5.12.D). FnIII 5-7 promoted low levels of dose dependent cell adhesion, with EC_{50} and B_{max} values lower than for tenascin-C (Figure 5.12.E). AD2AD1 coating permitted moderate levels of adhesion, exhibiting EC_{50} and B_{max} values comparable to those for FnIII 5-7 (Figure 5.12.F). Coating with BCD promoted higher

levels of adhesion than AD2AD1 at 1 μ M coatings, but the EC₅₀ and B_{max} values were much higher than for AD2AD1 (Figure 5.12.G). Finally, the BAD2AD1CD protein coating caused the highest level of cell adhesion from the spliced FnIII proteins at 1 μ M, but the EC₅₀ and B_{max} values showed very similar levels as for the BCD protein (Figure 5.12.H)

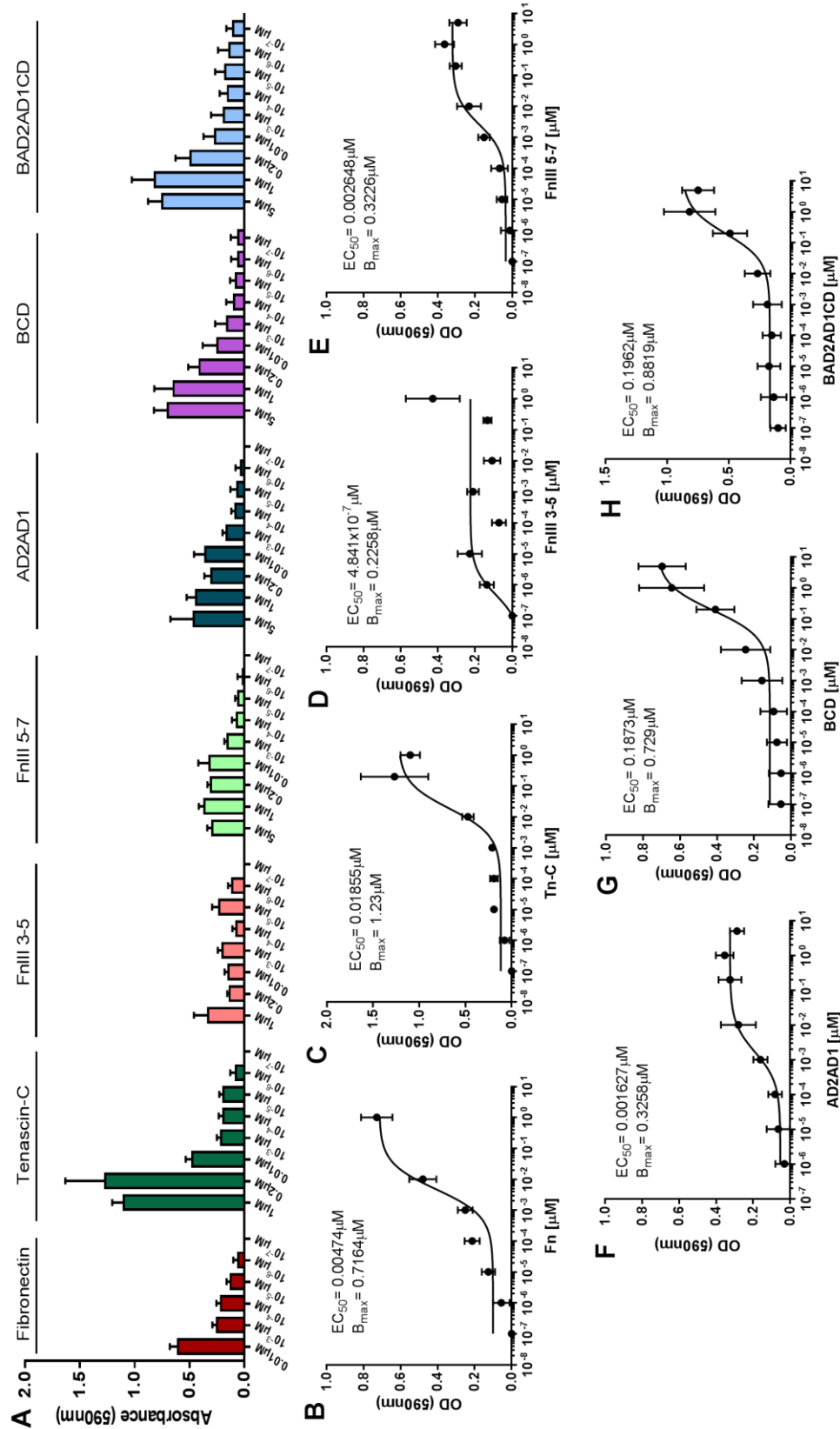


Figure 5.12. Tenascin-C promotes higher levels of MDDC adhesion than the alternatively spliced FnIII proteins. 96-well plates were coated with the stated concentrations of each protein for 16h at 4°C. Wells were washed and blocked with 10% BSA prior to addition of MDDCs at 2×10^5 cells/well for 60 minutes at 37°C. Cells were fixed and stained with 0.1% crystal violet before being solubilised in 10% acetic acid. Absorbance at 450nm was measured using the Fluostar plate reader (A). EC_{50} and B_{max} values were calculated for each substrate: fibronectin (B), tenascin-C (C), FnIII 3-5 (D), FnIII 5-7 (E), AD2AD1 (F), BCD (G) and BAD2AD1CD (H) ($n=4$). Data are shown as mean $\pm$ SEM, with background levels of cell adhesion to uncoated, and 10% BSA blocked wells subtracted.

These adhesion assays revealed that tenascin-C promoted higher levels of MDDC adhesion than fibronectin, and that the alternatively spliced FnIII proteins promoted moderate levels of tenascin-C adhesion but to lower levels than seen with the full length tenascin-C.

The absolute levels of adhesion and the differences in MDDC morphology caused by plating on different substrates were observed by comparing the adhesion data from 0.01 μ M coatings fibronectin, tenascin-C and FnIII coatings; as this was the highest concentration at which all substrate coatings were performed; and where the greatest differences in adhesion levels were observed. The levels of MDDC adhesion to tenascin-C and fibronectin are equally high, and are significantly greater than with any of the recombinant FnIII proteins. Furthermore, there is no significant difference in the levels of adhesion between coating with FnIII 3-5, FnIII 5-7, AD2AD1, BCD or BAD2AD1CD, as all induced some moderate levels of adhesion at this coating concentration (Figure 5.13.A).

Morphologically, when plated on fibronectin, tenascin-C, FnIII 5-7 and AD2AD1, MDDCs display more cellular processes, than those adhered to FnIII 3-5, BCD, BAD2AD1CD or BSA substrates; where the cells are visibly rounded and appear to show lower overall adhesion (Figure 5.13.B-I).

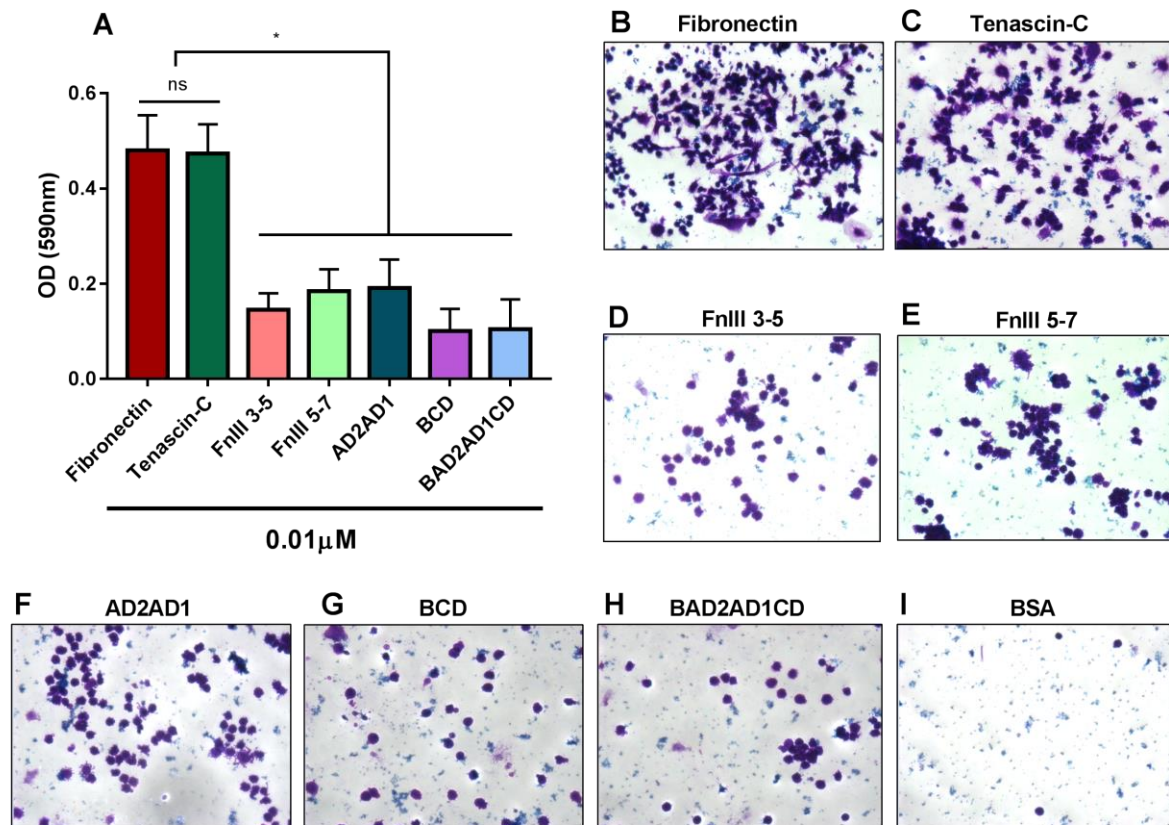


Figure 5.13. Tenascin-C fragments promote less adhesion than full length tenascin-C in MDDCs. 96-well plates were coated with the 0.01μM of each protein as indicated, for 16h at 4°C, or were left uncoated (BSA). Wells were washed and blocked with 10% BSA prior to addition of MDDCs at 2×10^5 cells/well for 60 minutes at 37°C. Cells were fixed and stained with 0.1% crystal violet before being solubilised in 10% acetic acid. Absorbance at 450nm was measured using the FluoStar plate reader (A), and images were taken with the Nikon Eclipse Ts2r light microscope at 40x objective (B-I). Data are shown as mean + SEM, from 4 separate experiments (n=4) with the BSA blank subtracted. One way Anova with multiple comparisons was performed, *p<0.05, non-significant (ns). Images are representative of 4 independent experiments.

To expand on the findings made in MDDCs, further analysis was performed to assess whether the adhesion effects of these recombinant FnIII proteins were conserved across MDDCs and macrophage cell types. Therefore, a cell adhesion assay was performed with protein substrates coated at 0.01μM as previously described for MDDCs.

In GM-CSF macrophages, adhesion was greatest to the tenascin-C coating, and was lower to fibronectin, FnIII 3-5, FnIII 5-7, AD2AD1, BCD and BAD2AD1CD where the adhesion levels were approximately equal. However, adhesion to all of the substrates was higher than the background levels (Figure 5.14.A). For M-CSF macrophages, there was a higher level of adhesion to tenascin-C than to fibronectin; though the absolute level of adhesion of both was

higher than in GM-CSF macrophages. FnIII 3-5, FnIII 5-7, AD2AD1, BCD and BAD2AD1CD proteins all promoted moderate levels of adhesion but they were not significantly different from one another (Figure 5.14.B).

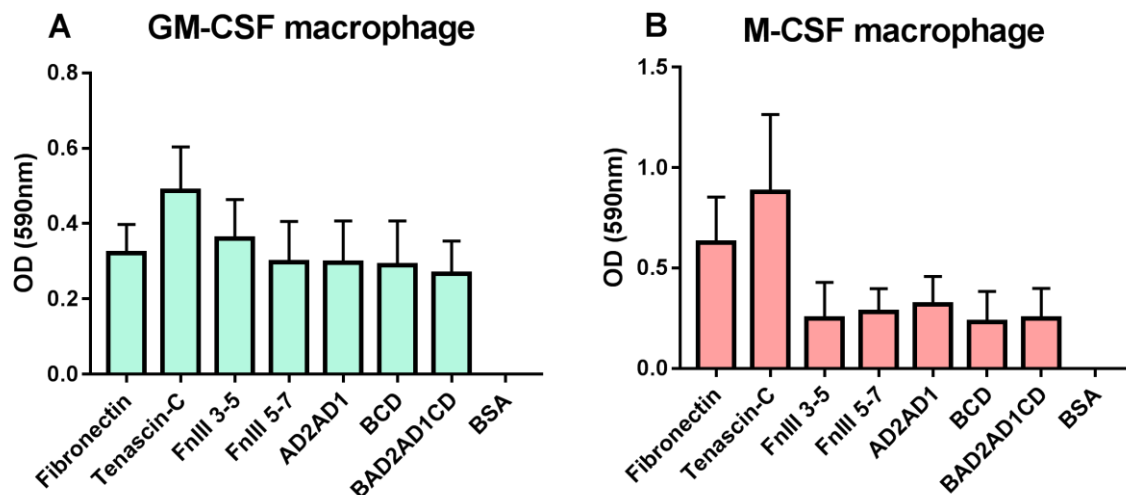


Figure 5.14. Tenascin-C fragments promote less adhesion than full length tenascin-C in M-CSF and GM-CSF macrophages. 96-well plates were coated with the 0.01 μ M of each protein as indicated, for 16h at 4°C, or were left uncoated (BSA). Wells were washed and blocked with 10% BSA prior to addition of GM-CSF macrophages (A) or M-CSF macrophages (B) at 2x10⁵ cells/well for 60 minutes at 37°C. Cells were fixed and stained with 0.1% crystal violet before being solubilised in 10% acetic acid. Absorbance at 450nm was measured using the FluoStar plate reader. Data are shown as mean + SEM, from 4 separate experiments (n=4) with the BSA blank subtracted.

In order to better visualise the adhesion of MDDCs to fibronectin, tenascin-C and the recombinant FnIII domains, the morphology and cellular distribution of vinculin and actin were observed in MDDCs that were coated on these proteins.

MDDCs plated on fibronectin exhibited a mixture of phenotypes. Some were small and rounded, in which actin and vinculin co-localised around the edges of the cells. Other cells were larger and more spread, staining strongly for either actin or vinculin within the cytosol, and at dendrite like projections. On tenascin-C coating, the MDDCs were larger and more spread than on fibronectin. Here, vinculin staining was concentrated in the centre of the cell and clustered in a ring around the edges, where it co-localised with actin.

MDDCs plated on FnIII 3-5, and AD2AD1 were larger and more spread than when cultured on fibronectin, with short fibrils of actin and clusters of vinculin localising to the cell edges and to regions which resemble migration fronts. In cells on FnIII 3-5, vinculin co-localised to actin; however actin was also detected separately from vinculin at the cell edges. On AD2AD1, faint vinculin and actin staining was detected in the cytosol, and little co-localisation was observed at the cell edges.

Cells cultured on FnIII 5-7, and BAD2AD1CD exhibited similar morphology to MDDCs, as cells were smaller and more rounded than those on fibronectin, tenascin-C FnIII 5-7 and AD2AD1; but they contained many short cell processes. On FnIII 5-7 and BAD2AD1CD, cells mainly stained positive for either actin or vinculin, but some cells were positive for both.

This contrasted with cells plated on BCD, where every cell showed co-localisation of actin and vinculin towards the edges of the cells. Cells plated on BCD appeared most morphologically similar to those cultured on FnIII 5-7 and BAD2AD1CD (Figure 5.15).

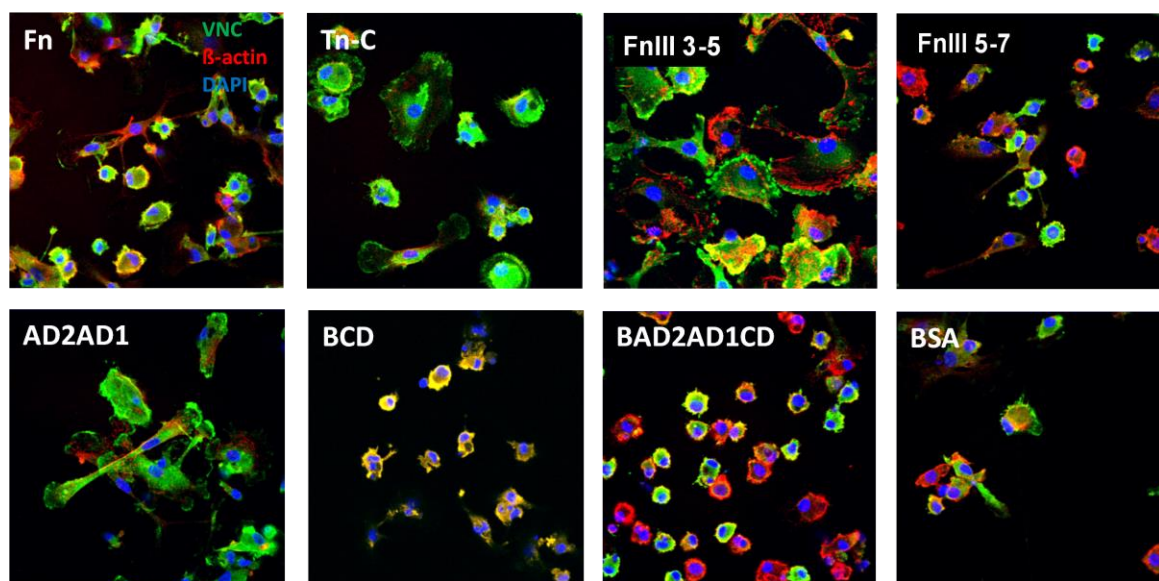


Figure 5.15. Assessing morphology and the distribution β -actin and vinculin in MDDCs cultured on fibronectin, tenascin-C, and recombinant FnIII proteins. Glass coverslips were coated with 0.01 μ M fibronectin, tenascin-C, FnIII 3-5, FnIII 5-7, AD2AD1, BCD, BAD2AD1CD or 10% BSA for 16h at 4°C. Coverslips were washed and blocked with 10% BSA prior to addition of MDDCs at 2×10^5 cells/well for 60 minutes at 37°C. MDDCs were then fixed, permeabilised in 0.02% Triton X-100, and stained for β -actin using 1:200 AlexaFluor 568nm labelled Phalloidin (Red) and 1:200 mouse anti-human vinculin primary antibody (Green). All slides were stained with DAPI diluted 1:250 (Blue), alongside the goat anti-mouse AlexaFluor 488nm secondary antibody at 1:1000. Representative images 3 separate experiments are shown. Images were visualized with the Olympus FV1200 IX83 Confocal System, with a 60x objective and 2x zoom.

5.6 FnIII-BCD promotes MDDC migration

I noted that MDDC morphology in cells adherent to tenascin-C, FnIII 3-5 and AD2AD1, resembled a migratory phenotype with numerous polarised vinculin rich lamella, raising the question of whether these proteins can also regulate MDDC migration. Therefore, I next examined MDDC cell migration in response to coated FnIII proteins, using a transwell migration assay.

Transwell membranes were coated with 0.01 μ M fibronectin, 0.1, 0.01, 10⁻³ μ M tenascin-C and FnIII 3-5, and 0.1, 0.01, 10⁻³, 10⁻⁴ μ M for FnIII 5-7, AD2AD1, BCD and BAD2AD1CD. Here 10% BSA coating served as a negative control. MDDC migration was induced through the 5 μ m transwell pore with 10% FBS RPMI-1640, and serum free media served as a passive migration control^{419, 420}. Quantification of migration was performed by counting migrated cells in 40x field images as described in section 2.12.5.

MDDC migration was induced through the pores of the transwell assay by creating a stable FBS concentration gradient. In the upper chamber, cells were plated in serum free media, and migration was encouraged through the transwell membrane pores into the lower chamber by the presence of 10% FBS media on the lower side. This type of active cell motility, termed in this study as cell migration, was specific in that it is stimulated by the presence of an FBS concentration gradient. The FBS that was used as a chemoattractant was heat inactivated, but contains a wide variety of cytokines, chemokines, growth factors and nutrients which the cells cultured in serum free media are lacking. For each different protein coating assessed, I also included a passive migration negative control where serum free media was present on either side of the transwell membrane; and no FBS gradient existed. This negative control measured the level of passive cell motility that is not induced by a specific stimulus, which in this study was termed passive chemotaxis.

As dendritic cell migration had previously been characterised on fibronectin⁴²¹, this coating served as a positive control. Blocking the transwell membrane with 10% BSA also served as a well characterised positive control for migration, as BSA adsorption to the TC plate prevented MDDC interaction with the plate, permitting greater cell motility.

The positive controls confirmed the efficacy of the assay, as MDDCs on fibronectin exhibited a high level of selective migration in response to FBS, as was expected. Similarly, when the cells were plated on BSA a high level of specific migration was detected, also as expected. For tenascin-C, FnIII 3-5 and FnIII 5-7, levels of migration towards the FBS were comparable to those for fibronectin, but unlike on fibronectin, this migration was accompanied by high levels of non-specific chemotaxis. Cells on AD2AD1 exhibited dose dependent migration towards FBS that was also accompanied by relatively high levels of passive chemotaxis. BCD coating permitted higher levels of dose dependent migration than was observed on fibronectin, and was accompanied by low levels of passive chemotaxis. Finally, cells on BAD2AD1CD permitted the highest overall levels of cell migration, but this was independent of the presence of the FBS stimulus (Figure 5.16.A). The images display the migrated and stained MDDCs on the underside of the transwell membranes that were coated with 0.01 μ M proteins, or 10% BSA (Figure 5.16.B).

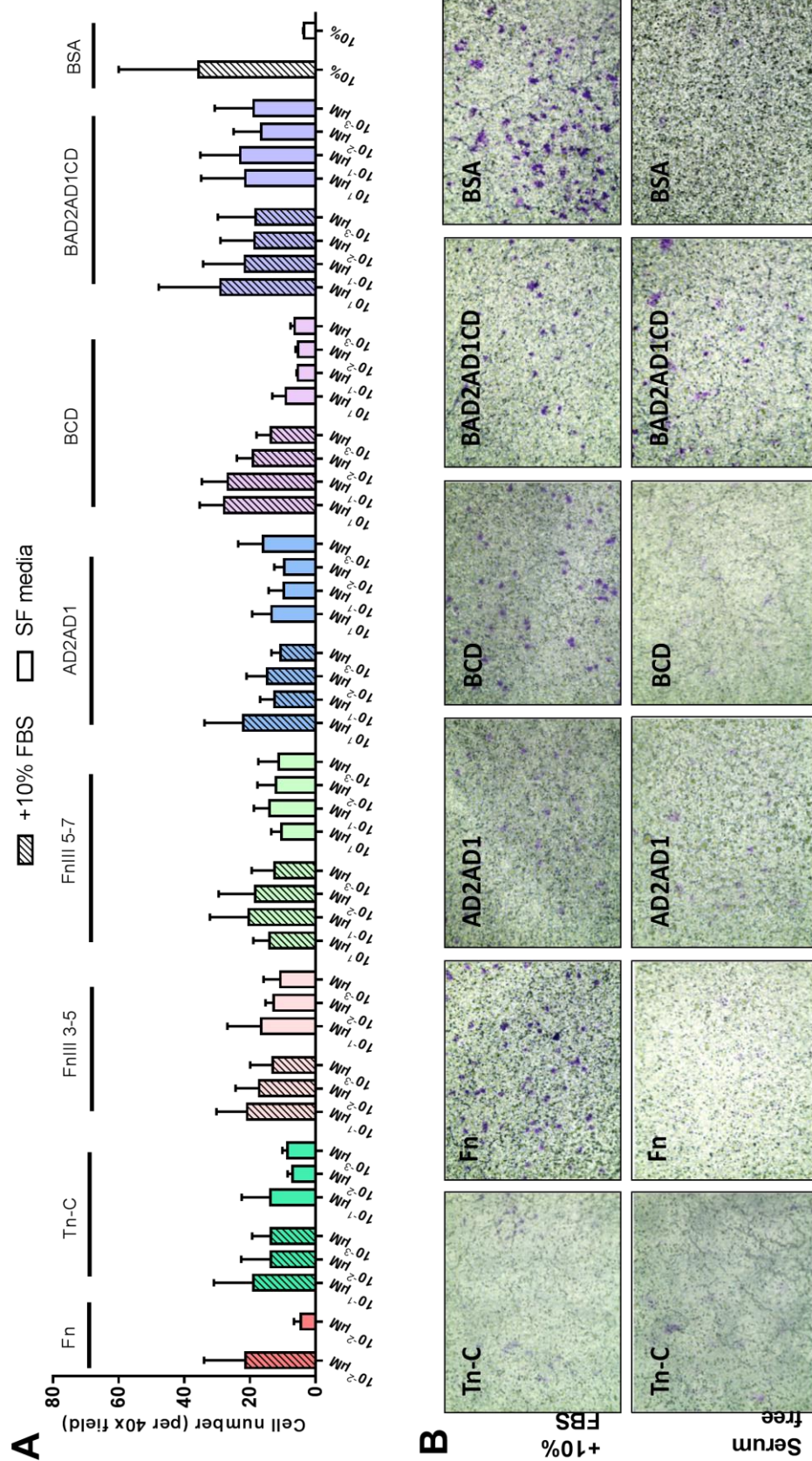


Figure 5.16. Titrating fibronectin, tenascin-C and tenascin-C fragment substrate coating concentrations for MDDC transwell migration assay. (A) proteins were coated on individual 24-well transwell inserts (5µm pore size), in 100µl volumes at 4°C for 16 hours, with fibronectin, tenascin-C, FnIII 3-5, FnIII 5-7, AD2AD1, BCD, BAD2AD1CD, and 10% for BSA at the concentrations as described on the graph. Wells were washed and blocked with 10% BSA prior to addition of 2x10⁵ MDDCs/well in serum free RPMI-1640 into the upper chamber, and 600µl of either serum free RPMI-1640 (clear bars on figure), or 10% FBS RPMI-1640 (hatched bars on figure) into the lower chamber, for 3 hours. Cells in the upper chamber were removed, and the membranes were stained with 0.1% crystal violet. Data are shown as mean + SEM, from 4 separate experiments. (B) The number of cells from 3 representative 40x fields per well were counted using the Nikon Eclipse Ts2R light microscope, with representative images displayed with the coating substrate and bottom chamber media indicated.

Based on the above data, I calculated the migration index as described in ^{419, 420}; which here describes the overall level of specific cell migration in response to the FBS chemoattractant. This analysis revealed that the highest levels of specific MDDC migration were observed on the fibronectin and BSA positive controls coatings; where the migration index was significantly higher than levels for tenascin-C. By contrast, the recombinant FnIII proteins FnIII 3-5, FnIII 5-7, AD2AD1 and BAD2AD1CD promoted no more specific MDDC migration than full length tenascin-C; setting BCD apart as a protein that was permissive of selective MDDC migration. Despite the low migration index of BAD2AD1CD, the overall levels of selective and passive migration on this substrate were the highest detected, indicating that BAD2AD1CD is permissive of cell migration but this was not selective in response to FBS as was the case for BCD (Figure 5.17.).

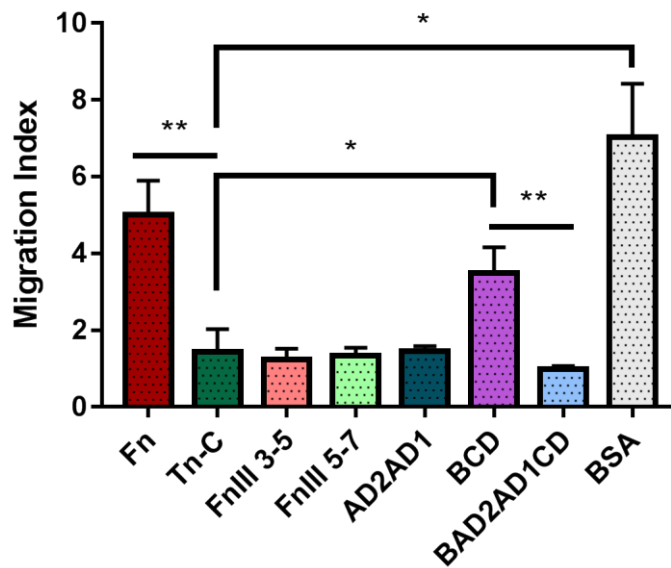


Figure 5.17. BCD supported selective MDDC migration in response to FBS, while BAD2AD1 supported active and passive migration to FBS. Fibronectin, tenascin-C (Tn-C), FnIII 3-5, FnIII 5-7, AD2AD1, BCD and BAD2AD1CD (0.01 μ M) were coated on transwell inserts (5 μ m pore size) for 16h at 4°C, control inserts were left uncoated (BSA). Wells were washed and blocked with 10% BSA prior to addition of 2×10^5 cells/well in serum free RPMI-1640 into the upper chamber, and either serum free RPMI-1640, or RPMI-1640 containing 10% FBS into the lower chamber, for 3 hours. After cell migration, the membranes were stained with 0.1% crystal violet. The number of cells on the underside of the membranes from 3 representative 40x fields per well were counted using the Nikon Eclipse Ts2R light microscope, and the migration index was calculated by dividing the number of passively migrated cells into the serum free RPMI-1640 by the number of cells migrated into the 10% FBS RPMI-1640 media. Data are shown as mean + SEM. (n=4); One way Anova, *p<0.05 and **p<0.01.

These data showed that the alternatively spliced FnIII domains of tenascin-C can modulate MDDC migration. Neither Tenascin-C nor any of the other recombinant FnIII proteins supported selective MDDC migration to FBS, except for BCD which stood out as a protein that permitted MDDC migration to FBS, but also reduced passive chemotaxis. In contrast, interruption of the BCD domains with AD2AD1, as in protein BAD2AD1CD, greatly reduced the ability of the BCD domains to support selective migration of MDDCs.

5.7 Summary

These data have shown that activated MDDCs and diseased fibroblasts regulate the expression of alternatively spliced FnIII domains that are functionally relevant to inflammatory processes; and can modify cytokine expression, cell adhesion and migration processes in innate immune cells and fibroblasts.

Resting MDDCs switch from expressing low levels of a larger tenascin-C isoform where FnIII-B,AD2,AD1,C,D are contiguous, to higher levels of a shorter isoform where FnIII-AD2,AD1 are omitted, and FnIII-B,C,D are contiguous; this confers a gain in function, allowing the FnIII-B,C,D containing proteins to stimulate cytokine expression in macrophages, and promote MDDC migration. Additionally, the morphology and distribution of actin and vinculin in MDDCs cultured on tenascin-C, was most similar to when cultured on FnIII 3-5 and AD2AD1; where the morphology resembled that of MDDCs that are spreading. On fibronectin, MDDC morphology was most similar when cultured on FnIII 5-7, BCD and BAD2AD1CD; however the distribution of actin and vinculin was dysregulated when FnIII-AD2 and AD1 interrupted the BCD domains.

In contrast, diseased fibroblasts such as RA-SFs express shorter tenascin-C isoforms richer in FnIII-B,C,D than the longer NSF variants where FnIII-B,AD2,AD1,C,D are contiguous. Here, the impact of these domains is different than on MDDCs and reveals cell-type specific functions. The FnIII-B,AD2,AD1,C,D domain supported fibroblast adhesion, while the disease associated FnIII-B,C,D supported cell detachment. All together these data reveal indicate that inflammation and disease associated tenascin-C splice variants modify immune cell and fibroblast behaviors in a cell-type specific manner during inflammation.

5.8 Discussion

This chapter has built on work detailed in chapter 4, where differences in the alternative splicing patterns of tenascin-C between resting and activated immune cells, and fibroblasts from different sources were observed. This work discusses the bacterial expression and purification of tenascin-C alternatively spliced FnIII domains that were shown to be differentially regulated during inflammation and disease. Here, the recombinant FnIII domains AD2AD1, BCD and BAD2AD1 were cloned, expressed, purified and used for the first time in cell based cytokine expression, cell adhesion and cell migration assays; revealing that alternatively spliced FnIII domains of tenascin-C can induce TLR4 dependent cytokine expression in macrophages, can promote fibroblast adhesion, and support MDDC cell migration.

5.8.1 *Expression and purification of recombinant proteins for cell assays*

In order to assess the functional relevance of the alternatively spliced FnIII domains that were selectively regulated in myeloid cells during inflammation and in fibroblasts during disease, recombinant proteins were cloned, expressed and purified using protocols previously described by the Midwood lab⁴³.

Previously the Midwood lab had expressed and purified active recombinant domains from *E.coli*; which corresponded to the constitutively expressed regions of tenascin-C, including the TA domain, EGF-L, FnIII 1-3, 3-5, 5-7, 1-8, and the FBG domain⁴³. Similarly, the group previously cloned, expressed and purified the FBG domains from tenascin- R, -W and -X⁴². These established methods were adapted for the cloning, expression and purification of the AD2AD1, BCD and BAD2AD1CD domains of tenascin-C that took place in this study.

The recombinant proteins generated for this study were purified by Ni-NTA chromatography^{43, 320, 380, 422}. Purification was performed using an N terminal 6xHis-tag, that

was engineered within each recombinant protein, and binds to the Ni-NTA column; being released by adding imidazole. An N-terminal His-tag was selected because of its small size (0.93kDa), compared to alternative tags including glutathione-S transferase (GST) (25kDa), maltose binding protein (MBP) (45kDa) or thioredoxin (TRX) (12kDa); which reduces the impact of steric hindrance on future binding interactions. This was particularly important given the relatively small size of some of the expressed and purified proteins such as AD2AD1 (19.94kDa). The 6xHis-tag is also functionally inactive, and does not need to be cleaved from the recombinant proteins, which reduces the changes of contamination caused by performing additional enzymatic digestion steps to separate the tag from the purified protein. The N-terminal positioning of the His-tag was selected, so that if it was later discovered that the His-tag impacted the functional assay, it could be removed using an exopeptidase; this is not possible if the His-tag is engineered on the C-terminus.

An advantage of expressing and purifying proteins from *E.coli* instead of in mammalian cells is that large protein yields can be quickly obtained following relatively simple protocols. However, significant drawbacks of bacterial expression include the additional steps required to remove contaminating endotoxins such as LPS, and the lack of glycosylation of the proteins, which has been shown to impact tenascin-C function¹²⁶. Furthermore, proteins expressed in *E.coli* are not always solubilised, and may require refolding. An advantage of using the GST, MBP or TRX tags is that they promote protein solubilisation. However, CD spectra analysis of the purified FnIII 5-7, AD2AD1, BCD and BAD2AD1CD, confirmed the presence of β -sheet secondary structure that was comparable to that observed from the structurally homologous FnIII-7 of tenascin-X⁴²³, and to the only crystal structure currently available for an FnIII domain of tenascin-C; FnIII-3⁴²⁴.

An alternative to adding exogenous purified proteins would be to overexpress proteins, or to add blocking antibodies to determine the contribution of specific domains within tenascin-C.

Previous studies that investigated the function of alternative splicing often used antibody perturbation assays; where antibodies were added to inactivate specific alternatively spliced FnIII domains through binding. These studies had the advantage of inhibiting very specific regions of tenascin-C that were expressed and trafficked by the source cells in a physiologically relevant manner; but unfortunately antibodies are not available or specific to all of the alternatively spliced FnIII domains, with specific antibodies available only to FnIII domains A2, B, C and D. This would have precluded any analysis of AD2 and AD1. Other studies had assessed functions by overexpressing individual FnIII domains in target cells; as was previously reported for FnIII-A1 and B,AD1,D^{188, 425}. Limitations of this technique in the context of this study would include complications with transfecting primary human immune cells, and the lack of control on levels of target protein expression in this method.

There advantages for using recombinant proteins when assessing functions in cell based assays include the ability to strictly control the quantity of target protein present in the assay; allowing for direct comparison between the activities of different proteins at the same concentration. Secondly, specific functions of the target protein could be confidently identified as they are presented to cells after purification, without contaminating factors. These methods for characterising the functions of alternatively spliced FnIII domains have been widely reported in the literature. Spliced FnIII domains were previously purified from either *E.coli* or mammalian cells, while proteins were most commonly purified with Ni-NTA chromatography or MonoQ chromatography^{34, 144, 166, 209, 299, 416, 426}.

5.8.2 *Cytokine expression is induced by alternatively spliced FnIII domains*

Prior to this study, the impact of alternatively spliced FnIII domains of tenascin-C on cytokine expression had not been assessed. Previous work by the Midwood lab established that the FBG domain of tenascin-C was an endogenous activator of TLR4, and induced expression of TNF α , IL-6 and IL-8 cytokines in macrophages, monocytes and fibroblasts^{27, 43, 257, 273}. This was effect as mediated by TLR4 binding to 3 distinct regions in the FBG domain⁴².

This study found that recombinant FnIII domains BCD and BAD2AD1CD did induce cytokine expression that appeared to be TLR4 dependent. However, bacterially expressed recombinant proteins corresponding to the constitutive FnIII domains of tenascin-C caused no induction in cytokine expression; as previously described in Midwood *et al.*,⁴³.

The controls for the cytokine expression assays gave unexpected results; working best for the TNF α and IL-6 assays, and worst for the IL-8 assay. PMB pre-incubation of FBG-C reduced TNF α and IL-6 release, perhaps indicating the presence of LPS in the control samples. However, cytokine expression stimulated by FBG-C was always reduced by PK pre-incubation, which contrastingly suggested that the induction effect by FBG-C was dependent its intact protein structure; and not due to LPS contamination. These findings were consistent with observations made by the Midwood lab, which previously described a reduction in TNF α release following treatment with PMB⁴³, and suggests the presence of a mechanism by which PMB treatment downregulates TNF α release.

TNF α release was not induced by the FnIII 5-7 negative control as previously described⁴³, or by AD2AD1; but was induced by BCD and BAD2AD1. The additional controls suggested this induction was dependent on the intact protein structure of BCD and BAD2AD1CD, and was TLR4 dependent; which implicated that the FnIII-B, C or D domains were critical in

inducing TNF α , but not FnIII AD2 or AD1, or the region spanning domains FnIII B-C as interruption of these domains by AD2AD1 did not abolish signalling. The PMB controls indicated that some of this activation could be due to contaminating LPS.

IL-6 release was induced by BCD, but not FnIII 5-7, AD2AD1 or BAD2AD1CD, suggesting that in contrast to the induction of TNF α , FnIII-B, C and D exhibited no activity individually, but that the region spanning FnIII-B and C may be required for IL-6 induction; in a manner that appeared to be TLR4 dependent. The structural confirmation of the FnIII domains is very important for determining their activity. Previous studies found that for FnIII-1c of fibronectin, mechanical stretch or MMP-2 cleavage exposed a C-terminal region of the domain that then induced cytokine expression via TLR2 or TLR4 activation in fibroblasts^{387,388,387, 389}. This resulted in NF-kB and p38 MAPK dependent IL-8 and TNF α release^{389, 427, 428}. Therefore, perhaps the removal of FnIII AD2AD1 confers a conformational change that permits the BCD domains to induce IL-6 release.

The positive and negative controls gave the least expected results from the IL-8 cytokine expression assay. Here, IL-8 release was induced by the negative control FnIII 5-7, but was dependent on the integrity of the protein structure, and not the result of contaminating LPS. This finding starkly contrasted with previous findings by the Midwood group⁴³; and indicates a failure of the control experiment. Such a non-specific induction may have resulted from denaturation of the FnIII 5-7 protein structure between purification and its use in cell assays; if this was the case, denaturation of the FnIII may have exposed cryptic binding sites that alter the binding activity of the protein, such as observed in denatured FnIII-1c³⁸⁹.

IL-8 release was also induced by AD2AD1 and BAD2AD1CD, but the controls indicated that this induction was not completely TLR4 dependent, or influenced by the integrity of the protein structure. Strangely, both of the recombinant proteins containing the AD2AD1

domains induced IL-8 release, however induction is unlikely to be promoted by denatured FnIII AD2AD1 domains; therefore this observation may be consistent with LPS contamination of the AD2AD1 and BAD2AD1CD samples.

Other studies have previously shown that alternatively spliced FnIII domains of fibronectin can induce TLR4 signalling. Previously, expression of cellular fibronectin containing the spliced EDA domain was shown to induce IL-1 α / β and MMP-1 expression in endothelial cells⁴²⁹; and also induced TLR4 dependent CXCL-3, IL-8 and TNF α cytokine release from HDFs⁴¹¹, T-cells⁴³⁰ and Mast cells⁴³¹; however, these effects were synergistic with the adjacent FnIII domains, and the constitutively expressed FnIII-1c⁴¹¹. Therefore, perhaps in this experimental model where only the alternatively spliced FnIII domains are added to stimulate macrophages, the spliced FnIII proteins may not be in their correct physiological conformation required to stimulate TLR4 signalling; due to the absence of the constitutive FnIII domains that are usually present alongside them.

Further investigation will be required to conclusively state that cytokine secretion induced by alternatively spliced FnIII domains was TLR4 dependent. Although for the TNF α and IL-6 assays, induction by BCD and BAD2AD1CD closely resembled what would be expected for TLR4 dependent cytokine release.

The exogenous addition of the recombinant proteins to the M-CSF macrophages was deemed a more physiologically relevant choice for stimulating the cells compared to coating plastic plates with the proteins, because it was previously shown in this study that tenascin-C expressed by myeloid cells was predominantly secreted to the supernatant. This suggests that myeloid expressed tenascin-C to induce paracrine signalling, but its presence in the milieu may also support autocrine signalling. Before commencing the assay, it was unknown if the small size of the purified recombinant proteins would make them biologically inactive. FnIII

domains are highly elastic, and may be present in a different steric conformation when present in the full tenascin-C molecule. A previous study found that tenascin-C FnIII proteins expressed as single domains were inactive, and therefore expressed doublets and triplets to assess cell functions²⁹⁹. Here it was speculated that the highly elastic FnIII domains were only functionally active when the domains were stretched into a conformation that more closely resembled that seen in full length tenascin-C.

FnIII domains have also been shown to induce TLR-independent cytokine expression. FnIII-3 was previously shown to induce IL-6 and IL-23 cytokine expression in murine dendritic cells via $\alpha 9$ integrin in a manner that was synergistic with TLR2 and TLR4. Interestingly, in the Midwood lab, treatment of human myeloid cells with FnIII 3-5, did not result in the expression of TNF α , IL-6 or IL-8⁴³, suggesting that $\alpha 9$ dependent signalling by FnIII-3 is not sufficient to induce cytokine expression in human macrophages. This difference in induction may result from the differences in species, or the protocols for differentiation that were used to generate the human and mouse cells for these studies.

As the endogenous activation of TLR4 by tenascin-C is mediated by the FBG domain⁴³, it could be possible for tenascin-C to induce integrin signalling simultaneously with TLR4 signalling; via interactions with integrin binding sites present in the constitutive and alternatively spliced FnIII domains. In addition to the constitutive FnIII integrin binding domains, FnIII-D contains an $\alpha 7\beta 1$ binding site¹³²; however in this study, cytokine expression induced by FnIII-D containing proteins was reduced by TAK242 treatment, suggesting that TLR4 and not integrins are facilitating cytokine expression.

5.8.3 *Spliced FnIII domains regulate cell adhesion in HDFs but not MDDCs*

In this study, HDFs exhibited greater overall adhesion to fibronectin than to tenascin-C; this is strongly supported by a wealth of previous reports showing that cell adhesion to the constitutive domains of fibronectin is syndecan-4^{432, 433, 434}, $\alpha 5\beta$ ⁴¹⁷ and $\alpha 4\beta 1$ ⁴¹⁸ integrin dependent. During fibroblast adhesion to fibronectin, syndecan-4 binds to the FnIII-13 (HepII) region of fibronectin, and promotes RhoA and FAK signalling, which promotes focal adhesion formation, cytoskeletal organisation, stress fibre assembly, and cell spreading^{415, 432, 433, 435}.

Apart from the ability of tenascin-C to interrupt cell adhesion to fibronectin, the constitutive regions of tenascin-C contain RGD sequences that permit $\alpha 8\beta 1$ integrin binding in FnIII-3⁴¹², and $\alpha \nu\beta 3$ integrin to bind in the FnIII-3 and FBG regions^{278, 279, 413}. The FnIII-7,8 domains were previously shown to promote embryonic skin fibroblast adhesion as efficiently as full length tenascin-C⁷⁶. Furthermore, it was previously shown that endothelial cells plated on tenascin-C substrate, upregulated the expression of fibronectin isoforms negative for EDA and EDB; and then incorporated this fibronectin into $\alpha 5\beta 1$ integrin adhesion beneath the endothelial cells to support greater adhesion⁴³⁶. Additionally, cell adhesion to tenascin-C was shown to block actin stress-fibre dependent expression of DKK1, and activate Wnt signalling in tumour cells; which promoted tumour cell survival, angiogenesis and micro-metastasis⁵¹.

The HDFs plated on tenascin-C appeared more rounded than those on fibronectin; which fits with previous descriptions describing focal adhesion formation on fibronectin substrates^{147, 437}; and less attachment to tenascin-C⁸². Furthermore, coating with FnIII 3-5 also greatly increased HDF adhesion relative to tenascin-C; and resulted in vinculin and β -actin co-localisation in these fibroblasts; suggesting integrin involvement in the adhesion. This is

likely explained by the presence of an RGD sequence in FnIII-3, which binds to $\beta 1$ integrins and supports focal adhesion formation^{149, 412, 416}.

FnIII 5-7 adhesion was not increased relative to tenascin-C indicating the absence of pro-adhesive sites in these domains. Cell adhesion to only the FnIII 5-7 domains has not previously been investigated; but a previous study did identify an $\alpha 8\beta 1$ integrin binding site within the overlapping FnIII domains 6,7,8 which promoted the adherence of myeloid cells in monolayer cultures⁴³⁸; but here did not promote adhesion in HDFs; perhaps due to differences in receptor expression due to cell lineage, or because the domain FnIII-8 is critical in promoting $\alpha 8\beta 1$ integrin binding.

The adhesion of HDFs to alternatively spliced tenascin-C FnIII domains was assessed. On AD2AD1 and BCD, adhesion was slightly elevated compared to tenascin-C, but adhesion was significantly higher on BAD2AD1CD. The ability of BAD2AD1CD, but not AD2AD1 or BCD to facilitate adhesion, indicated that the domains responsible are either not in the correct structural conformation in the smaller AD2AD1 or BCD proteins, or that there is a pro-adhesive region that spans the B-AD2 or AD1-C boundaries. The mechanism by which BAD2AD1CD promotes adhesion is not clear. Therefore it appears that the omission of FnIII-AD2 and AD1 from the tenascin-C transcript may reduce fibroblast adhesion. No functional links have previously been made between FnIII AD2, AD1 and cell adhesion. However, the expression of FnIII-AD2 and AD1 containing tenascin-C isoforms has been previously associated with the tumour migration front, and is elevated in aggressive breast cancers; where its overexpression alongside FnIII-B,AD2,D promoted breast cancer invasive properties¹⁸⁹. Therefore perhaps in this context, inclusion of FnIII-AD2 promoted tumour cell interaction with the ECM, which could have facilitated tumour cell invasion. Furthermore, domains B and D are known to modify neurite outgrowth behaviours via contactin/F3³⁴ and $\alpha 7\beta 1$ integrin interactions³⁴. However, little is known about the role of adjacent FnIII

domains on mediating these behaviours, and whether their functions are interrupted or modified by the presence of adjacent FnIII domains.

This study showed that actin and vinculin did not co-localise in fibroblasts coated on BAD2AD1CD, suggesting that focal adhesions had not formed; but actin clusters were observed within the centre of the cell and were surrounded by a ring of vinculin, indicating that cytoskeletal reorganisation may be in progress⁴³⁹; and therefore the adhesion of fibroblasts to BAD2AD1CD domains, may entail integrin involvement.

The structural organisation of FnIII domains and the presence of adjacent domains may play a role in determining whether BAD2AD1CD is adhesive, and yet AD2AD1 and BCD are not. For example, the adhesive activity of FN-EDA is enhanced by the presence of the flanking domains FnIII-11 and 12, which do not promote cell adhesion individually. Furthermore, the presence of low levels of fibronectin EDA complemented the activity of plasma fibronectin and synergistically promoted the formation of stress fibres and focal contacts^{440, 441}.

The adhesion of immune cells to alternatively spliced tenascin-C FnIII domains had not previously been investigated. This study showed that MDDCs, M-CSF and GM-CSF macrophages adhered to fibronectin, and MDDCs may have formed focal adhesions on this substrate. MDDC adhesion to fibronectin substrates was found in another study, to be integrin dependent⁴⁴², and myeloid cells are known to form focal adhesions on fibronectin substrates, where podosomes display a ring of vinculin surrounding distinct clusters of actin^{421, 442}. Furthermore, the literature describes a negative association between myeloid cell adhesion to fibronectin, and cell survival; as fibronectin induced signalling by VLA5 induced apoptosis in myeloid cells⁴⁴³, and by $\alpha 4\beta 1/\alpha 5\beta 1$ in erythrocytes⁴⁴⁴.

Before this study, very little was known about myeloid cell adhesion to tenascin-C. Here tenascin-C promoted adhesion of MDDCs, M-CSF and GM-CSF macrophages; but FnIII 3-5

did not, suggesting that the mechanisms for promoting adhesion to tenascin-C are shared across these cell types, but is not entirely dependent on the FnIII-3 RGD sequence and interactions with $\alpha 8\beta 1$ or $\alpha v\beta 3$ integrins^{412, 278, 279, 413}. It is not known if tenascin-C promoted greater adhesion due to the presence of a constitutively expressed epitope, or due to the conformation of alternatively spliced FnIII domains when included in the full-length protein.

MDDC cell adhesion was not supported on FnIII 5-7, AD2AD1, BCD or BAD2AD1CD substrates, highlighting the presence of different cell adhesion mechanisms in myeloid cells and fibroblasts. The lack of adhesion of MDDCs, GM-CSF and M-CSF macrophages to alternatively spliced FnIII domains, suggests that despite the activated myeloid cells switching to expression of tenascin-C isoforms richer in FnIII B-C-D due to the downregulation of AD2 and AD1, this has little impact on MDDC adhesion.

While tenascin-C is frequently thought of as an anti-adhesive protein, another study has shown that mAbs to the FnIII 7-8 region increased the number of non-adherent myeloid cells in culture; with adherent myeloid monocultures predominantly expressing large 280kDa isoforms containing multiple alternatively spliced FnIII domains, while non-attached myeloid cells predominantly express a smaller 260kDa isoform⁴³⁸, indicating that larger tenascin-C variants support myeloid cell adhesion. Furthermore, the presence of the YTITIRGV and YTIYLNKD sequences in FnIII-A2 and FBG domains respectively, stimulated myeloid tumour cell adhesion to fibronectin, by activating $\beta 1$ integrin^{445, 446}. Therefore the role of tenascin-C and alternatively spliced FnIII domains in myeloid cell adhesion is currently not well understood; but may be supported by integrin dependent signalling, and this should be investigated in future.

Immunofluorescence microscopy revealed that MDDCs plated on tenascin-C, were larger and more spread than when plated on fibronectin. On tenascin-C substrate, vinculin staining was

concentrated in the centre of the cell and clustered in a ring around the edges, where it co-localised with actin. This is not consistent with the formation of focal adhesions, and more closely resembles cells that are spreading; therefore perhaps tenascin-C was promoting less permanent cell-substrate interactions that permit the MDDCs to loosely adhere, and roam over the surface of the plate. Additionally, this spreading phenotype may have been promoted by the activation of the TLR4, due to the presence of the FBG domain of tenascin-C.

Altogether, alternative splicing of tenascin-C appeared to have no significant impact on MDDC adhesion, and the adhesive effects of tenascin-C may instead be supported by constitutively expressed regions.

5.8.4 MDDC migration is promoted following cell activation by FnIII BCD, and inhibited in resting cells by FnIII BAD2AD1CD

The impact of tenascin-C and its alternatively spliced isoforms on cell migration have been extensively studied in stromal cells such as fibroblasts and breast tumour cells, but never before in MDDCs. MDDCs interpret external cues which permit them to migrate; this migration is dependent on actin polymerisation and myosin contraction for locomotion⁴⁴⁷, and takes place on both adhesive and non-adhesive substrates⁴⁴⁸. Traction force studies on dendritic cells classified the cells as amoeboid-type, as a result of their high level of motility and rapid changes in morphology, which exert relatively small but constant forces during migration⁴²¹. This study showed that fibronectin supported high levels of MDDC migration. It was previously shown that when migrating on fibronectin, DCs exert short-lived stresses on the leading edge filopodia and rapidly turnover traction forces in a way that is different from fibroblasts⁴²¹; which form focal adhesions at the leading edge, which eventually disassemble at the trailing edge⁴⁴⁹.

In contrast to fibronectin, tenascin-C was a poor substrate for facilitating MDDC migration. MDDC migration on tenascin-C had not previously been evaluated, but in stromal cells, the literature almost unanimously describes tenascin-C as a pro-migratory protein; enhancing neural crest cell migration^{450, 451}, retinal endothelial cell migration⁴⁵² and tumour cell migration^{51, 187}. In some cases the mechanism by which tenascin-C promoted migration was found; for example, tenascin-C promoted melanoma cell migration via activation of EGF receptor with the EGF-L domains⁴⁵³, and promoted pancreatic adenocarcinoma invasion by stimulating annexin-II dependent hedgehog signalling⁴⁵⁴.

The FnIII 3-5 and FnIII 5-7 constitutive domains did not promote MDDC migration; and therefore suggests that in MDDCs, $\alpha\text{v}\beta\text{3}$ integrin does not promote migration as was previously postulated for myofibroblasts by the constitutive FnIII domains, and the FBG domain⁴⁵⁵. Here, the AD2AD1 protein did not promote MDDC migration but previously FnIII AD1 expression was reported at sites of high cell motility, and was shown to correlate with invasive capabilities of QT6 tumour cells when migrating through a fibronectin coated filter^{94, 188, 205}. BAD2AD1CD also did not support high levels of specific migration to the FBS stimulus, but did facilitate high levels of non-specific MDDC migration; perhaps by preventing the cells from strongly adhering to the substrate surface.

The BCD protein permitted high levels of specific MDDC migration that were interrupted by the inclusion of the AD2AD1 domains. Currently, FnIII domains B, C and D have no defined functions in MDDCs. However, FnIII B and D have been described to regulate neurite outgrowth in an $\alpha\text{7}\beta\text{1}$ dependent manner¹³², while pathological expression of alternatively spliced FnIII containing tenascin-C in tumours is also known to impact cell migration, as antibody perturbation of FnIII B in breast cancer attenuated the migration and proliferation of intra-ductal breast cancer¹⁸⁷.

FnIII B, C and D are more likely to be expressed contiguously in activated MDDCs, GM-CSF and M-CSF macrophages, as the AD2 and AD1 domains are down-regulated. This study therefore suggests a new mechanism by which activated myeloid cells may promote cell migration in an autocrine manner, by transiently secreting tenascin-C rich in FnIII BCD into the surrounding milieu, to promote MDDC migration. This contrasts with the tenascin-C isoforms expressed by resting cells, where very low levels of tenascin-C that contain a longer isoform are less permissible of migration.

Here it is shown that interruption of the contiguous BCD domains with AD2AD1 significantly reduces MDDC migration; and that activated MDDCs may promote their own migration during inflammation by omitting the FnIII-AD2,AD1 domains from the tenascin-C they express.

5.8.5 *Summary*

This chapter has detailed methods for the cloning, expression and purification of functionally active alternatively spliced FnIII domains. This study has inferred that in macrophages, alternatively spliced FnIII proteins can induce the release of TNF α , IL-6 and IL-8 in a manner that may be dependent on TLR4, but currently the mechanism for this cytokine release is unknown.

I found that tenascin-C FnIII-B, C and D domains which are overexpressed in HDFs and RA-SFs relative to NSFs and OA-SFs do not promote cell adhesion when expressed contiguously, but when the domains are interrupted with FnIII-AD2 and AD1, as is seen in NSFs and OA-SFs, cell adhesion is increased; suggesting that in RA, synovial fibroblasts are less adherent, and express tenascin-C isoforms that better support myeloid cell migration. In contrast, the tenascin-C isoforms expressed in normal and OA synovial fibroblasts are more adhesive to fibroblasts; and support less myeloid cell migration.

I also found that tenascin-C alternative splicing in activated MDDCs, GM-CSF and M-CSF macrophages does not significantly impact myeloid cell adhesion, but that alternatively spliced FnIII domains may support cytokine expression during inflammation. Additionally, switching from expressing a long tenascin-C isoform at rest, to a tenascin-C isoform lacking FnIII AD2 and AD1, where FnIII B, C and D are contiguous, may promote MDDC cell migration in response to cell activation; and in a paracrine manner, support less fibroblast adhesion.

Chapter 6 Discussion and Conclusions

6.1 Tenascin-C alternative splicing during inflammation

This thesis has characterised and discussed the patterns of tenascin-C expression and alternative splicing in cells activated with inflammatory stimuli, or in cells derived from normal and diseased tissues. This study then assessed the functional consequences of these inflammation associated alternatively spliced isoforms. Here I discuss the potential consequences of alternative splicing for individual cell types, and then speculate on the role for tenascin-C splicing in RA.

The functional relevance of the alternatively spliced FnIII domains of tenascin-C should really be discussed in context with the rest of the constitutive portions of the tenascin-C protein; within which the spliced domains are inserted. To date, many studies have identified immunomodulatory functions of tenascin-C in vitro and in vivo, with few ever investigating the role of alternatively spliced FnIII domains; which until now were not understood to be differentially regulated during inflammation.

In this study, the tenascin-C expression and alternative splicing in response to inflammation was separated into distinct cell-type response categories which are broadly discussed below:-

6.2 The role of tenascin-C expression and alternative splicing on fibroblasts during inflammation

This study has shown that the functional consequences of cells expressing tenascin-C are not only mediated by the total levels of expression during inflammation, but also the manner by which tenascin-C is alternatively spliced; offering another mechanism by which the functional effects of tenascin-C can be fine-tuned during inflammation.

It is known that during tissue repair following injury or infection, tenascin-C expression is transiently increased at the site of inflammation, and is downregulated when inflammation and tissue repair has been resolved⁴³. The data in this thesis supported these observations, and identified transient expression of tenascin-C in response to IL-1 β stimulation, as was previously reported in human synovial³¹⁹, and conjunctival fibroblasts⁴⁵⁶.

This study revealed that HDFs overexpressed FnIII-B and D domains compared to the other FnIII domains. FnIII-B and D have previously been implicated in processes associated with cell plasticity and integrin mediated adhesion and cell migration. The alternative splicing pattern of tenascin-C was not affected by stimulation with IL-1 β ; however the total levels of expression increased. This suggests that different mechanisms regulate tenascin-C expression and alternative splicing in HDFs, with more work required to elucidate these mechanisms. However, the types of tenascin-C expressed by HDFs do change in a context specific manner, for example, during stages of tissue repair and enhanced fibrosis, the elevated levels of TGF β were previously shown to promote expression of small tenascin-C variants lacking the FnIII A1-D domains³²⁴; which are commonly associated with dense tissues and regions of cell condensation. This modification of the variants expressed by fibroblasts in response to different inflammatory cues, suggests that tenascin-C isoforms are expressed in a context specific manner.

This study found that tenascin-C FnIII-B, C and D domains which are overexpressed in HDFs, do not promote HDF cell adhesion when expressed contiguously, but when the domains are interrupted with FnIII-AD2 and AD1, the cell adhesion was increased. The mechanisms by which HDFs adhered to these substrates was not explored, however, both constitutive and alternatively spliced FnIII domains have been reported in other studies to modify fibroblast adhesion via integrin interactions¹³². This study did not provide evidence that during acute inflammation, fibroblast expressed tenascin-C isoforms modify cell adhesion; but it did reveal that the abundance of tenascin-C was increased.

Modification of cell adhesive properties by alternative splicing is a mechanism that is shared amongst other ECM proteins; in fibronectin, the alternatively spliced EDA domain was previously shown to enhance the adhesion of various cell types compared to fibronectin without EDA^{440, 441}.

In this study, elevated tenascin-C expression was detected following inflammatory stimulation; and it has long been known that tenascin-C regulates cell adhesion. Incorporation of tenascin-C into fibronectin matrices typically reduced fibroblast adherence to the matrix. For example, cell adhesion could be supported on fibronectin, but not on tenascin-C or tenascin-C/fibronectin mixed substrates⁸². Therefore both the regulation of total tenascin-C expression, in HDFs may serve as a mechanism by which HDFs can modify their adhesion to the ECM.

This study found that the contiguously expressed alternatively spliced FnIII domains B, C and D expressed by HDFs, made fibroblasts less adherent and supported greater dendritic cell migration. No previous studies have examined the effects of alternatively spliced FnIII domains on myeloid cell adhesion, however it was previously shown that overexpression of FnIII-D/ B,D/ B,AD1,D in human breast cancer cell lines, or FnIII B and B,D in fibroblasts

increased proliferative and invasive qualities of breast cancer cells and induced 2 and 4-fold increases in MMP-13 and TIMP-3 expression respectively¹⁸⁷; revealing that in other cell types, spliced FnIII domains do modify cell migration.

This study showed that activated HDFs express more tenascin-C, and therefore tenascin-C expression by HDFs during inflammation may serve to exacerbate the already existing inflammatory response; previous studies have found that tenascin-C promoted TLR4 dependent upregulation of IL-6 and MMP-3 in cardiac myofibroblasts²⁵⁷, and triggered the expression of TNF α , IL-6 and IL-8 in synovial fibroblasts; via signalling with the constitutively expressed FBG domain⁴³. Additionally, tenascin-C also stimulated cytokine synthesis and MMP expression in synovial fibroblasts via activation of α 9 integrins via the constitutive FnIII-3 domain²⁷⁷. MMPs are key regulators of tumour metastasis⁴⁵⁷, and tenascin-C upregulates expression of the collagenase MMP-9²⁷⁰, which may contribute to degradation of the basement membrane by breaking down gelatine and type IV collagen^{458, 459}. Tenascin-C expression also plays a role during tissue repair; TGF β is a potent inducer of tenascin-C expression in fibroblasts^{92, 403}, and has also been shown to bind tenascin-C in tissues where the elevated local concentrations of TGF β may drive tissue fibrosis during the resolution of inflammation⁴⁶⁰. Hence, elevated tenascin-C expression by HDFs during inflammation may further exacerbate the pre-existing inflammatory response and modify tissue repair and fibrotic mechanisms.

See the Figure 6.1 below, for a summary of the roles of tenascin-C expression and alternative splicing in dermal fibroblasts during inflammation.

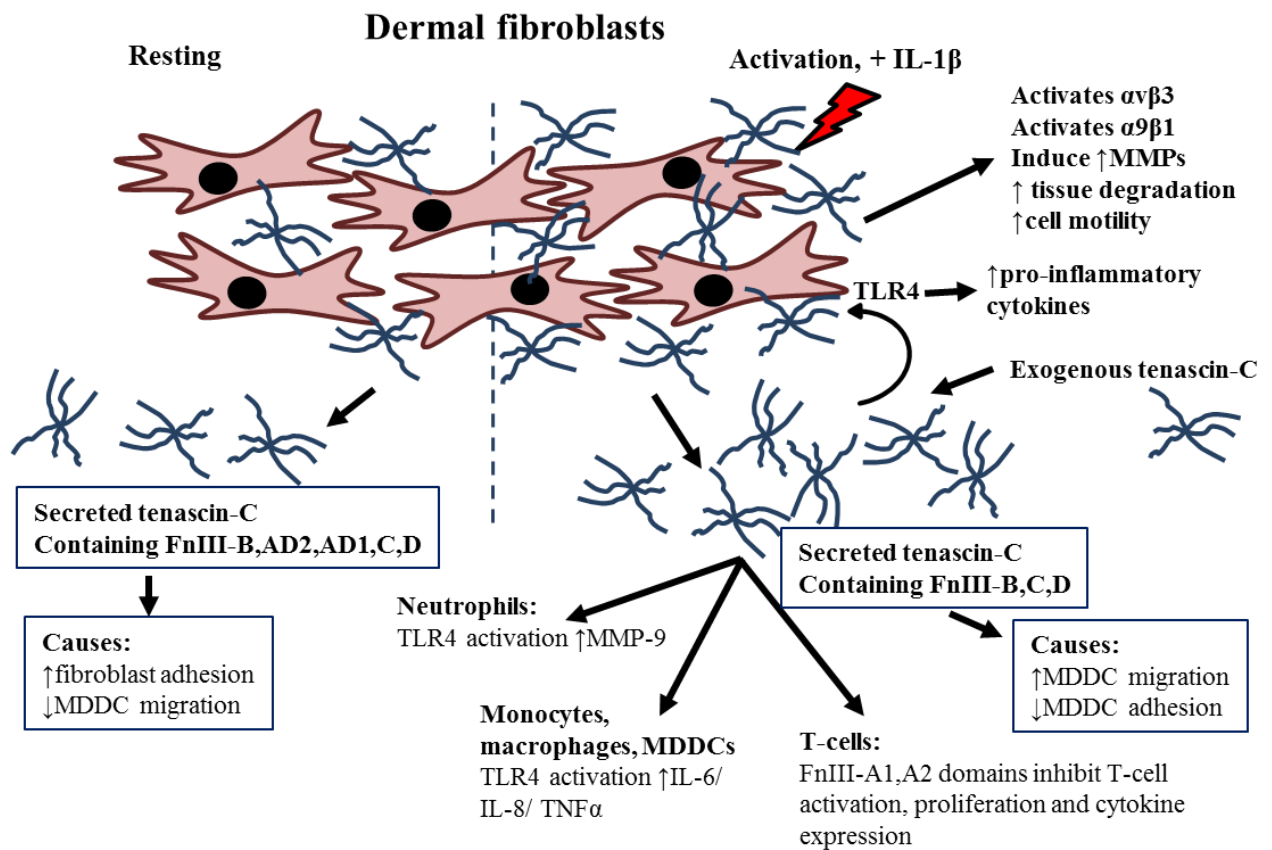


Figure 6.1. Summary of the roles of tenascin-C expression and alternative splicing on dermal fibroblast function during inflammation. Findings arising from this study are separated by boxes.

6.3 Tenascin-C splicing and innate immunity

This study found that resting macrophages and MDDCs expressed low levels of large tenascin-C isoforms containing FnIII A1-D; but switched to expressing smaller tenascin-C variants lacking FnIII AD2 and AD1, following TLR4 dependent LPS stimulation. This resulted in expression of inflammation specific transcripts where domains FnIII B,C,D were expressed contiguously. This suggested that innate immune cells regulate the effects of tenascin-C by changing the total levels of expression, and by modifying its alternative splicing pattern.

The soluble tenascin-C secreted by activated macrophages and dendritic cells may regulate intracellular inflammatory signalling pathways. In human glioblastoma cells, tenascin-C downregulated Wnt inhibitor DKK-1, and increased Wnt signalling by stabilising β -catenin²⁸². Furthermore, tenascin-C interacts with Wnt3a, and human adenocarcinoma cells plated in the presence of Wnt3a demonstrated enhanced Wnt signalling. However, when the cells were incubated with soluble tenascin-C, Wnt signalling was reduced²⁸³.

Therefore perhaps cell associated tenascin-C sequesters Wnt ligands and retains them close to the cell membrane to enable the activation of signalling; while perhaps soluble tenascin-C diffuses from the point of expression, and binds free Wnt ligands to reduce Wnt signalling. As Wnt and $\text{Nf-}\kappa\text{B}$ pathways exhibit significant cross-talk, Wnt signalling by tenascin-C may exert positive and negative modulation of inflammatory responses²⁸⁴.

Tenascin-C is a damage associated molecular pattern (DAMP); DAMPs are endogenous molecules that trigger the same pattern recognition receptor signalling triggered by pathogen associated molecular pattern (PAMP) signalling. The sensing of DAMPs like tenascin-C by pattern recognition receptors ensures that inflammation is stimulated in both sterile injury, and in response to infection. Tenascin-C's FBG domain activates TLR4, but the

constitutively expressed domains FnIII 1-8 did not⁴³. Furthermore, FBG domain mediated signalling of tenascin-C is an important driver of pattern recognition receptor signalling responses in dendritic cells. TLR4 dependent activation of bone marrow derived macrophages with LPS, results in rapid upregulation of tenascin-C mRNA which induces miR-155 activation; which when not matured in the absence of tenascin-C signalling prevents efficient translation of LPS-dependent pro-inflammatory cytokines like TNF α ²⁷⁵. In addition, tenascin-C signalling by TLR4 drives inflammasome priming, which results in elevated expression of specific cytokines including IL-1 β and IL-18, and induces pyroptosis which is a form of programmed cell death²⁵⁹. Inflammasomes require 2 stimuli to be instigated, firstly a primer promotes pattern recognition receptor dependent cytokine production, and this is then followed by inflammasome assembly, caspase-1 mediated maturation and subsequently the release of IL-1 β and IL-18²⁶⁰.

In this thesis I revealed findings that suggest the alternatively spliced FnIII domains also exhibit the ability to induce IL-6, IL-8 and TNF α cytokine release in primary human macrophages; in a manner that for FnIII-BCD and BAD2AD1CD appeared to be TLR4 dependent. Recombinant FnIII proteins corresponding to tenascin-C isoforms likely to be expressed at rest, and following LPS mediated activation, exhibited different cytokine release profiles. The resting tenascin-C variant (FnIII-B,AD2,AD1,C,D) induced TNF α release, while the inflammation associated variant promoted strong release of IL-6, IL-8 and TNF α ; to levels which were comparable to those induced by the FBG domain. In this manner, perhaps the inflammation specific FnIII domains act synergistically with the FBG domain to induce greater cytokine synthesis; to drive processes such as T-cell polarisation during inflammation. Dendritic cells isolated from tenascin-C deficient mice exhibited diminished IL-6 and IL-23 cytokine release following LPS stimulation, and a reduced capacity to drive Th17, but not Th1, Th2 or Treg cell polarisation²⁶¹. Th17 cells are the dominant cellular

component in many inflammatory autoimmune diseases, and are a key driver of pathology in RA²⁶². Furthermore, it was shown that stimulation of DCs with exogenous tenascin-C promoted their ability to drive Th17 cell generation; which was abrogated by IL-6 and TLR4 blocking antibodies revealing that IL-6 secretion triggered by TLR4 activation was an important factor in driving Th17 cell polarisation²⁵⁶. The large conformation of tenascin-C, and its multitude of ligand binding sites, may permit for tenascin-C to induce inflammatory cytokine expression synergistically via different pathways. Alternatively spliced FnIII domains have widely been shown to induce integrin signalling pathways; and could also promote the formation of pathogenic Th17 cells in arthritis, via induction of $\alpha 9$ dependent Th17 associated cytokines in dendritic cells and macrophages in synergy with TLR2/4 signalling²⁸⁰.

The data in this thesis found that the alternatively spliced FnIII domains tested did not regulate MDDC or macrophage adhesion, however the shorter variant expressed during inflammation (FnIII-B,C,D) did support greater MDDC migration; and may therefore promote greater penetration of MDDCs in inflamed tissues following PAMP/DAMP sensing. However, the mechanism by which this is mediated is not yet understood, and should be investigated further. The ability of differential tenascin-C splicing to modulate adhesion or migration processes has only been recognised in one previous study, where it was found that adherent myeloid cultures predominantly expressed large 280kDa tenascin-C isoforms, whereas non-attached cells expressed a smaller 260kDa isoform⁴³⁸; the 20kDa difference in mass described between the adhesive and non-adhesive isoforms may correspond to the ~20kDa mass of the combined FnIII AD2 and AD1 domains; but could also be a product of post translation modifications.

Total tenascin-C expression in monocytes, macrophages and dendritic cells has been associated with areas of immune infiltration during inflammation, and accumulates at areas of

macrophage accumulation and IL-1 β and TNF α synthesis⁴⁶¹, supporting the notion that tenascin-C regulates immune cell migration. Furthermore, the effects of tenascin-C on myeloid cells appear to be cell type specific; as the absence of tenascin-C reduced macrophage migration but not neutrophil migration in wounded mouse corneas³⁰². Other studies report an inhibitory effect of tenascin-C on monocyte chemotaxis *in vitro*²⁹¹, while *TNC* -/- mice exhibit increased migration of monocytes and macrophages in the stroma of mammary tumours⁴⁶². Tenascin-C stimulation induced MMP expression in macrophages²⁶⁹; which may assist in permitting cell infiltration into tissues. Tenascin-C is capable of supporting immune cell migration, and expression of inflammation specific alternatively spliced isoforms may regulate pro- and anti- migratory effects.

Therefore following LPS induced activation, MDDCs switch from expressing low levels of a pro-adhesive tenascin-C isoform, to transiently secreting high levels of tenascin-C that does not support adhesion, and promotes MDDC migration. Secretion of the activated MDDC tenascin-C isoforms into the surrounding ECM may support greater MDDC cell migration, and disrupt the adhesion of nearby fibroblasts to permit transmigration of the immune cells in response to the sensing of PAMPS and DAMPS.

See Figure 6.2 below, for a summary of the roles of tenascin-C expression and alternative splicing in MDDCs during inflammation.

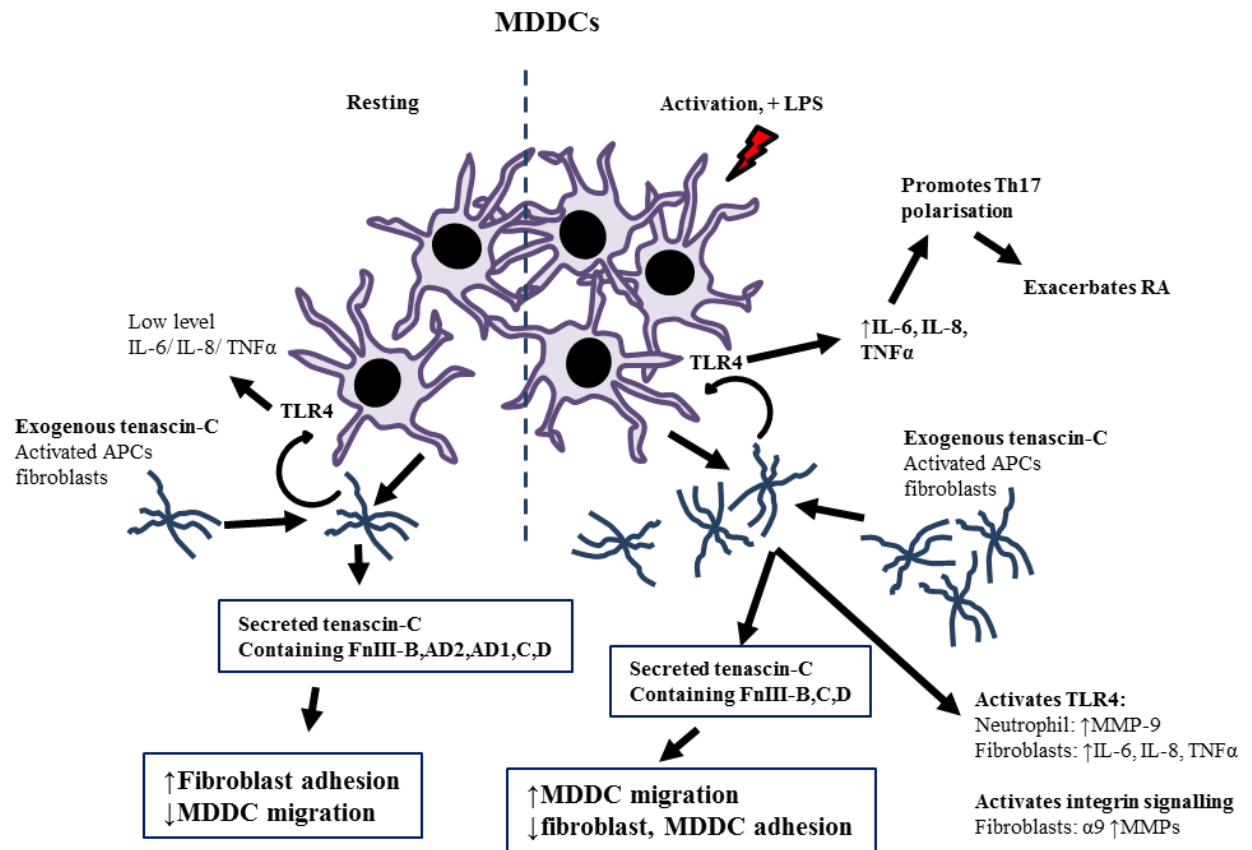


Figure 6.2. Summary of the roles of tenascin-C expression and alternative splicing on MDDC function during inflammation. Findings arising from this study are separated by boxes.

6.3 Tenascin-C splicing and adaptive immunity: T-cells

The data in this thesis revealed that resting CD4⁺ and CD8⁺ T-cells expressed negligible basal levels of tenascin-C, and showed transient induction of tenascin-C mRNA following activation; but exhibited no detectable levels of tenascin-C protein expression. This indicates that the tenascin-C protein detected at regions of high CD4⁺ T-cell density in the lymph nodes^{81, 292}, and activated dendritic cells²⁷³, is not directly expressed by T-cells, but rather by other cell types in the surrounding tissue. This is supported by the current understanding that tenascin-C functions as a driver of T-cell mediated adaptive immunity by its expression in activated APCs.

In this study, the FnIII A1 mRNA was never detected in resting or activated T-cells; which perhaps suggested a potent deleterious function of FnIII-A1 in modifying T-cell behaviours. A previous study showed that the recombinant FnIII-A1,A2 domains inhibited T-cell cytokine expression and proliferation in response to anti- CD3 and IL-2, or fibronectin mediated stimulation²⁹⁹; however, in this study the other FnIII domains exerted no observable modulatory effects on T-cell function. Therefore T- may selectively restrict the splicing of FnIII A1 domain due to its deleterious auto-inhibitory effects.

However, the splicing pattern of tenascin-C is unlikely to be sufficient enough to inhibit T-cell polarisation, as LPS activated MDDCs also express FnIII-A1 containing tenascin-C variants, and can polarise T-cells. Therefore the functional roles of FnIII-A1 containing tenascin-C variants on T-cell activity require further investigation.

The low expression of tenascin-C by T-cells during acute inflammation does not support an autocrine model of tenascin-C signalling, but may support the idea that tenascin-C expressed by local fibroblasts or antigen presenting cells impacts T-cell behaviour in a paracrine manner. Supporting this idea, this study showed fibroblasts, macrophages and MDDCs to

increase tenascin-C expression during inflammation, where it was predominantly secreted in a soluble form; but in fibroblasts was also incorporated into the local ECM. Exogenous tenascin-C has previously been shown to directly stimulate, or inhibit T-cell activation. Tenascin-C induced expression of IL-13, IFN γ , IL-5 and immunoglobulin E in mouse splenocytes when cultured on fibronectin⁵³, and induced Ca²⁺ influx in jurkat T-cells²⁹⁷. In vivo, tenascin-C -/- mice were protected from bronchial asthma due to a dampened Th2 response, and exhibited lower levels of Th2 CD4+ T-cell cytokine secretions, such as IL-13, IL-5 and IL-4 compared with wild type mice⁵³.

In contrast, tenascin-C inhibited T-cell activation by natural antigens²⁸⁸, anti-CD3 and fibronectin²⁸⁹, anti-CD28, ICAM-1 and laminin²⁹⁰. It also significantly inhibits mitogen-induced and anti-CD3 dependent proliferation, and IFN γ production in lung tumour lymphocytes¹⁵⁵. Altogether, tenascin-C has been shown to modulate Th2 in asthma⁵³, Th17 in RA³⁰³, and Th1 and Th17 in murine encephalomyelitis³⁰⁰ showing that the tenascin-C expressed in these pathologies induces context specific immunomodulatory effects on T-cells, which could be fine-tuned through the inclusion of different alternatively spliced FnIII domains.

It is possible that expression and alternative splicing of tenascin-C by fibroblasts and APCs during inflammation, impacts the surrounding environment to regulate lymphocyte migration. A Leu1677Ile single-nucleotide polymorphism in the FnIII-D domain of tenascin-C is associated with a high risk of asthma; partly via causing FnIII-D domain instability that affects its elasticity, and promotes airway stiffness²⁹⁸; suggesting that alternatively spliced FnIII domains facilitate normal immune cell migration by maintaining permissive tissue structures.

In inflamed tissues, differences in tenascin-C expression, ECM incorporation and proteolytic degradation induced by nearby fibroblasts and antigen presenting cells may modify T-cell migration during inflammation. Secreted tenascin-C accumulates at sites of leukocyte accumulation in patients with myocarditis⁴⁶³, and supported primary human blood and tonsillar lymphocyte adhesion and rolling²⁹³; linking tenascin-C accumulation with elevated T-cell motility.

Selective secretion or deposition of tenascin-C into the ECM may confer or block permissible migration routes during inflammation. Around the lymph nodes, fibroblasts express tenascin-C that concentrates around the reticular fibres, where it is proposed to form a pathway that supports lymphocyte migration in the lymph node²⁹⁵. Conversely, a jurkat and glioma experimental model revealed that fibronectin-rich ECM activates extracellular signal-regulated kinase activation to drive amoeboid-like T-cell migration; however inclusion of tenascin-C in the fibronectin matrix prevented extracellular signal-regulated kinase signalling on T-cells, so migration does not occur²⁹⁶. T-cell migration could also be impacted by the induction of tenascin-C degrading enzymes during the resolution of inflammation, for example MMP-19 expression in the bronchioles of mice cleave tenascin-C to reduce tissue levels, preventing tenascin-C accumulation in the bronchioles after allergen induced asthma⁴⁶⁴. In these ways, incorporation of tenascin-C expressed by fibroblasts or antigen presenting cells into the ECM may promote or prevent T-cell migration; and the manner by which tenascin-C is incorporated into the ECM may be modified in a context specific manner by alternatively spliced FnIII domains, though more work is required to understand how this is impacted by alternative splicing.

See Figure 6.3 below, for a summary of the roles of tenascin-C expression and alternative splicing in T-cells during inflammation.

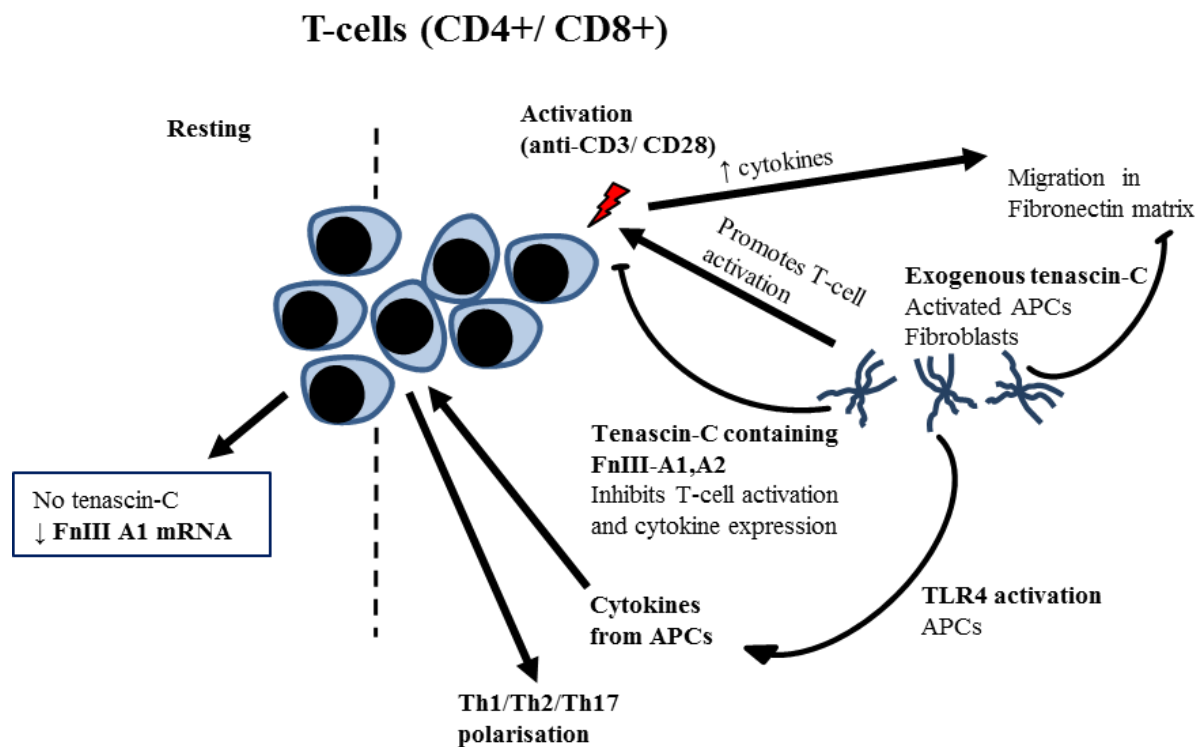


Figure 6.3. Summary of the roles of tenascin-C expression and alternative splicing on T-cell function during inflammation. Findings arising from this study are separated by boxes.

6.4 Tenascin-C and innate immunity: Neutrophils

Other studies have shown tenascin-C to be functionally relevant in neutrophil mediated inflammatory processes. However, this study showed that neutrophils did not express tenascin-C at rest or following acute inflammatory activation with LPS or GM-CSF; suggesting that like in T-cells, neutrophil functions are largely affected by tenascin-C isoforms not expressed by neutrophils, but perhaps instead expressed by local cells such as fibroblasts and APCs.

Little is known of the functional relevance of tenascin-C alternative splicing on neutrophils, as most previous studies investigated the role of total tenascin-C. It is known that in concanavalin-A induced murine hepatitis, tenascin-C knockout mice showed reduced neutrophil and lymphocyte infiltration into the liver, and downregulated the expression of IFN γ , TNF, IL-4 and TGF β ⁵²; indicating that here tenascin-C is vital for promoting neutrophil migration to sites of inflammation, and in inducing the expression of cytokines important in mediating the resolution of tenascin-C. However, the effect of full length tenascin-C on myeloid cells is cell type specific; as the absence of tenascin-C reduced macrophage migration but not neutrophil migration in wounded murine corneas³⁰².

Tenascin-C is known to modify neutrophil responses in vitro via TLR4 signalling. Here, bone marrow derived neutrophils upregulated MMP-9 expression in a TLR4 dependent manner²⁶⁵. Adhesion molecules are important in promoting leukocyte attachment to vascular epithelium, and MMPs are also important for facilitating leukocyte transmigration across vascular barriers; where elevated MMP-9 expression was shown to mediate leukocyte transmigration in livers following ischaemic reperfusion injury^{266, 267}. Previously tenascin-C has been shown to upregulate MMP-9 synthesis in fibroblasts²⁶⁸, macrophages²⁶⁹ and tumours²⁷⁰. The TLR4 dependency of MMP-9 expression by neutrophils was also confirmed by showing lower MMP-9 levels in TLR4 knockout mice after experimental stroke²⁷¹. IL-6 regulated MMP-9

activity in neutrophils²⁶⁶, and IL-6 levels are significantly reduced in tenascin-C knockout mouse livers following ischaemic reperfusion injury²⁶⁵.

All together, these data suggest that tenascin-C expressed by fibroblasts and myeloid cells during inflammation drives TLR4 dependent MMP-9 synthesis, which assists leukocyte infiltration during inflammation. See Figure 6.4 below, for a summary of the roles of tenascin-C expression and alternative splicing in T-cells during inflammation.

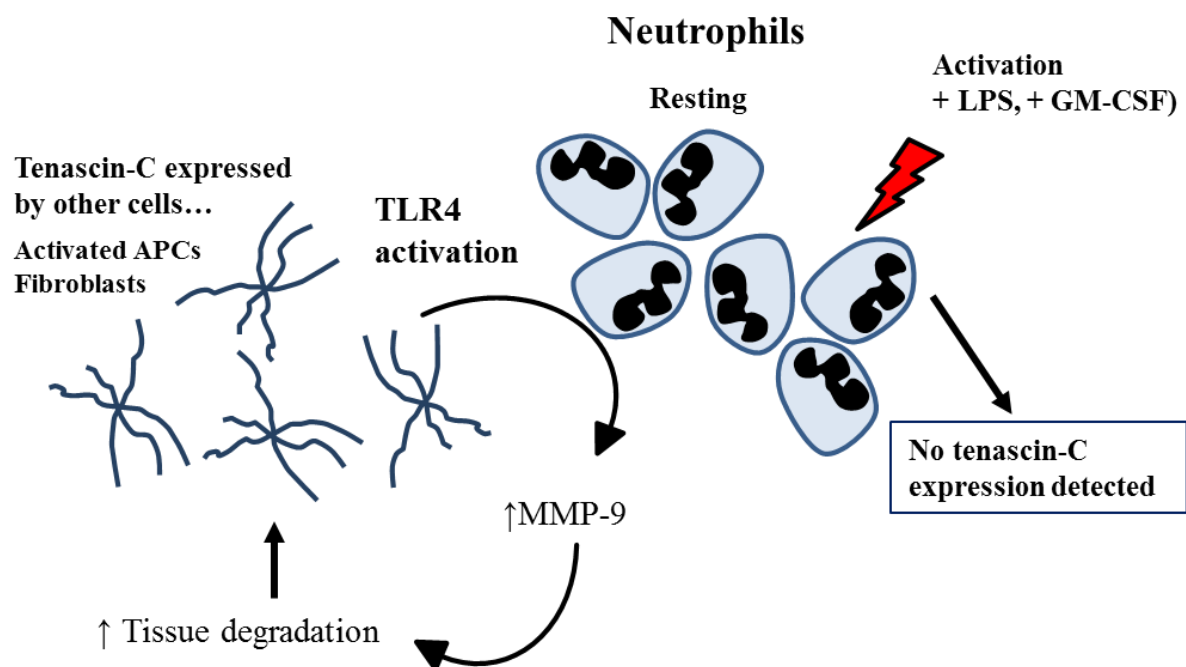


Figure 6.4. A summary of the roles of tenascin-C expression on Neutrophil function during inflammation. Findings arising from this study are separated by boxes.

6.5 Tenascin-C splicing in rheumatoid arthritis

Rheumatoid arthritis is a progressive, inflammatory autoimmune disease characterised symptomatically by painful swollen joints, as well as other systemic symptoms. RA is characterised by inflammation of the synovial membrane, and destruction of the joint cartilage and bone; in a process mediated by elevated expression of inflammatory cytokines and cartilage degrading enzymes such as MMPs.

This study identified elevated levels of tenascin-C expression in RA-SFs relative to NSFs, and further identified fundamental differences in the alternative splicing patterns between RA-SFs, and those isolated from normal and OA joints. RA-SFs expressed greater levels of shorter FnIII-B and D containing tenascin-C isoforms, whereas both NSFs and OA-SFs expressed similar tenascin-C isoforms that contained all of the spliced FnIII, except for A1 which was ever detected.

The elevated levels of tenascin-C expression in RA-SFs, was supported by previous observations where tenascin-C was detected in the synovial fluid of RA patients but here it did not correlate with synovial levels of CRP³⁰⁶, but did correlate with the severity of joint erosion³⁰⁷. Recently, a subset of autoantibodies against a citrullinated FBG epitope could be detected in patients, years before the onset of RA symptoms and may serve as an early indicator for clinical diagnosis³⁰⁸. In addition to elevated synovial levels in RA, circulating serum levels of tenascin-C were elevated in RA patients. The ELISA used to detect this protein recognised the FnIII-C domain, further confirming that tenascin-C variants containing alternatively spliced FnIII domains are secreted in a soluble form, and are detected distally from the joint³⁰⁷. Furthermore, tenascin-C levels in patient serum correlated with levels of C-reactive protein (CRP) in a number of diverse conditions including malaria, cancer and sepsis³⁰⁹; indicating that elevated serum tenascin-C levels are associated with pathological inflammation generally and are not disease specific.

The expression of alternatively spliced FnIII domains of fibronectin, have already been shown to be of significance in RA. For example, the spliced EDA domain is expressed in RA-SF -like cells, where EDA containing fibronectin was shown to be more adhesive to synovial cells than plasma fibronectin^{465, 466}; furthermore its presence in the synovial fluid also predicted joint destruction in RA⁴⁶⁷.

In this study, elevated tenascin-C expression and a distinctly different tenascin-C alternative splicing pattern was maintained in RA-SFs relative to NSFs beyond 6 passages of cell culture; long after removal of the cells from the diseased RA joint. This suggested that the elevated levels of tenascin-C expression in RA-SFs, were not the result of signalling induced by RA specific endogenous factors left over from the joint; but instead indicates that transcriptional regulation of tenascin-C expression and alternative splicing was fundamentally altered in the RA joint. The method by which this could be achieved is not yet known, though perhaps an unknown epigenetic regulation mechanism or miRNA regulation could alter the transcriptional activity to promote constitutively elevated expression of RA-specific tenascin-C isoforms.

Persistent tenascin-C expression correlates with excessive matrix deposition in a number of fibrotic diseases, such as chronic liver disease^{249, 250}, but also pulmonary fibrosis; a complication associated with RA²⁵¹. Tissue levels of tenascin serve as predictive markers of fibrosis^{468, 469}, and this is supported by findings showing that mice lacking tenascin-C are protected from excessive fibrosis in bleomycin-induced interstitial lung disease⁴⁷⁰; implicating tenascin-C as a key promoter of ECM deposition and fibrosis in vivo. Furthermore, anti-TNF α treatment is currently the most efficacious treatment for reducing the symptoms of joint inflammation and destruction in RA, but it does not reduce the rate of lung disease, and fibrotic nodules may get worse, despite anti-TNF α treatment. It is possible that during anti- TNF α treatment in RA, tenascin-C expression is still elevated and despite not

driving joint destruction due to TNF α signalling blockade, it may be possible for the molecule to exert other fibrotic effects; therefore new treatments are required that will address the underlying and prolonged expression of inflammatory mediators in RA.

The data in this thesis showed that inflamed RA-SFs expressed elevated levels of FnIII-B and D containing tenascin-C variants, and did not contain FnIII AD2 and AD1. Similarly to this, activated MDDCs expressed larger tenascin-C variants also lacking FnIII AD2 and AD1; meaning that during chronic inflammation and in acute inflammation, tenascin-C is alternatively spliced and is more likely to express the FnIII-B,C,D domains contiguously. This study showed that FnIII-B,C,D did not support fibroblast or MDDC cell adhesion, but that it did promote MDDC migration; which may facilitate antigen presenting cell migration into the inflamed joint, and reduce the adhesion and condensation of fibroblasts; both of which may contribute to the immune cell recruitment and degradation of the tissue structure observed in RA. In contrast with RA-SFs, the NSFs and OA-SFs, express lower levels of larger tenascin-C variants containing the FnIII-AD2 and AD1 located between domain FnIII-B and C; this supported greater fibroblast adhesion, and did not promote antigen presenting cell migration.

Previous in vitro studies have shown tenascin-C to both positively and negatively modulate T-cell maturation and activation; and therefore tenascin-C's role in RA must be highly context specific. In RA, elevated tenascin-C levels drive chronic inflammation by triggering Th17 generation via TLR4 dependent cytokine expression⁴³, and also by supporting $\alpha 9\beta 1$ ²⁸⁰ integrin signalling, resulting in the expression of Th17 associated cytokines, and MMPs; that contribute to joint degradation.

Tenascin-C may drive RA pathology by two key pathways. Firstly, total tenascin-C expression drives FBG dependent inflammation in RA by triggering TLR4⁴³. Inflammatory

effects of tenascin-C have been reported for tenascin-C in joint inflammation; for example, in zymosan induced synovial inflammation tenascin-C deficient mice were protected from prolonged synovitis and erosive joint disease to a greater extent than wild type mice⁴³. During the erosive arthritis, tenascin-C deficiency caused a reduction in levels of IL-6 and IL-23 expression, which prevented Th17 polarisation, resulting in lower IL-17 expression. This reduced IL-17 expression has also been observed in experimental murine encephalomyelitis; and was accompanied by a decrease in IFN γ producing Th1 cells, implicating tenascin-C in the polarisation of Th17 and Th1 cells in this model of inflammation³⁰⁰. One of the main mechanisms by which tenascin-C drives Th17 cell polarization, is by promoting the induction of IL-6 and IL-23 cytokine expression by antigen presenting cells via the TLR4 pathway. In this study I revealed evidence that the recombinant FnIII AD2,AD1, B,C,D and B,AD2,AD1,C,D proteins each induced TLR4 dependent cytokine expression in primary human M-CSF macrophages. The expression of the tenascin-C isoforms containing FnIII-B,C,D by RA-SFs, and activated antigen presenting cells, promoted high levels of IL-6 release, which may further support Th17 polarisation.

Secondly, tenascin-C alternative splicing may also contribute to RA pathology. Inclusion of alternatively spliced FnIII into the tenascin-C isoforms may also support integrin mediated signalling that acts synergistically with TLR4 signalling to promote T-cell polarisation. In murine synovial fibroblasts and macrophages, tenascin-C induced $\alpha 9$ integrin-mediated signalling²⁷⁷, that is vital for the formation of pathogenic Th17 cells in arthritis, where $\alpha 9$ signalling induced expression of Th17 associated cytokines by dendritic cells and macrophages acts in synergy with TLR2 and TLR4 signalling²⁸⁰. Furthermore, tenascin-C activated $\alpha \nu \beta 3$ in peritoneal macrophages to induce expression of IL-6, IL-1 β and TNF α ; in an NF- κ B dependent manner²⁸¹.

Due to these findings, I propose a model where in RA, expression of alternatively spliced FnIII domains by RA-SFs and activated antigen presenting cells further supports IL-6 and other cytokine accumulation in the joint via TLR4 and/or integrin dependent cell signalling. This potentiates Th17-cell polarisation and drives the inflammatory pathology in the RA joint. Furthermore, tenascin-C signalling may upregulate expression of ECM degrading MMPs, which further potentiate the tissue destruction and liberation of DAMPS from the ECM by establishing a destructive and inflammatory positive feedback loop; which exacerbates the RA pathology (Figure 6.5).

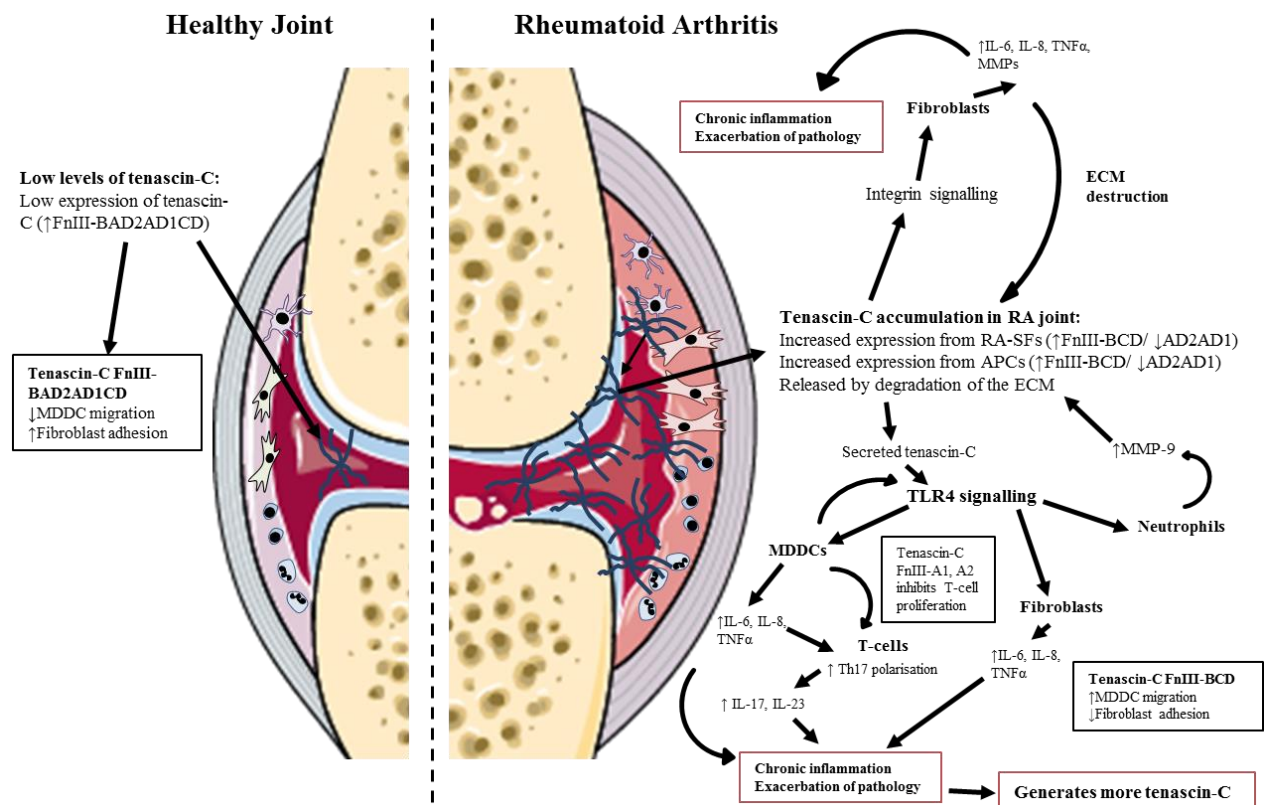


Figure 6.5. Summary of activity for tenascin-C expression and alternative splicing in the rheumatoid arthritis joint. The cartoon images of the healthy joint and RA joint are modified from “normal joint”, and “rheumatoid arthritis”, and were used under Creative Commons. The original author of the source image is “Servier Medical Art”.

This study showed that RA-SFs express tenascin-C isoforms that are distinct in their overall composition, from NSF and OA-SFs, and that alternative splicing of tenascin-C may act as a mechanism by which supports Th17 cell generation synergistically with the constitutively expressed FBG region of tenascin-C; and supports lesser cell adhesion and promotes greater antigen presenting cell migration during inflammation in RA. Therefore these findings suggest tenascin-C alternative splicing is functionally relevant in RA; and warrants further functional investigation.

6.6 Conclusions

In conclusion, this study expanded on the previous findings by the Midwood group, and generated novel data which supported the hypothesis that immune cells and fibroblasts express inflammation and disease specific tenascin-C isoforms, that directly impact cell behaviours that facilitate adhesion and migratory processes during inflammation.

The most significant findings of the thesis included the detailed standard curve qPCR characterisation of tenascin-C expression and alternative splicing in resting and activated primary human immune cells; and in healthy and diseased synovial fibroblasts. This revealed a cell lineage specific pattern of tenascin-C expression and alternative splicing in resting cells that was transiently induced and modified in activated monocytes, macrophages and MDDCs; but that was disease specific in synovial fibroblasts from RA and OA patients.

This study revealed for the first time, inflammation specific tenascin-C alternative splicing in activated macrophage and dendritic cells, and in diseased RA-SFs compared to NSF. The acute and chronically inflamed cell types both switched from expressing longer tenascin-C variants, to expressing shorter variants lacking FnIII-AD2 and AD1, so that domains FnIII-B, C, D were contiguous. This switch in expression supported less cell adhesion and promoted greater dendritic cell migration. Furthermore, the alternatively spliced FnIII domains

activated inflammatory cytokine expression in macrophages that could act in synergy with previously defined FBG dependent signalling, to support pathogenic generation of Th17 cells in RA.

6.7 Future work

More work is required to assess resting and activated expression and splicing of tenascin-C in other immune cell types including B-cells, eosinophils, basophils and mast cells as well as lymphoid stem cells and myeloid progenitor cells; in order to form a complete characterisation of the expression and alternative splicing of tenascin-C in the immune system. Further assessment of tenascin-C splicing in myeloid and lymphoid cells isolated from RA synovial membranes would complement previous studies revealing elevated tenascin-C expression²⁷³, and would reveal potential differences in the tenascin-C alternative splicing pattern in immune cells during acute and chronic inflammation; and if PAMP or DAMP signalling impacts tenascin-C splicing.

Due to the technical difficulties and limitations of the methods initially employed to investigate tenascin-C alternative splicing, this thesis described a new method to quantify the individual spliced FnIII domains by standard curve qPCR. However, limitations of this method include the inability to infer whether individual FnIII domains were present in the same mRNA transcripts and new techniques should be employed to confirm the contiguous expression of domains FnIII-B,C,D in activated MDDCs and RA-SFs. A new standard curve qPCR method could be performed on exon spanning regions of the alternatively spliced FnIII mRNA, to determine which spliced FnIII domains are expressed together in the same transcript. Alternatively methods entailing the PCR amplification of the entire alternatively spliced FnIII cassette, and subsequent Northern blotting and in-situ hybridization with FnIII specific probes, may reveal more information about the content of spliced tenascin-C mRNA transcripts. A better understanding of the FnIII content of individual alternatively spliced

tenascin-C isoforms expressed during inflammation would ensure that recombinantly synthesised FnIII proteins would contain spliced FnIII domains in a relevant context.

The current ability to characterise tenascin-C protein expression was restricted by the lack of specific antibodies to all of the alternatively spliced FnIII domains. Tenascin-C expression may be further quantified with an ELISA targeting FnIII-C domain; while the alternatively spliced FnIII content of specific tenascin-C protein isoforms could be determined by Southern blotting, and subsequent FnIII domain detection using commercially available antibodies to FnIII-A2, B, C, D; unfortunately specific antibodies to domains FnIII-A1, A3, A4, AD2 and AD1 are not yet available.

This study screened the levels of tenascin-C biosynthesis, but did not investigate the mechanisms by which tenascin-C expression or splicing is regulated. A better understanding of how the cell-type specific, and inflammation specific alternative splicing patterns are regulated, may reveal novel mechanisms by which pathological tenascin-C splicing is regulated during pathologies such as inflammation and cancer; and may identify upstream therapeutic targets which can modify tenascin-C expression and splicing in disease.

An aspect of this study that warrants further investigations are the functional roles of alternatively spliced FnIII domains during inflammation and disease. Further studies should observe the adhesive and migratory properties of normal and RA fibroblasts and immune cells on different ECM protein and FnIII domain coating combinations, and investigate the manner by which alternatively spliced FnIII domains impact tenascin-C integration into the ECM. The mechanisms by which FnIII adhesion and migratory effects observed in this thesis should be identified; for example, the role of integrin mediated signalling could be investigated by assessing the levels of phosphorylated vs non-phosphorylated FAK, Rho and myosin, and could use antibody perturbation experiments to disrupt FnIII domain functions.

Re-cloning and expression of total tenascin-C with and without the FnIII-A1-D domains, as well as expression of shorter proteins such as FnIII-A1-D, with and without FnIII-AD2,AD1, or FnIII-A1 should be performed to identify the functional roles of splice variants expressed by NSF, and of the entire alternatively spliced FnIII domains in the presence of the constitutive tenascin-C domains. In future studies, recombinant FnIII domain could be expressed in mammalian cells, so that the role of post-transcriptional modifications such as glycosylation could also be investigated.

Finally, the crystal structure of the alternatively spliced FnIII domains has not yet been solved, and would assist future research investigating FnIII domain interactions with other ECM components and cell receptors.

6.8 Clinical applications of this research

It is important to understand the cell type specific expression and functions of alternatively spliced tenascin-C isoforms during inflammation, as this knowledge could be incredibly useful in the development of diagnostic tests for auto-immune diseases, and could also identify FnIII modules suitable for pharmacological targeting for the treatment of auto-immune diseases.

Previous studies in tumours highlighted the importance of characterising the expression of alternatively spliced tenascin-C isoforms so that diagnostic markers and therapeutic targets could be identified. To date, this analysis had not been performed in the study of inflammation and autoimmunity, though the findings of this thesis may provide a basis on which to develop new diagnostic tests and therapeutics that specifically target or modify the behaviour of MDDCs, macrophages, and RA-SFs for the treatment of RA.

The targeting of alternatively spliced FnIII domains of tenascin-C has already shown to be useful in the treatment of cancer. Early studies identified stark correlations between the elevated expression of specific alternatively spliced FnIII domains, and aggressive tumours; an observation which combined with their low expression in normal healthy tissues, raised interest in pharmacological targeting of alternatively spliced FnIII domains, for the purposes of delivering highly targeted radiotherapies and chemotherapies, specifically to the tumour site.

The characterisation of alternatively spliced tenascin-C isoforms in cancers has already led to the development of two immunotherapeutic antibody conjugates that are currently in Phase III development²⁹; the first targeting FnIII-A1 (F16) to deliver IL-2, ¹³¹I, and ¹²⁴I for the treatment of lung cancer, breast cancer, Hodgkin's lymphoma^{214, 192, 215, 216, 218}, and the second targeting FnIII-C/D (81C6) to deliver ¹³¹I for the treatment of glioblastoma multiforme^{213, 214}, (See Section 1.8).

Also in tumours, the surveillance of alternatively spliced FnIII domains has shown to be useful in cancer patient diagnosis and stratification. Driemel *et al*, demonstrated that the sensitivity and specificity of conventional HE staining in atypical oral brush biopsies could be improved in combination with mAb BC-2 IHC staining; which allowed for the detection of FnIII-A1/A4 containing tenascin-C isoforms¹⁹¹. There is also a correlation between FnIII-AD2 and AD1 expression and invasive ER negative breast cancer in younger women, hence FnIII-AD2/AD1 prevalence may serve as a predictor of tamoxifen resist breast cancer¹⁸⁸. Furthermore, serum levels of FnIII-C are significantly elevated in colorectal cancer¹⁹⁶, and also predicted hepatic recurrence¹⁹⁷; while urine levels of FnIII-B predicted the presence of presence urinary bladder cancer, and indicated the muscle invasive status²⁰⁰. Altogether, studies in cancers have revealed that surveillance of tenascin-C alternative splicing is a useful tool for patient diagnosis and treatment.

This study identified several FnIII domains that are expressed by activated immune cells and RA-SFs, and identified behaviour modifying functions of these domains. The applications of these findings may include the development of novel diagnostic tests for RA. It was previously shown that tenascin-C levels are elevated in the synovial fluid of RA patients³⁰⁶, and that there are raised circulating levels in RA³⁰⁷. Furthermore ELISA tests detecting the FnIII-C epitope confirmed that large tenascin-C variants containing alternatively spliced FnIII domains can be detected in circulation, distally from the joint; therefore surveillance of synovial or circulating levels of FnIII-B, C or D as identified by this study, may have potential for the diagnosis of RA, in distinguishing RA from OA, or perhaps in predicting the disease stage in RA.

There may also be potential for monitoring of alternatively spliced FnIII domains B,C,D and the early diagnosis of RA. The results of this study may indicate that an epigenetic switch is activated in RA-SFs but not NSF or OA-SFs, resulting in the expression of an RA associated tenascin-C splicing pattern. However, not much is known as to how early this switch in isoform expression occurs; and perhaps similarly to the observations by Schwenzer *et al.*, where a citrullinated FBG epitope of tenascin-C could predict RA years before the onset of symptoms³⁰⁸; perhaps early switches in the alternative splicing pattern of tenascin-C could also predict early RA onset, however further investigation on this topic is required.

The second major application of the findings presented in this study may be in the identification of elevated FnIII-B,C,D expression as a biomarker that could be used to deliver antibody conjugated therapeutics to the RA synovium. Additionally, FnIII-B,C,D domains may serve as a viable therapeutic target for the treatment of RA, as disruptive immunotherapeutic antibodies could be generated that attenuate the mechanisms of FnIII-B,C,D activity on MDDCs, macrophages and fibroblasts in the RA synovium. Such blocking antibodies may serve to decrease FnIII-B,C,D induced cytokine expression and cell migration

which may positively impact RA symptoms for patients. The targeting of FnIII-C,D domains may even be possible with the antibody 81C6, which is a constituent of the antibody conjugate Neuradiab that is currently licensed for the treatment of grade IV glioblastoma^{213, 214}.

Anti-tenascin-C antibodies are currently in development for the treatment of RA, but this targets the constitutively expressed FBG epitope and aims to disrupt TLR4 signalling in the RA synovium. While these therapies hope to reduce the destructive cycle of cytokine expression in the RA joint, they may have off target effects on tenascin-C expressed in other tissues. In contrast to this, therapeutic antibodies targeting FnIII-B,C,D domains offer greater specificity, and could complement other RA therapies to positively impact patient treatment.

*“Enough of Science and of Art;
Close up those barren leaves;
Come forth, and bring with you a heart,
That watches and receives.”*

- William Wordsworth, *The Tables Turned*

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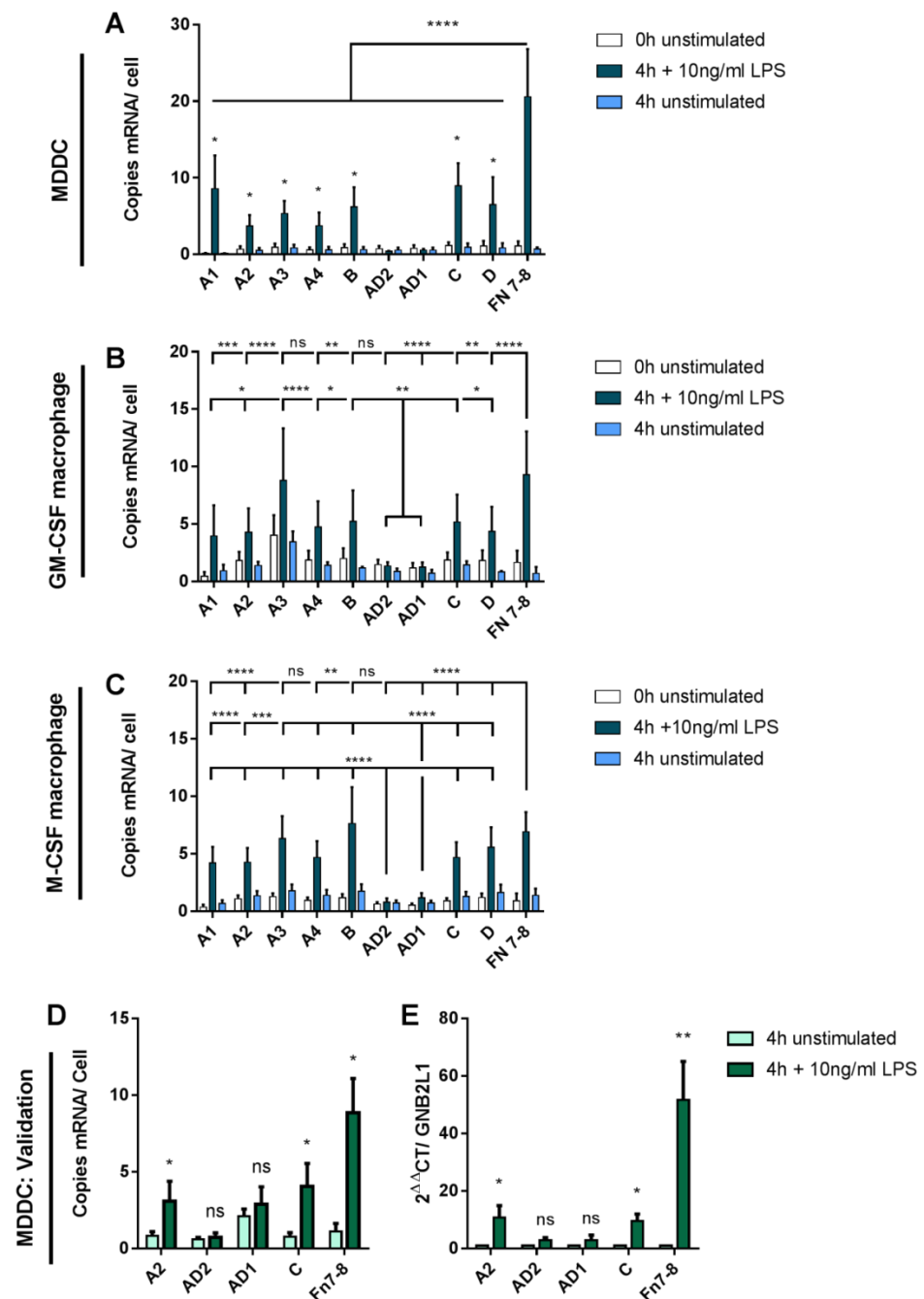
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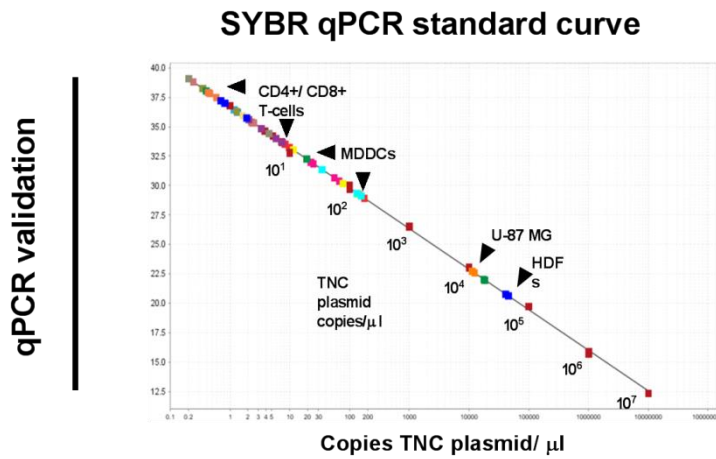
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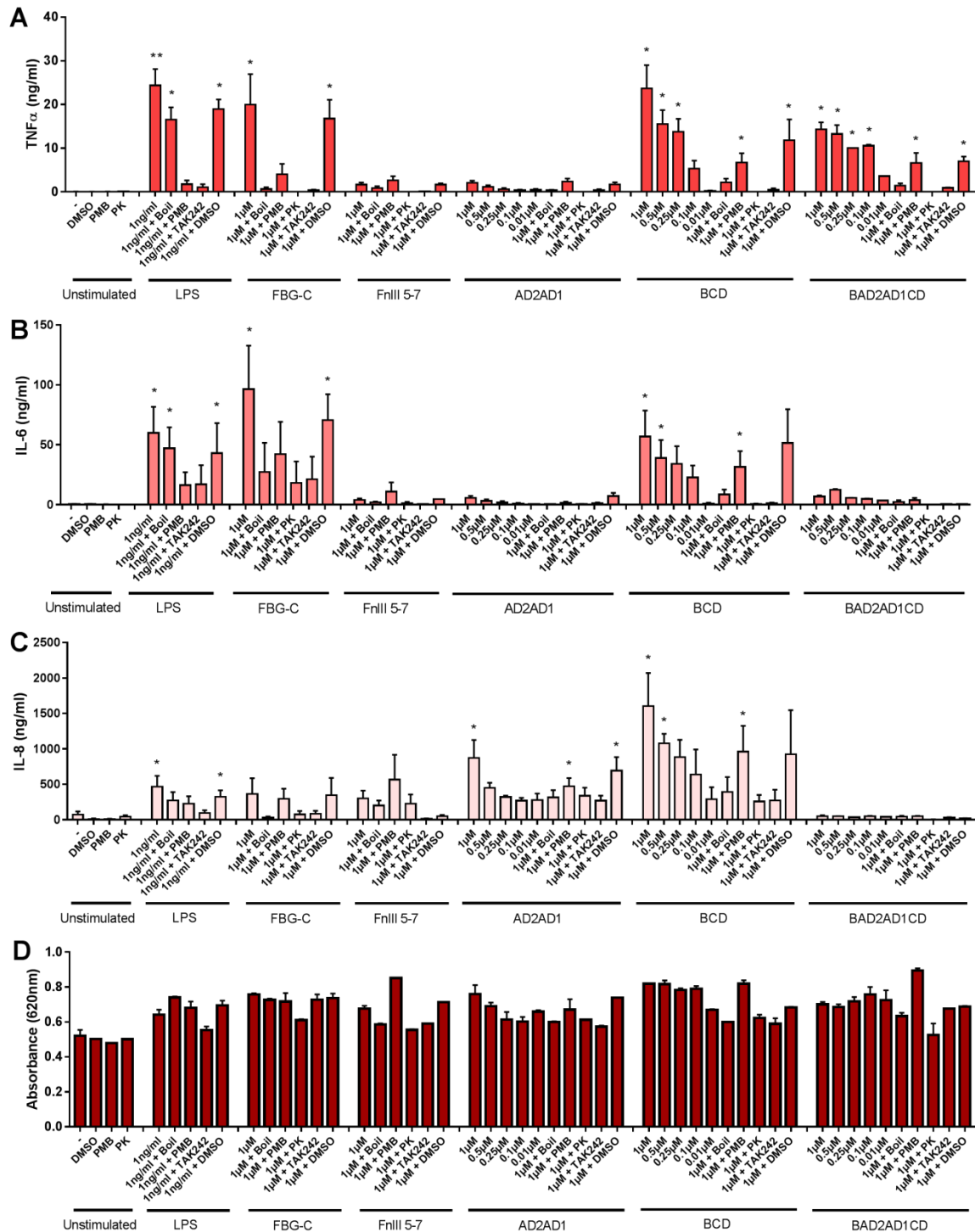
Appendix



Appendix Figure 4.8. LPS stimulation induces transcription of all alternatively spliced FnIII mRNAs except AD2 and AD1 in MDDC, GM-CSF and M-CSF macrophages. Primary human MDDCs, GM-CSF and M-CSF macrophages were plated and rested for 16 hours and were then stimulated, or not-stimulated with 10ng/ml LPS for 0 and 4 hours. The absolute abundance of alternatively spliced FnIII mRNAs and FnIII 7-8 in response to LPS stimulation was quantified by standard curve qPCR in MDDCs (n=6) (A), GM-CSF macrophages (n=3) (B), and M-CSF macrophages (n=3) (C). Key, (white bars: 0h unstimulated/ dark blue bars: 4h + LPS/ light blue bars: 4h unstimulated). The absolute abundance of alternatively spliced FnIII in MDDCs was validated in MDDCs rested or stimulated with 10ng/ml LPS for 4 hours, by standard curve qPCR (n=5) (D) and $\Delta\Delta C_t$ qPCR normalized against control gene GNB2L1 (n=5) (E). Data are shown as mean +SEM. 2-way ANOVA tests with multiple comparisons were performed within individual cell types (A-C) and un-paired t-tests were performed between stimulated and unstimulated samples (D, E). Statistical significance is displayed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and non-significant (ns).



Appendix Figure 4.9. Alternatively spliced domain FnIII-A1 is absent in CD4+ and CD8+ T-cells. Primary human T-cells were harvested, purified and plated for 16 hours to rest. Following this, cells were stimulated with anti-CD3/CD28 dynabeads® at a 1:1 cell:bead ratio, and mRNA was extracted at 0h, 2 day and 7 day time points. The abundance of FnIII A1 mRNA was quantified by standard curve qPCR in CD4+ T-cells (n=5), and in CD8+ T-cells (n=5). A representative standard curve showing quantification of FnIII-A1 mRNA in samples is displayed.



Appendix Figure 5.7. Cytokine expression is induced in M-CSF macrophages by recombinant FnIII BCD fragments. M-CSF macrophages were plated and rested for 16 hours prior to stimulation with 1ng/ml LPS, 1 μ M FBG-C or 5-7, or 1, 0.5, 0.25, 0.1 and 0.01 μ M AD2AD2, BCD or BAD2AD1CD for 24 hours. Controls included boiling of the stimulus (+Boil; 90°C for 30mins), 30 minute pre-incubation with polymyxin-B (+PMB) or 6 hour pre-incubation with TAK242 or DMSO. Cytokine release was quantified by ELISA for TNF α (A), IL-6 (B) and IL-8 (C); and cell viability was assessed by MTT (D). Data are shown as mean + SEM from n=4 independent experiments. One way Anova with multiple comparisons was performed and statistical significance is displayed as *p<0.05, ** p<0.01.



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Tenascin-C: Form versus function

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Keywords: biosynthesis, cancer, development, glycosylation, matrix assembly, proteolytic cleavage, splicing, tenascin-C, therapeutics, transcription

Abbreviations: AD1/AD2, additional domain 1/ additional domain 2; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; ASMCs, aortic smooth muscle cells; BDNF, brain derived neurotrophic factor; bFGF, basic fibroblast growth factor; BHKs, baby hamster kidney cells; BMP, bone morphogenetic protein; *c*, charged; CA19–9, carbohydrate antigen 19–9; CALEB, chicken acidic leucine-rich EGF-like domain containing brain protein; ccRCC, clear cell renal cell carcinoma; CEA, carcinoembryonic antigen; chRCC, chromophobe-primary renal cell carcinoma; CNS, central nervous system; CRC, colorectal carcinomas; CTGF, connective tissue growth factor; DCIS, ductal carcinoma in-situ; ECM, extracellular matrix; EDA-FN, extra domain A containing fibronectin; EDB-FN, extra domain B containing fibronectin; EGF-L, epidermal growth factor-like; EGF-R, epidermal growth factor receptor; ELISPOT, enzyme-linked immunospot assay; FBG, fibrinogen-like globe; FGF2, fibroblast growth factor 2; FGF4, fibroblast growth factor 4; FN, fibronectin; FNIII, fibronectin type III-like repeat; GMEM, glioma-mesenchymal extracellular matrix antigen; GPI, glycosylphosphatidylinositol; HB-EGF, heparin-binding EGF-like growth factor; HCEs, immortalized human corneal epithelial cell line; HGF, hepatocyte growth factor; HNK-1, human natural killer-1; HSPGs, heparan sulfate proteoglycans; HUVECs, human umbilical vein endothelial cells; ICC, immunocytochemistry; IF, immunofluorescence; IFN γ , interferon gamma; IGF, insulin-like growth factor; IGF-BP, insulin-like growth factor-binding protein; IHC, immunohistochemistry; IL, interleukin; ISH, in situ hybridization; LPS, lipopolysaccharide; mAb, monoclonal antibody; mitogen-activated protein kinase, MAPK; MMP, matrix metalloproteinase; MPNSTs, malignant peripheral nerve sheath tumors; *M_r*, molecular mass; NB, northern blot; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; NK, natural killer cells; NSCLC, non-small cell lung carcinoma; NSCs, neural stem cells; NT, neurotrophin; PAMPs, pathogen-associated molecular patterns; PDGF, platelet derived growth factor; PDGF-R β , platelet derived growth factor receptor β ; pHo, extracellular pH; PIGF, phosphatidylinositol-glycan biosynthesis class F protein; PLC γ , phospholipase-C gamma; PNS, peripheral nervous system; pRCC, papillary renal cell carcinoma; PTPR ζ 1, receptor-type tyrosine-protein phosphatase zeta; RA, rheumatoid arthritis; RCC, renal cell carcinoma; RD, rhabdomyosarcoma; RGD, arginylglycylaspartic acid; RT-PCR, real-time polymerase chain reaction; SB, Southern blot; SCC, squamous cell carcinoma; siRNA, small interfering RNA; SMCs, smooth muscle cells; SVZ, sub-ventricular zone; TA, tenascin assembly domain; TGF β , transforming growth factor β ; TIMP, tissue inhibitor of metalloproteinases; TLR4, toll-like receptor 4; TNF α , tumor necrosis factor α ; TSS, transcription start site; UBC, urothelial bladder cancer; UCC, urothelial cell carcinoma; VEGF, vascular endothelial growth factor; VSMCs, vascular smooth muscle cells; VZ, ventricular zone; WB, immunoblot/ western blot.

Tenascin-C is a large, multimodular, extracellular matrix glycoprotein that exhibits a very restricted pattern of expression but an enormously diverse range of functions. Here, we discuss the importance of deciphering the expression pattern of, and effects mediated by, different forms of this molecule in order to fully understand tenascin-C biology. We focus on both post transcriptional and post translational events such as splicing, glycosylation, assembly into a 3D matrix and proteolytic cleavage, highlighting how these modifications are key to defining tenascin-C function.

The Multifunctional Nature of Tenascin-C

Tenascin-C was independently and concurrently characterized in the 1980s by several research groups with interests in the fields of cancer, matrix biology and embryonic/neural development. Tenascin-C is highly expressed in the developing embryo in a strictly regulated spatio-temporal pattern, but most healthy adult tissues exhibit negligible tenascin-C levels. Here, expression is constrained to sites where high cell turnover, plasticity and tissue-remodeling are obligatory; such as stem cell niches and the central nervous system (CNS), in addition to regions which undergo significant tensile stress; such as tendons, ligaments and smooth muscle fibers. However, transient expression of tenascin-C is observed at sites of active tissue remodeling in the adult, such as during the healing of wounds, and persistent tenascin-C expression is detected in pathological states such as cancer or rheumatoid arthritis (RA) (reviewed in¹⁻³)

Tenascin-C consists of 4 distinct domains, which can interact with pathogenic components, matrix constituents, soluble factors and cell surface proteins; conferring upon

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tenascin-C the ability to bind to more than 25 different molecules identified thus far.³ For example, the tenascin assembly (TA) domain forms inter-molecular hydrophobic interactions and disulphide bridges, the epidermal growth factor-like (EGF-L) repeats act as a low affinity ligand for the EGF-receptor (EGF-R), inducing mitogen-activated protein kinase (MAPK), and phospholipase-C (PLC) γ signaling, the fibronectin (FN) type III-like repeats (FNII) interact with proteins as diverse as integrins, aggrecan, and perlecan, as well as binding to growth factors including members of the platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and transforming growth factor- β (TGF β) families (Table 1), and the fibrinogen-like globe (FBG) binds to integrins, receptor-type tyrosine-protein phosphatase zeta (PTPR ζ 1) and activates Toll-like receptor-4 (TLR4). This enables tenascin-C to drive a range of processes from oligomerization to induction of mitogenic responses, cell migration, cell attachment, cell spreading, focal adhesion, cell survival, matrix assembly, neurite outgrowth and potentiation and protease and pro-inflammatory cytokine synthesis (reviewed in ³).

This collection of functions is reflected in the wide ranging consequences of genetic deletion of tenascin-C in mice. Reported abnormalities include reduced FN during dermal wound healing,⁴ hyperactivity,⁵ reduced kidney regeneration,⁶ reduced haematopoiesis,⁷ increased tumor monocyte population,⁸ abnormal tumor organization and angiogenesis,^{9,10} and aberrant immune responses,¹¹⁻¹³ among many others (reviewed in ^{14,15}). Genetic variation at the human tenascin-C gene locus is associated with 6-fold increase in risk of Achilles tendon injury,¹⁶ non-syndromic hearing loss,¹⁷ and increased risk of developing adult asthma.¹⁸

Together these data point toward very diverse biological and pathological roles for tenascin-C. Here, we discuss some of the mechanisms that exist in order to dictate how this extracellular matrix (ECM) glycoprotein can exert so many different effects.

Transcriptional Regulation of Tenascin-C

One of the foremost means of regulating tenascin-C function is via control of its expression at the transcriptional level; and the cloning, and subsequent characterization, of the tenascin-C gene has begun to shed some light on the molecular mechanisms that underpin this tightly regulated control.

Cloning the TNC gene

The tenascin-C gene (*TNC*) is a large intron rich gene, which in humans spans 97.68 kb of DNA on the antisense strand of chromosome 9, at locus 9q32-34/9q33.1^{19,20} and of which only ~7.9% is protein coding (reviewed in ²¹). The first partial gene sequence derived from chicken tenascin-C identified several transcripts of various lengths prompting the speculation that the transcript is alternatively spliced.²² The first human tenascin-C

exon sequence was published using cDNA clones isolated from U-373MG glioblastoma cells, identifying a clone with 8 consecutive FNIII like repeats, and another clone containing the same repeats but with a 1.9 kb insert between FNIII 5 and 6; providing clear genetic evidence of alternative splicing within the tenascin-C transcript.²³

Transcriptional regulators of tenascin-C expression

Expression of tenascin-C is regulated in a stimulus specific manner in humans, mice, rats and chickens; the promoter elements of which are well conserved up to around 250 bp upstream of the transcription start site (TSS), including a TATA box located 21 nucleotides downstream of the TSS.²⁴ Regulation of the tenascin-C promoter is influenced by many transcription factors (Table 2)(reviewed in²¹). These data illustrate how tenascin-C transcription may be induced or repressed in response to different subsets of stimuli including pathogen-associated molecular patterns (PAMPs), cytokines, reactive oxygen species, growth factors and mechanical stress. More about the transcriptional regulation of all tenascin family genes is described in detail by Chiovaro et al in this issue. However, it is likely that not all of the transcription factors regulating tenascin-C expression are known; and our understanding of the details of this large *TNC* locus is only in its infancy. Moreover, in addition to simply turning *TNC* on and off, some stimuli specifically induce particular forms of tenascin-C, and this is discussed in more detail below.

Post Transcriptional Regulation of Tenascin-C

Alternative splicing allows a single gene to encode multiple proteins by the inclusion or exclusion of selected exons into the mature mRNA, dramatically increasing the size and diversity of the proteome. Up to 95% of the ~21,000 protein coding genes in humans are alternatively spliced,²⁵ and 85% of these have a minor splice isoform with an expression frequency exceeding 15%. As a result this moderate number of genes is able to produce >290,000 non-redundant peptide combinations.²⁶ Following on from the first observations by Jones et al.²² and Gulcher et al.²³ that tenascin-C is subject to alternative splicing, many studies have expanded on this theme revealing that post transcriptional modification of tenascin-C has a profound effect on tenascin biology.

Tenascin-c exon structure

The first *TNC* human exon sequences published showed the gene to comprise 28 exons (accession number NM_002610). However, 2 further alternatively spliced FNIII domains (additional domains 1 and 2: FNIII AD1²⁷ and AD2²⁸ (accession numbers U88892.1 and EU295718.1 respectively) were later discovered. FNIII AD1 was identified within human U251 glioma cDNA clones as a single exon between FNIII repeats B and C. This study also showed that while AD1 was present in human glioblastoma, neuroblastoma and osteosarcoma tumor cells, it

Table 1. Interactions of tenascin-C with growth factors and growth factor receptors. For studies on EGF-L repeats,¹⁹⁸⁻²⁰⁰ FNIII 4–5,²⁰¹ and FBG.²⁰² Vascular endothelial growth factor (VEGF), phosphatidylinositol-glycan biosynthesis class F protein (PIGF), bone morphogenetic protein (BMP), neurotrophin (NT), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), insulin-like growth factor (IGF), insulin-like growth factor –binding protein (IGF-BP), connective tissue growth factor (CTGF), heparin-binding EGF-like growth factor (HB-EGF), hepatocyte growth factor (HGF), chicken acidic leucine-rich EGF-like domain containing brain protein (CALEB)

Tenascin-C Domain	Growth Factor/ Receptor Family	Growth Factor/ Receptor	Interaction Binding Affinity (+) No Binding Affinity (–)
EGF-L Repeats	EGF-R	EGF-R	+
FNIII 4–5	FGF	FGF-1/ -9/ -16/ -19/ -20/ -21	-
		FGF-2/ -4/ -6/ -7/ -8/ -10/ -17/ -18	+
	PDGF/VEGF	PDGF-AA/ -AB/ -BB/ -DD	+
		VEGF-A165	+
		VEGF-A121	-
		VEGF-B/ -C	+
	PIGF	PIGF-1	-
		PIGF-2/ -3	+
	TGFβ	BMP-2	+
		BMP-4/ -6/ -7	-
		TGFβ-1/ -2	+
		TGFβ-3	-
	Neurotrophic Factors	NT-3	+
		BDNF	+
		NGF	-
	IGF	IGF-1/ -2	-
		IGF-BP3/ -BP5	+
	CCN	CTGF	-
	EGF	EGF	-
		HB-EGF	-
	S1 Plasminogen	HGF	+
FBG	EGF	CALEB-80	+
		CALEB-140	-

was absent in healthy human lung fibroblasts and umbilical vein endothelial cells (HUVECs) providing the first indication that tenascin-C splicing may play a role in tumorigenesis.²⁷ Human FNIII AD2 was discovered between FNIII B and AD1 in oral mucosa carcinoma samples and was named so on account of being in the same respective location as the avian FNIII AD2; situated between FNII B and AD1, in addition to it sharing 70% amino acid and 55% nucleotide sequence homology with the avian FNIII AD2.^{28,29} Thus human *TNC* contains 30 exons (Fig. 1).

Tenascin-C in other vertebrate species including chickens, rats, mice and pigs, exhibits similar domain organizations to the human protein (Fig. 2). Each contains a TA domain, a series of EGF-L and FNIII like domains and an FBG domain. However, a number of differences exist between orthologs. For example, all vertebrate tenascin-C contains 13.5 EGF-L repeats, with the exception of mammals which possess 14.5 EGF-L repeats.³⁰ Furthermore, tenascin-C from humans, rats, mice and chickens each contain 8 constitutive FNIII repeats, with an additional cassette of FNIII which may be alternatively spliced between FNIII domains 5 and 6. However, the number of alternatively spliced FNIII repeats varies by species; with chickens and mice, rats, and humans having 6, 7 and 9 respectively.^{28,31,32}

Theoretically there are 511 possible human tenascin-C splice isoforms; this figure was calculated via the equation $\frac{9!}{(9-r)!(r)!}$

as follows,

$$\begin{aligned} & \left(\frac{9!}{(9-1)!(1)!} \right) + \left(\frac{9!}{(9-2)!(2)!} \right) + \left(\frac{9!}{(9-3)!(3)!} \right) \\ & + \left(\frac{9!}{(9-4)!(4)!} \right) + \left(\frac{9!}{(9-5)!(5)!} \right) + \left(\frac{9!}{(9-6)!(6)!} \right) \\ & + \left(\frac{9!}{(9-7)!(7)!} \right) + \left(\frac{9!}{(9-8)!(8)!} \right) + \left(\frac{9!}{(9-9)!(9)!} \right) \end{aligned}$$

where 9 represents the total number of alternatively spliced FNIII, “r” represents the total number of alternatively spliced FNIII included in a single variant, and “!” denotes a factorial e.g. $4! = 4 \times 3 \times 2 \times 1$. Despite this large theoretical variation, the actual number of recorded splices does not exceed 100. This may partly be explained by the finding that some alternatively spliced FNIII domains are preferentially expressed together. Human FNIII AD2 is observed to be included in the transcript only when linked to AD1, FNIII C only when alongside D and interestingly FNIII A4 and C have never been observed to be linked together in mice or humans.^{28,31,33} This partnering of multiple alternatively spliced FNIII repeats significantly reduces the possible number of tenascin-C splice variants which are observed.

Alternatively spliced isoforms of tenascin-C have been shown to play integral roles in many different processes. Here we focus

Table 2. Transcriptional regulators of tenascin-C expression

Stimulus	Transcription Factor/ Promoter	+ve/–ve regulation	Features of Study	Reference
PAMPs				
Lipopolysaccharide (LPS)	NF-κB	+ve	Identified 33 binding sites in <i>TNC</i> locus. In primary human monocyte derived dendritic cells tenascin-C expression was PI3K/Akt dependent	Goh et al. ²⁰³
Cytokines				
TNFα/ Sphingomyelinase	Postulated ATF-2/c-jun	+ve	JNK/SAPK-1 pathway activation increased tenascin-C expression in human epidermal keratinocytes.	Latijnhouwers et al. ²⁰⁴
IL-4/ IFN-γ	Postulated STATs	+ve	Activate JAK-STAT pathway	Latijnhouwers et al. ²⁰⁴
IL-13	unknown	+ve	Cultured human dermal fibroblasts, regulation was PI3K/Akt and/or PKC pathway dependent	Jinnin et al. ²⁰⁵
TNFα	NF-κB	+ve	TNFα driven tenascin-C expression was NF-κB p65 subunit (RelA) dependent in cultured human articular chondrocytes	Nakoshi et al. ²⁰⁶
Growth Factors				
TGFβ	Smad3/4, Ets1 Sp1, p300/ CREB-binding protein	+ve	Utilized WS-1 human dermal fibroblasts.	Jinnin et al. ²⁰⁷
PDGF	Sp1 Ets1/Ets2	+ve	Cultured human dermal fibroblasts, PI3K/Akt dependent	Jinnin et al. ²⁰⁸
	Fli1	–ve	Overexpression in human dermal fibroblasts inhibits effects of PDGF on tenascin-C expression	
Mechanical Strain				
Mechanical strain	NF-κB	+ve	Induced during mechanical strain via ROS in rat neonatal cardiac myocytes. Inhibited by anti-oxidants	Yamamoto et al. ²⁰⁹
Cyclic strain	MKL1	+ve	Binds CARG box (c-fos promoter)	Asparuhova et al. ²¹⁰
Biomechanical stretch	NFAT5	+ve	Activates <i>TNC</i> expression following mechanical stress in vascular smooth muscle cells (VSMCs). May improve migratory activity in VSMCs	Scherer et al. ²¹¹
Other/ Unknown				
	Evx-1	+ve	Dependent on 89 bp region containing TRE/ AP-1 site in chicken tenascin-c promoter	Jones et al. ²¹²
	Strain responsive element		Chicken tenascin-C promoter	Chiquet-Ehrismann et al. ²¹³
	TATA box, Sp1, NF-1, C/EBP, AP-1, AP-2, <i>Krox-20</i> , <i>Pax</i>	+ve/ unknown	Sequence analysis of the promoter region identified multiple putative transcription factor binding sites	Gherzi et al. ²¹⁴
	NF-1, TN control-element	+ve	+ve regulation in mouse NIH-3T3 fibroblasts, C6 rat glioma and N2A mouse neuroblastoma cells	Copertino et al. ²¹⁵
	OCT	+ve	Required for +ve regulation by Brn2 in mouse N2A, inactive in C6 glioma	Copertino et al. ²¹⁵
	Krox24/EGR-1 element	+ve/–ve	+ve regulation in mouse C6 glioma –ve regulation in mouse N2A neuroblastoma No effect in mouse NIH-3T3 fibroblasts	Copertino et al. ²¹⁵
	OTX2	–ve	Homeodomain protein involved in anterior head formation. Represses tenascin-C expression in OTX2 transfected cells; U87-MG glioma cells, C6 rat glial tumor cells, O1 human primary glioblastoma, MRC-5 human fibroblasts, NIH-3T3 mouse fibroblasts and SEK-MEL-28 human melanoma cells	Gherzi et al. ²¹⁶
	NF-κB/c-Jun		NF-κB and c-Jun synergistically trans-activate the tenascin-C promoter with c-Jun binding	Mettouchi et al. ²¹⁷

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Table 2. Transcriptional regulators of tenascin-C expression (*Continued*)

Stimulus	Transcription Factor/ Promoter	+ve/–ve regulation	Features of Study	Reference
Denatured type-I collagen	Unknown	+ve	at a GCN4/AP-1 site in rat REF and RF3T3 fibroblast cell lines Rat aortic A10 VSMCs cultured on denatured type-I collagen express tenascin-C in ERK1/2 and β 3 dependent. Promoter for transcription factor is in –43 to –165 bp 5' of TSS	Jones et al. ²¹⁸
	Fli1	+ve	Overexpression in human dermal fibroblasts results in +ve regulation. Modest activation observed with Ets1 and Ets2	Shirasaki et al. ²¹⁹
Denatured type-I collagen	Prx1	+ve	Prx1/2 expression increases when rat aortic A10 VSMCs are cultured on denatured collagen substrate. Prx1 expression enhances <i>TNC</i> promoter 20-fold	Jones et al. ²²⁰
Focal Adhesion Kinase	Prx1	+ve	Mouse fibroblast cell-lines. FAK induces Prx1, promoting tenascin-C dependent migration	McKean et al. ²²¹
	GATA-6	–ve	Overexpression inhibited basal and decreased IL-4/TGF β induced tenascin-C mRNA/protein levels in human foreskin fibroblasts	Ghatnekar and Trojanowska. ²²²
Notch2	RBPJK	+ve	Required for Notch-2 dependent transactivation of <i>TNC</i> promoter	Sivasankaran et al. ²²³

on how splicing controls tenascin-C action during development and in cancer.

Tenascin-C splicing - development

Tenascin-C is abundantly expressed during embryogenesis in neuroectodermal tissues, and subsequently in several non-neural sites where high cell-turnover, tissue remodeling and epithelial-mesenchymal interactions occur (reviewed in²⁴). Typically, the overall expression of tenascin-C decreases with increasing age, generally peaking during embryonic development and shortly after birth before decreasing into adulthood when expression is restricted to a few sites at relatively low levels.^{34,35} Prior to the development of tools permitting the detection of specific alternatively spliced FNIII repeats, many studies reported the presence of high and low molecular mass (*Mr*) isoforms of tenascin-C, differentiated by their apparent *Mr* upon PAGE (PAGE) under reducing conditions, at various stages of embryogenesis in chickens, mice and rats. Further analysis utilizing in situ hybridization (ISH) and immunostaining/protein gel blotting (WB) with cDNA probes and monoclonal antibodies (mAbs) respectively, could distinguish between 'long' and 'short' tenascin-C forms; revealed that differently size splice variants are expressed in cell and tissue specific patterns that change over the course of development. These studies are described below and summarized in Table 3.

Large versus small

A good example of this derives from an elegant series of studies in the developing chick which revealed 4 main isoforms; Tn260, Tn230, Tn200 and Tn190, which have apparent *Mr* of 260, 230, 200 and 190 kDa respectively.^{36,37} Tn260 was rarely expressed in contrast to Tn230, Tn200 and Tn190, which were widely expressed during embryonic and early postnatal

development.^{38,39} High *Mr* variants (Tn230/200) were associated with regions of active tissue remodeling, cell migration and cell division; evidenced by their presence in epithelial substratum for migrating neurons, embryonic skin fibroblasts, whole brain, cerebellum, chord glia, Bergmann glia, endoderm-derived epithelium at developing lung bronchioles, growing wing bud tips, base of feather buds, major blood vessel endothelium, kidney, lung, osteoblasts and regions of osteogenesis; where the expression of tenascin-C is only required transiently.^{22,34,40–49} In these regions, large *Mr* tenascin-C is expressed by migrating glia and Bergmann glia in the developing chick spinal cord and cerebellum respectively; expression in the latter of which facilitates granule cell migration.⁴⁶ In contrast, lower *Mr* isoforms (Tn190/200) are observed to be expressed more stably in dense connective tissue in areas such as gizzard tendons, intramuscular connective tissue, aortic mesenchyme, articular cartilage, inner layer of perichondrium and zones of active chondrocyte proliferation, where cell condensation and differentiation are more prevalent.^{22,39,41,44–47,50–52}

Similar patterns of tenascin-C expression are observed in mice and rats, although significantly less is known about the presence of alternatively spliced tenascin-C during human fetal development. WB and real-time PCR (RT-PCR) techniques revealed that smaller *Mr* tenascin-C variants are the predominant isoforms expressed in E14 mouse gut mesenchyme,⁵³ and in the developing thymus and skin from E17 to P6 respectively.³⁵ Furthermore, smaller isoforms are observed to be persistently expressed into adulthood in thymus, colon, cerebellum, lymph nodes and splenic tissues.^{34,35} In contrast, the larger *Mr* isoforms are the predominant variants expressed in embryonic mouse kidney and in developing rat lung.^{40,54,55} Small isoforms are also implicated in regulating development of rat lungs, where the expression of a small

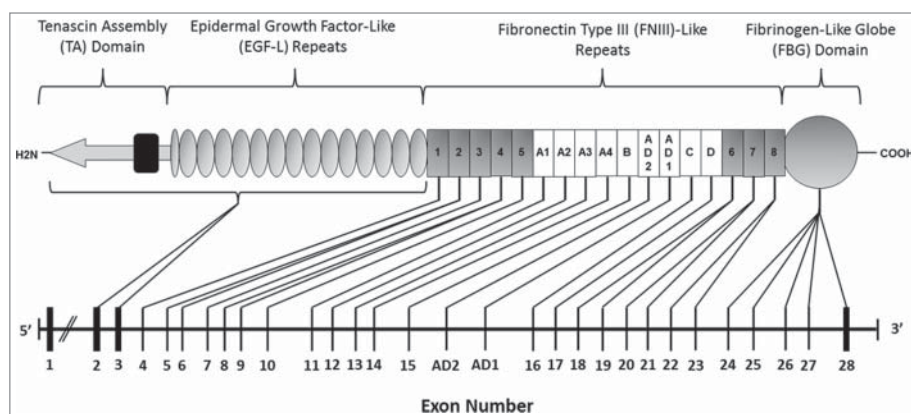


Figure 1. The exon structure of *TNC* with corresponding protein domains in tenascin-C. The human tenascin-C protein comprises 4 domains: a TA domain, 14.5 EGF-L repeats, up to 17 FNIII like repeats and an FBG domain. Eight of the FNIII repeats are constitutively expressed (FNIII 1–8 (gray), and 9 can be alternatively spliced (FNIII A1–D (white)). The *TNC* gene comprises 30 exons (1–28, plus AD1 and AD2). All exons are translated excluding the first. Exon 2 encodes the start sequence for translation of mRNA, and together exons 2 and 3 code for the signal peptide, the TA domain and all the EGF-L repeats. The 8 constitutively expressed FNIII repeats are coded for between exons 4–10 and 18–23, and the 9 alternatively spliced FNIII from exons 11–17. Each alternatively spliced FNIII repeat is encoded by its own exon. In contrast only the constitutive FNIII repeats 1 and 3 are encoded by a single exon; the remainder of the modules FNIII 2 and 4–8 are encoded by 2 exons each. Alternative splicing of FNIII domains within the tenascin-C pre-mRNA transcript means that the human *TNC* exon sequence varies in size from a maximum of 9154 bp to a minimum of 6251 bp. The FBG domain is coded for by exons 23,27,28,248.

variant is shown to be preferentially induced by TGF β in explant cultures,⁵⁵ and low *Mr* isoforms have the effects of inhibiting lung branching morphology and avascularization.⁵⁴

These data imply that predominance of the larger isoform at sites of active tissue remodeling aid cell migration and dynamic tissue organization, while the smaller, pro-adhesive isoforms

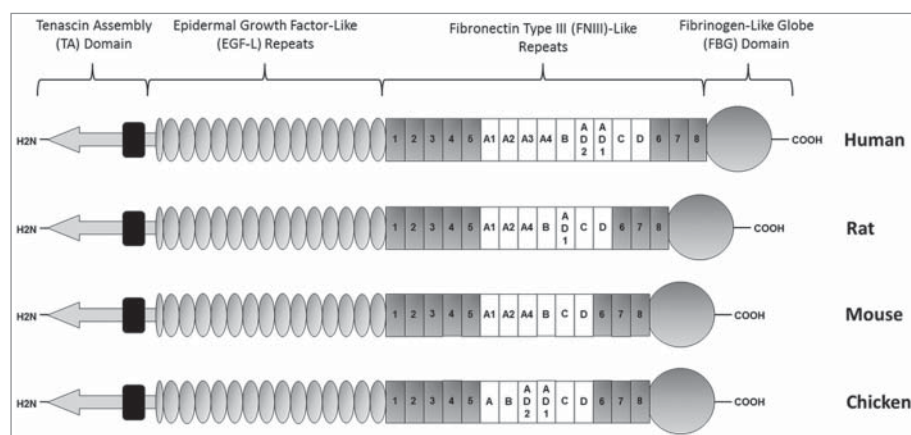


Figure 2. Schematic representation of human, rat, mouse and chicken tenascin-C. While each species contains 8 constitutively expressed FNIII repeats, the number and content of alternatively spliced FNIII repeats varies. Human tenascin-C contains 9 alternatively spliced FNIII repeats, rat 7, and mouse and chicken 6 each. Alternatively spliced repeats are typically more homologous than constitutive repeats. For example, constitutive mouse FNIII repeats share on average 44% nucleotide sequence identity to each other, in contrast to the alternatively spliced FNIII which share 52% identity. Of the mouse alternatively spliced FNIII, A2 and D share the lowest nucleotide identity at 41%, while A1 and A4 share 80%. Analysis of human tenascin-C also noted 80% amino acid sequence homology between the first 4 alternatively spliced modules (A1, A2, A3 and A4), in contrast to the other alternatively spliced FNIII repeats raising the possibility that these domains are the result of gene duplication of an ancestral FNIII module. The absence of any comparable homology between avian alternatively spliced FNIII repeats, allows for speculation that any such duplication occurred after the divergence of avian and mammalian lineages.^{19,33}

The splicing pattern within developing tissues was also observed to vary over time, as well as by location within the organism. For example, there is a shift in the relative abundance of splices away from large isoforms, in favor of smaller ones with increasing age in developing mouse kidney, cerebellum, skeletal muscle, stomach, bladder, duodenum, ileum, jejunum and colon.^{34,40,56,57} In embryonic chicken brain and gizzard, the relative proportion of smaller variants also increases with developmental age,^{22,43} as is also true in embryonic mouse intestine, gut mesenchyme, and chick cerebellum.^{40,44,53}

The smallest tenascin-C isoform, with no alternatively spliced FNIII included, is known to bind strongly to FN and the glycosylphosphatidylinositol (GPI)-anchored neural cell adhesion molecule contactin/F11^{58–60} and to promote cell attachment and the formation of focal adhesions. This is in contrast to larger tenascin-C isoforms, which prevent focal adhesion formation and drive cell migration toward the end of development and into adulthood. Indeed, in the prenatal chick brain, larger isoforms are abundantly expressed from E6 although the relative occurrence of smaller isoforms increases from E6 to E15;⁴³ and at postnatal day 3 only a single 7.2 Kda message encoding a 220 kDa peptide is detected.⁴⁴ Increased cell migration in the developing CNS was found to correlate with accumulation of long tenascin-C, but not short tenascin-C isoforms;^{50,57,62,63} indicating that long tenascin-C splices facilitate neurite motility in development.⁶⁴

These studies provided a wealth of information about tenascin-C splicing during development, but it is worth mentioning that 2 isoforms containing a different repertoire of alternatively spliced FNIII repeats can still exhibit the same molecular mass, as each alternatively spliced FNIII repeat is approximately the same size (89–92 amino acids), with a mass of ~10 kDa each. Moreover, analysis of tenascin-C forms based purely on *Mr* does not allow distinction between

Table 3. Association of 'long' and 'short' tenascin-C splice variants with stages of embryonic development

Species	Size of splice variants detected (kb, kDa, if known)	Tissue or cell type	Features of study	Reference
Chicken	Small (150 kDa, 170 kDa, 190 kDa, 200 kDa) and large (220 kDa)	Embryonic skin fibroblasts, breast muscle	E11 fibroblasts predominantly express Tn220 in ratio 4:1:1 with Tn200/190 respectively, and express 7x more tenascin-C than muscle cells. E11 myoblasts express Tn220, 200 and 190 in ratio 2:1:1 respectively	Chiquet and Fambrough. ⁴²
	Small and large	Embryonic brain, gizzard, wing and skin fibroblasts	E10 skin fibroblasts predominantly express Tn220, but also Tn200 isoform. Doublet at ~190 kDa predominant in E11 brain, gizzard and wing. Bands also detected include 210, 220 kDa and ~400 kDa in brain	Erickson and Taylor. ²²⁴
	Small (170 kDa) and large (195 kDa, 205 kDa and 220 kDa)	Sterna	Identified 6-armed oligomer from E17 sterna. Reducing-PAGE gave prominent major bands at 195/205 kDa, and minor bands at 220/170 kDa	Vaughan et al. ¹⁷⁶
	Small (190/ 200 kDa)	Embryonic chick retina	In E8 retina Tn 190/200 abundant along with ligand contactin/F11 in inner and outer plexiform layers. Identified possible binding site for HSPGs within FNIII-5	Vaughan et al. ¹⁷⁶
	Small (180 kDa, 160 kDa) and large (200 kDa, 220 kDa, 250 kDa).	Brain	Identified novel 250 kDa chondroitin sulfate containing isoform. Larger isoforms are expressed extensively at E6 to E15, but prevalence of smaller isoforms increases over this time	Hoffman et al. ⁴³
	Small (220 kDa, 200 kDa/ 7.2 kDa) and large (240 kDa, 220 kDa/ 8 kb)	Embryonic gizzard, brain, liver	7.2 kb and 8 kb mRNA isoforms increased in expression in E9/E15 gizzard and brain respectively. Corresponding peptides of 220/ 240 kDa and 200/220 kDa were identified in gizzard and brain respectively	Jones et al. ²²
	Small (190 kDa) and large (230 kDa/ 200 kDa)	Embryonic chick fibroblasts	Identified 3 cDNA clones generated from E11 skin fibroblasts with open reading frames of 1808, 1626 and 1535 amino acids, which correspond with in vitro translated tenascin identified at 200/ 180 and 170 kDa	Spring et al. ³⁸
	Small (190 kDa) and large (230 kDa/ 200 kDa)	Primary chick fibroblasts	Large isoform associated with gizzard smooth muscle layer and connective tissue below villi epithelium. Shorter isoform predominant in tendons and intramuscular connective tissue. Transfection with middle-T polyomavirus antigen induces preferential secretion of large but not small isoforms	Matsuoka et al. ²²⁵
	Small (6.6 kb, 6.4 kb) and large (220 kDa/ 7.2 kb)	Cerebellum	Total tenascin-C increased E8 to E15, then decreased until barely detectable at P3. Seven.2 kb mRNA prominent in E6–15 cerebellum while 6.4 kb decreases over this time. ISH probe for FNIII-B,C hybridized only to 7.2 kb message in CNS, and was absent in non-neural tissues (chondroblasts, tendons and lung mesenchyme)	Prieto et al. ⁴⁴
	Large	Embryonic skin fibroblasts	Proteolytic cleavage of the 230 kDa variant isolated from E11 skin fibroblasts by pronase, and detected by mAb Tn68, produced C-terminal heparin binding peptide fragment specific only to cleavage of large isoform	Chiquet et al. ³⁷
	Small (190 kDa) and large (200 kDa and 220 kDa)	Embryonic cornea	Close association between 220 Kda isoform expression and embryonic corneal cell migration in E3–19	Kaplony et al. ⁵⁰
	Small (190 kDa) and large (230 kDa)	Embryonic lung bud/ bronchiole tube epithelium	Used ISH to detect mRNAs corresponding to Tn190 in tips of budding bronchioles but not older epithelia or dense mesenchyme. Tn230 probes had identical association to Tn190	Koch et al. ²²⁶

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Table 3. Association of 'long' and 'short' tenascin-C splice variants with stages of embryonic development (*Continued*)

Species	Size of splice variants detected (kb, kDa, if known)	Tissue or cell type	Features of study	Reference
Mouse	Small (200 kDa, 190 kDa) and large (220 kDa)	Neural crest	Identified Tn230, 200, 190 in E3 neural crest cell conditioned media. Identified neural crest as major expresser of tenascin-C in developing spinal cord until E18 after which all splices not expressed	Tucker and McKay. ⁴⁸
	Small (190 kDa, 200 kDa) and large (230 kDa)	Periosteal cells, osteoblast enriched cultures and endochondral bone.	Used universal cDNA probe to detect tenascin-C in osteogenic and chondrogenic regions. Full length tenascin-C present only in osteogenic regions and expressed by osteoblasts. Periosteal cultures express 3 isoforms but enriched osteoblast cultures express Tn230 only	Mackie and Tucker. ⁴⁵
	Small (190 kDa)	Embryonic chick brain	Identified Tn190 as ligand to contactin/F11. Contactin/F11 binds FNIII-5,6 via first 3 Ig-domains and binding efficacy is reduced by incorporation of alternatively spliced FNIII	Zisch et al. ⁵⁸
	Tn190, 200, 230	Embryonic knee cartilage	IHC revealed Tn190 present in E11 articular cartilage. Peripheral articular cartilage and fibrocartilage expresses Tn200, and Tn230 is expressed in perichondrium	Pacifici et al. ⁵¹
	Small and large	Whole chick embryos	ISH revealed large and small tenascin-C abundantly expressed in embryo at E3–7. From E10–15 expression was spatially regulated; chord glia, Bergmann glia, endoderm-derived epithelium at growing tips of lung bronchioles, endothelium of major vessels, and osteogenic regions predominantly express large isoform. Small isoform associated with cartilage deposition and chondrocyte proliferation e.g. surrounding E10 notohord	Tucker. ⁴⁶
	Small and large	Embryonic aorta and adjacent mesenchyme	Identified 3 isoforms containing 0, 1 or 3 alternatively spliced FNIII repeats, in haematopoietic progenitor/primordial germ cell migratory pathways of which the smallest was most prominent	Anstrom and Tucker. ⁵²
	Small (210 kDa) and large (260 kDa)	Gut mesenchyme	Small isoform predominant at E14, but after birth abundance of large isoform increases	Aufderheide and Ekblom. ⁵³
	Small (190 kDa) and large (220 kDa)	Embryonic intestine, brain	190 kDa isoform more prevalent than 220 kDa isoform in mouse ileum, but relative concentration is the same from E14 to adulthood. Adult brain expresses single 160 kDa isoform. Developmental appearance of increasing concentration gradient of all tenascin-C from crypt to villus in ECM at epithelial-mesenchyme interface. Proposed to facilitate epithelial shedding in the villus	Probstmeier et al. ²²⁷
	Small (5.5 kb) and Large (7 kb)	Brain, submandibular gland, thymus, lung, heart, spleen, kidney, liver, pancreas, esophagus, stomach, intestine, bladder, skin and skeletal muscle	Two isoforms observed between E17 and P6 in skeletal muscle, stomach, cerebellum, bladder, duodenum, jejunum, ileum, and colon. Large (7 kb) isoform observed in lung, kidney and cerebrum. Small isoform observed in thymus and skin. Expression of all variants was decreased at P32, but small (5.5 kb) message continued to be transcribed in thymus, colon and cerebellum	Saga et al. ³⁴
	Small (6 kb) and large (8 kb)	Kidney, intestine	Large variant predominant in newborn mouse kidney but postnatally small variant increases in abundance. E13 intestines express small isoform, and by birth the larger isoform is predominant	Weller et al. ⁴⁰
	Small (190 kDa, 200 kDa/ 6 kb) and large (225 kDa, 240 kDa/ 8 kb)	Cerebellum	Expression of larger isoforms down regulated faster than smaller isoforms from P0 to >P60	Bartsch et al. ⁵⁷

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Table 3. Association of 'long' and 'short' tenascin-C splice variants with stages of embryonic development (*Continued*)

Species	Size of splice variants detected (kb, kDa, if known)	Tissue or cell type	Features of study	Reference
Rat	Small (200 kDa/ 6 kb) and large (230 kDa/ 8 kb)	Thymus, spleen, lymph nodes, lung, skin, cerebellum	Small isoform abundantly expressed in adult thymus, weaker expression in cerebellum, skin and none in spleen, testes, skeletal muscle, liver, kidney and heart. WB revealed 200 kDa tenascin in adult spleen and lymph nodes while thymus also contained 230 kDa isoform	Ocklind et al. ³⁵
	Small (200 kDa) and large (250 kDa)	NIH-3T3 cells	TGFβ1 and FGF induce expression of small and large isoforms respectively in mouse embryonic fibroblast cell line	Tucker et al. ⁴⁷
	Small (6.5 kb) and large (7.2 kb/ 280 kDa)	Lung	7.5 kb mRNA more abundant than 6.5 kb in developing rat lung. Corresponds with prominent 280 kDa isoform detected from E17. All tenascin-C expression increases at early postnatal age and decreases to levels found in adult by P21. Bacterially expressed FNIII-6,8 peptide inhibited lung branching morphogenesis though only slightly more than FNIII1-5 and A-D	Young et al. ⁵⁴
	Small (6.4 kb/ 180 kDa) and large (7.3 kb/ 230 kDa)	Lung explant culture	TGFβ preferentially induces expression of 180 kDa isoform containing 1 alternatively spliced FNIII, over 230 kDa isoform containing 5 in dose dependent manner. Strong expression of both occurs from P2 to P30, and decreases from P30 into adulthood, although 230 kDa isoform more abundant from E19 onwards.	Zhao and Young. ⁵⁵
	Small (180 kDa) and large (230 kDa)	Cultured lung epithelial, fibroblast and endothelial cells	The conditioned medium of lung fibroblasts and endothelium both expressed both 180 and 230 kDa variants, whereas lung alveolar cells expressed very little total tenascin-C	Zhao and Young. ²²⁸
	Range from small (190 kDa) to large (280 kDa)	Cortex, thalamus and cerebellum	E16 and P7 cortex, thalamus, cerebellum. All tissues expressed isoforms ranging from 190–280 kDa. Large variant is most prominent at E16 and P7 in all tissues. Expression of total tenascin-C increased most in P7 cortex and thalamus, but no major shift in the ratio of isoform expression was observed	Götz et al., 1997 ⁷⁹

tenascin-C that has been alternatively spliced and tenascin-C that has been proteolytically clipped. As it became apparent that each alternatively spliced FNIII repeat had unique functions, analysis of the precise FNIII repeats that make up each isoform became increasingly important.

Analysis of specific FNIII repeats

The pattern of expression of specific FNIII domains in developing tissues and the functional consequences of the expression of these different isoforms are described below and summarized in **Tables 4 and 5** respectively. In both the text and tables individual splice variants separated by (,) are included in the same transcript, while those separated by (/) are not.

Developing mouse

Tenascin-C is abundantly expressed in the mouse olfactory bulb, ventricular zone (VZ) and sub ventricular zone (SVZ) of the CNS during development and postnatally⁶⁵⁻⁶⁷ where it has been shown to orchestrate the development of niches permitting the development of neural stem cells (NSC) altering their response to FGF-2 and BMP-4.⁶⁸ Embryonic and postnatal day

6 mouse cerebellum express 4 and 27 different major mRNA isoforms respectively, of which the largest decrease in expression faster than the shorter variants during neural development.^{31,56} Similarly, NSCs express 20 isoforms which are differentially regulated by Pax6 and Sam68 transcription factors.^{69,70} In NSCs, expression of the largest isoform comprising FNIII-A1,A2,A4,B,C,D was demonstrated to contain *N*- and *O*-linked natural killer-1 (HNK-1) carbohydrate epitopes;⁷¹ first described on tenascin-C by Kruse et al.⁷² HNK-1 epitopes; otherwise known as CD57 are named after the HNK-1 mAb, and are found on the surface of a subset of T-lymphocytes and natural killer (NK) cells, but also on a number of cell adhesion molecules distributed throughout the nervous system including myelin-associated glycoprotein,⁷³ neural cell adhesion molecule,⁷⁴ and L1 cell adhesion molecule.⁷⁵ HNK-1 has functions in cell-cell and cell-substrate interactions, and promotes NSC proliferation via modulating the expression of the EGF-R.⁷¹

Further functional studies demonstrated how tenascin-C splice variants are capable of modulating the response of neurons during CNS development. In E18 rat and P6 mouse neurons, FNIII-A1,A2,A4,B,D coated plates, supported initial

Table 4. Associations between specific alternatively spliced FNIII repeats and developing tissues. Individual splice variants separated by (,) are included in the same transcript, while those separated by (/) are not

Cell/Tissue Type	Alternatively spliced FNIII repeats	Associations	Method of Identification	Reference
Chick embryo	A,B	FNIII-A,B containing tenascin-C synthesized by migrating glia and osteoblasts at sites of epithelial-mesenchymal interactions in feather buds, kidney, bronchiole tips and tendons	ISH, IHC, RT-PCR	Tucker et al. ⁴⁶
Embryonic mouse cerebellum	A1,A2,A4,B,D/ A1,A2,A4,D/ D/ no FNIII	Splices contained all FNIII or excluded C, B-C, A1-C or A1-D. Expression of isoforms containing 6, 5, 4, 1, 0 alternatively spliced FNIII decreased from E14 to adulthood, although expression of larger isoforms decreased faster than shorter isoforms	ISH, RT-PCR, Northern Blot (NB), sequencing, Southern blotting (SB)	Dörries and Schachner. ⁵⁶
Rat aortic smooth muscle cells (ASMCs)	Full length/ D/ none	Treatment with PDGF-BB subunit homodimer or Angiotensin II induced expression of mRNAs containing all, one or no variable FNIII repeats. Tenascin-C also inhibited cell adhesion of ASMCs to FN	Radiolabelling, WB, cell adhesion assays, RT-PCR	LaFleur et al. ²²⁹
Embryonic chick spinal cord, tendons, base of feather buds, bronchiole tips, skin fibroblasts.	A/ B/ C/ A,B/ AD2/ AD1	First report of FNIII-AD1/ AD2/ C in chicken. ISH cDNA probes for FNIII-A/ B/ C hybridize in E7 bronchiole tips, ligamentum flavum, kidney mesenchyme, FNIII-A,B in E7 aorta endothelium, spinal-chord ependyma and E10 spinal cord, tendons and base of feather buds. FNIII-C absent in spinal cord. Identified FNIII-AD1 and AD2 in E11 skin fibroblasts by RT-PCR	ISH, WB, IHC, RT-PCR	Tucker et al. ²⁹
Chick embryonic lung bud tips, feather buds, bone	AD1/ AD2	AD2 observed in E10 bronchiole bud tips. AD1 expression more widespread and abundant in developing bone (where 85% tenascin-C contained AD1)	Quantitative ISH, WB, IHC, RT-PCR and SB	Derr et al. ⁴⁹
Human Fetal Membranes	Predominant isoforms D/ A1,A2,A3,A4/A1,A2,A4	Identified 8 mRNA isoforms associated with processes analogous to tissue remodeling and wound response prior to labor and delivery in normal membranes Speculated that inclusion of FNIII A3 provides substrate for MMP-2, 3, 7 digestion prior to membrane rupture	Sequencing, SB, RT-PCR	Bell et al. ⁹⁶
Mouse postnatal day 6 cerebellum	27 isoforms ranging from 1–6 FNIII repeats	Identified 27 tenascin-C isoforms (22 of which were novel) in P6 cerebellum. Cerebellum confirmed as major expresser of tenascin-C in P6 brain. Only splice containing FNIII-D found in adult brain	RT-PCR, SB	Joester and Faissner. ³¹
Mouse embryonic whole tooth	D/ A1,D/ B,D/ B,C,D/ A1,A2,A4,B,D	E13 whole tooth expresses multiple alternatively spliced isoforms	ISH with cDNA probes, RT-PCR, IHC	Sahlberg et al. ⁸⁰
Mouse embryonic dental papilla mesenchyme	D/ B,D/ A1,A2,A4,B,D	E12 dental mesenchyme expresses FNIII-D following induction by FGF-4 and TGFβ. TGFβ induces expression of long mRNA containing 5 alternatively spliced FNIII. Propose mesenchyme becomes sensitive to epithelial induction of tenascin-C during E11	ISH with cDNA probes, RT-PCR, IHC	Sahlberg et al. ⁸⁰
Mouse NSCs	20 isoforms identified, novel A1,A4,B,D	Identified 20 isoforms in embryonic forebrain derived NSCs. Transcription factor Pax6 overexpression induced isoforms with 4, 5, and 6 alternatively spliced FNIII repeats, but downregulated smaller ones	RT-PCR, gene overexpression	Von Holst et al. ⁶⁹

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Table 4. Associations between specific alternatively spliced FNIII repeats and developing tissues. Individual splice variants separated by (,) are included in the same transcript, while those separated by (/) are not (*Continued*)

Cell/Tissue Type	Alternatively spliced FNIII repeats	Associations	Method of Identification	Reference
Mouse embryonic NSCs	Full length	Identified N- and O-linked HNK-1 epitopes expressed in NSC rich regions is almost exclusively expressed on full length tenascin-C. Small interfering RNA (siRNA) knockdown showed HNK-1 on full length tenascin-C promotes NSC proliferation via modulating EGF-R expression	Immunocytochemistry (ICC), WB, siRNA knockdown, RT-PCR, liquid chromatography MS/MS	Yagi et al. ⁷¹
Rat Hippocampal Neurons	AD1, various others.	FNIII AD1 detected in VZ and area dentate of rat brain. Observable shift from short to long isoform expression in rat hippocampus from embryonic day 16, to postnatal day 5	ICC, RT-PCR, ISH	Garwood et al. ³²

attachment.⁷⁶ FNIII-B,D/ D,6 and 6 containing isoforms facilitate neurite outgrowth in P6 granule cell neurons and E18 rat hippocampal, P0 mouse dorsal root ganglia explants respectively, and even increase the proportion of neurite bearing cells in culture.^{76,77} Mercado et al.⁷⁸ demonstrated that the FNIII-D repeat is strongly inductive of neurite outgrowth on account of 2 short sequences within the VFDNFVLK amino acid sequence, which permit interactions with $\alpha 7$ and $\beta 1$ subunit containing integrins. The FNIII-A1,A2,A4 region in contrast is anti-adhesive and was demonstrated to promote neuronal migration and repulsion in rat E18 and P0 mouse root dorsal ganglia neurons,^{76,77} and E16 rat cortical and thalamic axonal outgrowth.⁷⁹

Non-neural sites exhibiting a diverse expression of splice variants include the developing tooth, which at E13 expresses D/ A1,D/ B,D/ B,C,D/ and A1,A2,A4,B,D.⁸⁰ Nearby dental mesenchyme papilla expressed FNIII-D following induction by FGF-4 and TGF β , the latter of which also induced the expression of a long mRNA variant containing 5 alternatively spliced FNIII repeats. Interestingly, the response of dental mesenchyme papilla to growth factor induction always occurred in E12 but not always in E11, suggesting that the dental mesenchyme becomes responsive to the induction of tenascin-C at some point during E11.⁸⁰ In the mammary gland, full length tenascin-C isoforms are highly prevalent during involution, and recombinant proteins containing FNIII A1-D inhibit milk protein synthesis, suggestive of a role for tenascin-C in facilitating the cessation of lactation in mammals.⁸¹

Developing chicken

In Situ hybridization (ISH) experiments using cDNA probes complementary to FNIII AD2 or C revealed a strict spatial pattern of tenascin-C splicing in the developing chick. These probes did not hybridize to chondrogenic or osteogenic regions; while AD2 cDNA probes did hybridize at regions of epithelial-mesenchymal interactions including lung bronchioles and the base of feather buds.^{28,48} In contrast probes for AD1 and A,B hybridize strongly in almost all regions where tenascin-C is detected in the E10 chick embryo, such as feather buds, lung bronchioles, tendons, ligamentum flavum, cartilage and bone; with exceptions

including spinal cord glia and sternal perichondrium.^{28,48} In developing bone, up to 85% of the tenascin-C transcripts detected via quantitative immunohistochemistry (IHC) included the AD1 repeat.⁴⁹ FNII-C localizes in a pattern which exclusively overlaps the expression pattern for FNIII-A,B, with the exception that FNIII-C cDNA probes never hybridize in the spinal cord, or aorta endothelium, indicating a more tightly regulated pattern of expression in this location.²⁹

Functionally, tenascin-C was shown to promote adhesion and outgrowth of E3 spinal cord, E8 sensory, E8–11 sympathetic and E6 retinal ganglion cell neurons in chickens.^{82–84} In CNS neuron neurons this effect was mediated by FNIII-A, but in peripheral nervous system (PNS) neurons was mediated by FNIII-A and 3. Cell adhesion to FNIII-A and 3 was inhibited by anti- $\beta 1$ -integrin antibodies, and arginylglycylaspartic (RGD) peptides respectively, illustrating that the adhesive effects of FNIII-A on neurons is $\beta 1$ -integrin dependent.⁸⁵

In chicken embryos, the expression of isoforms containing FNIII-AD2/AD1/ and C are dramatically up-regulated at sites of active tissue remodeling and FN expression within the developing feather-bud and sternum. In vitro these variants have been shown to decrease cell attachment and organization of actin microfilament bundles in myoblasts cultured on FN.⁸⁶ However, cells bound to a FNIII-AD2/AD1/C containing substrata developed stronger adhesions than those bound on tenascin-C containing no alternatively spliced FNIII, but did not form focal adhesions as is the case on FN. Instead they formed F-actin microfilament bundles at non-uniform adhesion points, giving the cells an irregular shape much like when adherent cells are cultured on thrombospondin-1.^{86,87} The introduction of alternatively spliced FNIII modules may conversely disrupt adhesions between tenascin-C and other ECM molecules, as was demonstrated by FNIII A/B/C splices binding less strongly to FN and the GPI-anchored immunoglobulin-superfamily neural cell adhesion molecule contactin/F11.⁵⁸ This is because in contactin/F11, the first 3-Ig domains preferentially bind to the uninterrupted FNIII-5,6 region;⁵⁹ as a result, contactin/F11-tenascin-C binding is attenuated by the inclusion of alternatively spliced FNIII in the tenascin-C molecule which interrupt this binding region.⁵⁸

Table 5. Functional consequences of specific alternatively spliced FNIII repeats during development. Individual splice variants separated by (,) are included in the same transcript, while those separated by (/) are not

Cell/Tissue Type	Alternatively spliced FNIII repeats inc.	Function	Method of Identification	Reference
Chick embryonic skin fibroblasts	A,B,C/ C/ none	FNIII-A,B,C/ C/ none containing fusion protein had no effect on promoting cell adhesion. FNIII-7,8 promoted adhesion as efficiently as full-length tenascin-C evidenced by perturbation with mAb Tn68	Generated fusion proteins, cell attachment assays, WB, electron microscopy, antibody perturbation experiments	Spring et al. ³⁸
Embryonic rat hippocampal and mesencephalon neurons	B,D	Promotes neurite outgrowth and cell adhesion to substratum. Effects inhibited by anti FNIII-B,D mAb J1/tn2	Antibody perturbation, cell substrate adhesion assay, ICC, rotary shadowing and electron microscopy	Lochter et al. ⁹²
Bovine aortic endothelial cells	A3/ D	Antibody perturbation revealed FNIII-A3/ D mediate loss of focal adhesions. Confirmed by addition of recombinant protein containing FNIII-A1,A2,A3,A4,B,C,D	Antibody perturbation experiment, focal adhesion assays	Murphy-Ullrich et al. ¹²⁷
Early postnatal mouse cerebellar cortex	A1,A2,A4/ B,D	FNIII-A1,A2,A4 promotes P6 granule cell neuron migration, but not outgrowth. B, C has no effect on migration but promotes outgrowth and increases the proportion of neurite bearing cells	Antibody perturbation experiment, cell migration assays, neurite outgrowth assays	Husmann et al. ⁷⁷
Rat Lung (Fetal/ Postnatal)	A-D	Inhibits lung branching morphogenesis and aveolarization	Produced spliced domain anti-serums, IHC	Young et al. ⁵⁴
Adult mouse mammary gland	A1,A2,A4,B,C,D	FNIII 1–3/ A1-D/ all (1–8) inhibit β -casein expression and milk production during involution of mammary gland	Generated recombinant FNIII fragments, ICC, NB, WB	Jones et al. ⁸¹
Chicken PNS and CNS neuron cultures	A	FNIII-A promotes PNS and CNS neuron adhesion, increasing proportion of cells with extending neurites. Adhesion effects inhibited by anti- β 1 integrin antibodies	Generated FNIII-A fusion protein, cell adhesion assays, antibody perturbation experiments	Phillips et al. ⁸⁵
Embryonic and Postnatal Mouse and Rat CNS Neurons	A1,A2,A4,B,D/ A1,A2,A3/ B,D/ D,6	FNIII-A1,A2,A4,B,D supported initial attachment in E18 rat and P6 mouse neurons. FNIII-A1,A2,A4 was repulsive to neurons, while B,D/ D,6/ and 6 promoted neurite outgrowth in E18 rat hippocampal and P0 mouse dorsal root ganglia explants	Cell binding assays, repulsion assays, neurite outgrowth assays, WB	Götz et al. ⁷⁶
Chicken Embryos	AD2/AD1/C	Decreased cell attachment and actin microfilament bundle organization on cells adherent to FN. Increased adhesion on AD2/AD1/C containing substrata without focal adhesion	Cell adhesion assays, immunofluorescence (IF), RT-PCR, SB, IHC, in-situ hybridization	Fischer et al. ⁸⁶
Rat cortical and thalamic explants	4,5/ A1,A2,A4/ D	Tenascin-C IHC and western blot staining identified isoforms ranging from 190–280 kDa in E16-P7 cortical tissue. mAb perturbation with J1/Tn1, J1/Tn2/ J1/Tn4 inhibited axon outgrowth by binding FNIII-A1,A2,A4/ D and 4,5 respectively. J1/Tn3 to EGF-L had no effect	IHC, ISH, mAb perturbation, WB	Götz et al. ⁷⁹
Rat embryonic cerebral cortical and hypothalamic neuronal cells	D/ A1,A4/ A4	Surface bound long and short isoforms promote E17 neurite process extension. Soluble long and short variants have no effect, or inhibit outgrowth respectively. FNIII-A1,A4/ D/ 6 are permissive and 6–8 are inhibitory. Different sites are masked/exposed when surface bound	Neurite outgrowth assays, antibody perturbation, generating recombinant FNIII proteins, ICC, WB	Meiners and Geller. ⁶⁴

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Table 5. Functional consequences of specific alternatively spliced FNIII repeats during development. Individual splice variants separated by (,) are included in the same transcript, while those separated by (/) are not (*Continued*)

Cell/Tissue Type	Alternatively spliced FNIII repeats inc.	Function	Method of Identification	Reference
Embryonic rat kidney	A1,A2,A4/ B,D	Tenascin-C expressed in kidneys from E14 past birth, strongest expression in cortical regions at newest growth. No alternatively spliced FNIII are implicated in kidney development in vitro	Antibody perturbation experiments (used 11 antibodies, 6 of which were novel)	Talts et al. ²³⁰
Rat embryonic cerebral cortical neurons, rat cerebral cortical astrocytes, baby hamster kidney cells (BHKs)	Full/ A1,A2,A3,A4/ B,C,D/ D	Bound human FNIII-A-D promotes neurite outgrowth. mAb perturbation revealed FNIII-D as outgrowth permissive region on FNIII A-D bound to astrocytes, and A1-4/ D as permissive on A-D bound BHKs. Bacterially expressed FNIII A1-A4 and B,C,D promoted astrocyte outgrowth on BHK cells	mAb perturbation, neurite outgrowth assays, binding assay, WB	Meiners et al. ⁹³
Rat embryonic cerebellar granule neurons	Full/ C/ D	Discovered FNIII-C mRNA in early postnatal rat cerebellum. FNIII-D permits neurite extension, C regulates orientation and growth	RT-PCR, neurite guidance assays, mAb perturbation, IF, WB	Meiners et al. ⁹⁰
Rat Embryonic Hippocampal Neurons	B,D/ D	FNIII-B,D/ D promote neurite process extension and outgrowth. The B,D effect was contactin/F3 dependent	Expressed hybrid-fusion proteins, Neurite outgrowth assays, RT-PCR	Rigato et al. ⁹¹
Early postnatal rat and mouse cerebellar granule neuronal cultures	D	Unique amino acid sequence VFDNFVLK within FNIII-D promotes neurite outgrowth in $\alpha 7/ \beta 1$ integrin subunit dependent manner	Antibody perturbation, synthesized recombinant wild type and mutant FNIII-D, neurite outgrowth assay, ICC, affinity chromatography, WB	Mercado et al. ⁷⁸
Embryonic rat hippocampal neurons	D	FNIII-D mediated E18 hippocampal neurite outgrowth in Ca^{2+} , PLC, contactin and $\beta 1$ integrin dependent manner	Neurite outgrowth assay, antibody perturbation, inhibitor experiments, pull down assay, WB, video microscopy	Michele and Faissner. x ⁹⁵
Embryonic rat retinal explant	B,D/ D,6/ A1,A2/ A1,D	FNIII-B,D fusion protein promoted strongest fiber outgrowth in E18 retinal explants, followed by A1,D. FNIII-A1,A2 is inhibitory. The FNIII-D responsible for outgrowth with effects modulated by neighboring FNIII	Generated alternatively spliced FNIII fusion proteins to human Ig-Fc fragment, antibody perturbation, axon/ neurite outgrowth assay	Siddiqui et al. ⁹⁴

Larger chick tenascin-C isoforms possess annexin II binding ability, and hence promote proliferation, cell migration and induce loss of focal adhesions hence encouraging cells cultured on high Mr tenascin-C to become motile.^{88,89} In addition, contactin/F11 binding to FNIII-5,6 was shown to be inhibited by heparin sulfate and dermatan sulfate.⁶⁰ All in all, the inclusion of adhesion modulating FNIII repeats such as AD2/AD1/C in the developing chick embryo create an environment conducive to tissue remodeling by permitting changes in motility, adhesion and cell shape.

Developing rat

AD1 containing variants are found in the highly plastic SVZ and dentate gyrus of the developing rat brain, with an observable shift in expression from shorter to longer isoforms as development progresses from embryonic day 1 to postnatal day 5.³² An early mAb perturbation study revealed that FNIII-4,5/A1,A2,

A4/ D but not the EGF-L was required for axonal outgrowth from E16 cortical and thalamic explants.⁷⁹ In vitro, FNIII-C facilitates the orientation of rat cerebellar granular neurons, but has no effect on outgrowth.⁹⁰ The variable rat FNIII B-D promotes contactin/F3 dependent neurite outgrowth when FNIII-C is excluded from the transcript; as its inclusion disrupts the contactin/F3 binding site formed between the adjacent FNIII-B and D.⁹¹ FNIII-B,D containing tenascin-C is widely reported to promote neuron outgrowth in embryonic rat hippocampal neurons, mesencephalic neurons, cortical astrocytes and retinal neurons.^{76,91-94} This repeat has a conserved function in P6 mouse cerebellar neurons, where it does not alter the rate of cell proliferation, but does increase the proportion of neurite bearing cells in culture.⁷⁷ Similarly to mouse neurites, FNIII-D also promotes rat neurite outgrowth in a manner dependent on Ca^{2+} , PLC, protein kinase-C and contactin.⁹⁵ The outgrowth of E18 rat

hippocampal neurons by FNIII-D was also inhibited by addition of anti- $\alpha 7 / \beta 1$ integrin mAbs, caffeine, thapsigargin, inositol triphosphate receptor, ryanodine, 3,4,5-trimethylbenzoic acid 8-(diethylamino)octyl ester and proved dependent upon Ca^{2+} mobilization from the endoplasmic reticulum.⁹⁵

Interestingly the abundance of FNIII-B,C in regions of high cell motility and turnover led some investigators to assess any potential functions of the repeat in developing organs outside of the CNS. However, despite a high abundance of tenascin-C in the E14 rat kidney mesenchyme, antibody perturbation of FNIII-B,C and A1,A2,A4 failed to exhibit any negative effect on the development of the kidney, suggesting that in an in vivo model at least, the presence of alternatively spliced FNIII in these tissues have no developmental function.⁹⁵ The model did not take into account the potential roles of variable FNIII repeats on in vivo specific processes in development, such as vascularization which would make good considerations for the future.

Developing human

A wealth of tenascin-C splice variants was found within human fetal membranes which encapsulate the developing fetus and amniotic fluid. Bell et al.⁹⁶ identified 8 different tenascin-C splice variants containing between 0 and 7 spliced FNIII repeats, which were speculated to promote spontaneous membrane rupture during labor by contributing to tissue remodeling processes in a manner analogous to a wound response. The predominant isoforms detected by RT-PCR contained FNIII-D/ and A1-A4, either with or without FNIII-A3.⁹⁶ The selective inclusion/exclusion of tenascin-C isoforms containing repeat FNIII A3, may provide functional significance with regard to mediating the susceptibility of tenascin-C to proteolytic degradation by matrix metalloproteinase (MMP) -2 and 3; both of which can cleave tenascin-C at a site located within the FNIII A3. MMP-7 also exhibits protease activity, and can digest tenascin-C between FNIII repeats A3-D;^{96,97} perhaps facilitating fetal membrane rupture prior to birth. In this way, 'long' tenascin-C isoforms would be susceptible to MMP-2, 3 and 7 mediated fragmentations, whereas 'small' tenascin-C isoforms lacking A3 would be resistant. This process may generate a novel regulatory pathway where FNIII A3 containing isoform dependent processes can be regulated by MMP-2 and MMP-3.⁹⁶

Tenascin-C splicing - cancer

Tenascin-C is abundantly expressed in the stroma of many solid tumors (reviewed in ^{1,98,3}). In addition to elevated tenascin-C in tumors, the splicing pattern often differs compared to healthy tissues. Studies examining 'long' and 'short' tenascin-C isoforms are summarized in Table 6, while Table 7 details specific alternatively spliced FNIII detected in tumors and Table 8 the functional consequences of particular FNIII repeats on tumor cell biology.

Tissue levels of large tenascin-C variants

What little tenascin-C is present in healthy or benign tissues generally consists of the small variant containing no alternatively spliced FNIII repeats (*Mr* 180–190 kDa). In contrast, a range of

larger isoforms with *Mr* 210, 220, 230, 250, 260, 280, 320 and 330 kDa, which would be predicted to contain one or more alternatively spliced FNIII repeats, have been described to be deposited in the ECM of a variety of human tumors including breast, colon, bladder, ovaries, prostate, pancreas, kidney, liver, uterus, brain, mouth, lung, skin, cartilage, connective tissues and peripheral nervous system (Table 6).

For example, total tenascin-C expression was observed to dramatically increase in invasive and intraductal breast carcinomas relative to normal breast tissues, with 2 major isoforms of 180 kDa and 250 kDa detectable via WB.⁹⁹ Subsequent analysis revealed that 85% of tenascin-C in normal breast tissue is a small 180 kDa isoform, but in malignancy the larger 330 kDa variant becomes predominant, with the small isoform always accounting for less than 40% of the total tenascin-C.¹⁰⁰ Similarly, elevated expression of large tenascin-C isoforms relative to smaller ones in lung cancer, was reported by Oyama et al.¹⁰¹ who found elevated mRNA isoforms encoding a large tenascin-C variant with 7 alternatively spliced FNIII repeats in 6 of 10 adenocarcinomas, and 3 of 3 squamous cell carcinomas (SCCs) tested.

However there are exceptions to this rule; the expression of tenascin-C in cancer is highly polymorphic, depending on the type of cell involved, and the stage of disease. This is evidenced by examples of cancers where the smallest tenascin-C isoform is predominantly expressed, such as in some renal, colon and breast carcinomas.^{99,102,103}

Association of large isoforms with disease progression

A number of studies have suggested that large tenascin-C isoforms may correlate with prognosis, invasion and cancer progression in the bladder, brain, colon, lung, breast, B-cell non-Hodgkin's lymphoma, and adipocytic tumors. Furthermore, the presence of these large isoforms in some cases can also indicate disease recurrence and chemotherapy resistance (Table 6).

In the case of non-small cell lung carcinoma (NSCLC), an 18-fold increase in the expression of large tenascin-C variants was predictive of disease recurrence.¹⁰⁴ Similarly, large 8 kb mRNA variants correlated with high stromal cellularity in breast cancer, and 330 kDa protein variants with extra domain-B containing FN (EDB-FN) positive colon cancer; linking large tenascin-C isoform expression with a tumor permissive stroma and the presence of invasive and angiogenic tumor marker protein EDB-FN, the expression of which is otherwise absent in healthy tissues.^{100,105} Likewise, in normal and benign breast cancer, the 190 kDa isoform was predominantly expressed, with a shift to expression of large 330 kDa variant in invasive disease. Furthermore in 3 of 16 cases of invasive disease, a number of intermediate splice variants were identified which suggested that while stromal cells are capable of synthesizing tenascin-C of 2 major isoforms, 190 and 330 kDa, epithelial cells can express tenascin-C isoforms of intermediate mass.¹⁰⁰

Again though, while elevated tenascin-C expression in tumors is often associated with poor prognosis and patient outcome, there are many examples of tumors where there is no such correlation. There is no correlation between tenascin-C expression and prognosis or disease stage in stomach adenocarcinoma;¹⁰⁶

Table 6. Association of 'long' and 'short' tenascin-C splice variants with cancer

Species	Alternatively Spliced FNIII Repeats (or size of splice variant if known)	Cell or Tissue Type	Features of Study	Reference
Human	Small (210 kDa) and large (230 kDa)	U-251MG Glioma	First identification of tenascin-C then called glioma-mesenchymal extracellular matrix antigen (GMEM). Identified major 230 kDa isoform and minor 210 kDa isoform. Did not specify whether difference in size of isoform is due to proteolysis or molecular heterogeneity	Bourdon et al. ²³¹
	Large (220, 230, 280 kDa) Large (320 kDa)	U-251 MG Glioma	Purified hexabrachions from conditioned media. Identified hexabrachions of different sizes, created by incorporation of different sized tenascin-C isoforms. In glioma, 220, 230 and 280 kDa tenascin-C isoforms were in roughly equal abundance	Aukhil et al. ²³²
	Small (180 kDa) and large (250 kDa)	Ductal and lobular breast carcinomas	Assessed distribution using mAbs in breast tissue from fetal, adult resting, lactating, aging parenchyma, fibrocystic, fibroadenomas, cystosarcoma phylloides and ductal and locular carcinomas. Total expression increased during fetal growth, gestation, hyperplasia, dysplasia, benign tumors, and much increased in infiltrating and intraductal breast carcinomas	Howeedy et al. ⁹⁹
	Small (0 AS-FNIII), large (7 AS FNIII – 1.9 kb insertion between FNIII 5- 6	Lung cancer tissues	6 of 10 and 3 of 3 adenocarcinomas and SCC respectively, exhibited elevated expression of large isoforms relative to small ones. Variant containing 7 FNIII comprised 27%, 24%, 54% and 42% of the total tenascin-C in adenocarcinoma, SCC, large cell carcinoma and small cell carcinoma respectively	Oyama et al. ¹⁰¹
	Small (~6 kb/190 kDa) and large (~8 kb/330 kDa)	Normal, hyperplastic and neoplastic breast tissue	Invasive carcinoma expressed 6 and 8 kb tenascin-C mRNA isoforms. Small variant accounts for 85% tenascin-C in healthy tissues, and < 40% total in malignant ones. Intermediate sized variants detected in 3 of 16 invasive carcinomas. In fibroadenomas, 8 kb isoform associated with high stromal cellularity, findings supported by intermediate protein bands also detected from these donors	Borsi et al. ¹⁰⁰
	Small (190 kDa) and large (280 kDa and 330 kDa)	Fibrosarcoma (HT-1080), Rhabdomyosarcoma (RD) and SV40 transformed fibroblast cell lines (WI-38-VA and AG-280), Melanoma (SK-MEL-28)	In WI-38-VA and SK-MEL-28 cell lines, only 330 kDa isoform visible in conditioned media. RD cells expressed low amounts of 330 kDa isoform in conditioned media and faintly in cell extract. HT-1080 and AG-280 expressed no detectable tenascin-C. Normal fibroblasts expressed 190 and 280 kDa isoforms in cell extracts and conditioned media	Carnemolla et al. ²³³
	Small (190 kDa) and large (280 kDa)	Transfected BHKs with long and short tenascin-C cDNAs using λ gt11 vector. Tenascin-C purified from SK-MEL-28 cells	Characterized binding locations of 11 mAbs to regions of tenascin-C, and found that mAbs BC-2, α -A2, α -A3, α -B and α -D bind to alternatively spliced FNIII repeats A1 and A4, A2, A3, B and D respectively	Balza et al. ²³⁴
	Small and large	Prostatic hyperplasia and carcinoma	WB and RT-PCR analysis identified small and large isoforms of tenascin-C protein	Ibrahim et al. ²³⁵
	Small (190 kDa) and large (250 kDa)	Invasive CRC	Tenascin-C absent in normal tissues. Variants purified from invasive CRC were 190 and 250 kDa in size, identical to sizes those found in human fetal fibroblasts	Sakai et al. ¹⁰³

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Table 6. Association of 'long' and 'short' tenascin-C splice variants with cancer (*Continued*)

Species	Alternatively Spliced FNIII Repeats (or size of splice variant if known)	Cell or Tissue Type	Features of Study	Reference
	Large (330 kDa)	CRC and colorectal adenomas	Large tenascin-C splice variant (330 kDa) was detected in 7 of 15 carcinomas. Presence correlated with expression of FN extra domain-B (ED-B)	Hauptmann et al. ¹⁰⁵
	Small (190 kDa)	Renal cell carcinoma (RCC) and Oncocytoma	Small 190 kDa isoform is predominantly expressed in RCC. Large isoform is almost absent	Lohi et al. ¹⁰²
	Small and large	Malignantly transformed fibroblasts	Normal human fibroblasts predominantly express smaller or larger tenascin-C isoforms when cultured in more acidic or basic culture medium respectively (pH 6.8–7.2) Malignantly transformed cells are resistant to external pH regulation of splicing and predominantly express large variant because a more basic cytosolic pH is maintained	Borsi et al. ¹⁶⁶
	Small (284 bp PCR product), and large (490, 556, 750, 1651 and 1924 bp) A4/ B/ C containing isoforms	Malignant and benign ovarian tumors	The smallest splice variant mRNA fragment (284 bp) found in all tumors tested. FNIII-B expression was widespread in all except smallest variant. Larger variants also expressed A4 and C. Nine/12 malignant compared to 1/6 benign tumors exhibited increase in intensity of larger ~490 and 556 bp products relative to smallest 284 bp	Wilson et al. ²³⁶
	Small and large	Endometrial adenocarcinoma	Total tenascin-C expression induced by 20 ng/ml TGFβ. Many individual splice isoforms identified, the most abundant contained none, 1 or 7 alternatively spliced FNIII repeats	Vollmer et al. ²³⁷
	Small (190 kDa/ 5.5 kb) and large (220, 250 kDa/ 7.5 kb)	Lung cancer	All 30 lung cancers tested expressed 190 kDa isoform (5.5 kb mRNA), and also in 28 cases a larger 250 kDa band (7.5 kb mRNA); sometimes accompanied by a 220 kDa band. Normal lung only expressed 5.5 kb mRNA transcript, but 190 and 250 kDa tenascin-C could be very weakly detected by WB	Kusagawa et al. ²³⁸
	Small (5.8 kb) and large (7.5 kb)	Skin (dermal keratinocytes and fibroblasts) – non cancerous	In keratinocytes treatment with IFNγ and TNFα slightly increased mRNA expression of large and small isoforms respectively, while IL-4 increased both small and large isoforms equally	Latijnhouwers et al. ²⁰⁴
	Small (220 kDa) and large (320 kDa)	Chondrosarcoma Clinical Specimens, and Cell Line JJ012	High small:large isoform ratio found in normal human articular chondrocytes. Low small: large isoform ratio found in chondrosarcomas and correlates with low survival. Determined via Semi-quantitative RT-PCR, IHC, survival analysis	Ghert et al. ²³⁹
	D (~250 kDa)	NSCLC	18-fold increase in large isoform expression observed in recurrent NSCLC compared to non-recurrent NSCLC, determined via quantitative RT-PCR and WB	Parekh et al. ¹⁰⁴
	Large isoforms (unspecified)	Neurofibromatosis, plexiform neurofibromas and malignant peripheral nerve sheath tumors (MPNSTs)	Relative expression levels of total tenascin-C in neurofibromatosis, plexiform neurofibromas and MPNSTs were 1: 2.98: 4.95, with larger spliced variants accounting for 27.6%, 54.1% and 60.3% respectively. Determined by RT-PCR	Lévy et al. ²⁴⁰

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Table 6. Association of 'long' and 'short' tenascin-C splice variants with cancer (*Continued*)

Alternatively Spliced FNIII Repeats (or size of splice variant if known)				
Species		Cell or Tissue Type	Features of Study	Reference
	Small (210 kDa) and large (260 kDa)	Amdc-s cells (NIH-3T3 transfected with human AdoMetDC), Ras-E4 (transfected with c-Ha-ras ^{Val12} oncogene) and Odc-n (NIH-3T3 cells transformed by overexpression of human ODC)	S-adenosylmethionine decarboxylase overexpressed in NIH-3T3 cells produced aggressive transformed cells. Amdc-s express 9.2-fold more tenascin-C than non-transformed cells, with the 260 kDa isoform abundantly expressed in Amdc-s, and the only isoform expressed in Ras-E4 cells; while Odc-n cells additionally expressed 210 kDa isoform	Paasinen-Sohns et al. ²⁴¹
Rat	Small (180 kDa) and large (220 kDa, very weak 280 kDa)	Hepatic and sarcoma derived cell linesx	Two hepatic and one sarcoma-derived cell lines shown to express major isoform 220 kDa isoform. Cell lines explanted into nude mice, epithelial sarcoma induced tenascin-C expression in stromal mouse tissue but no longer expressed tenascin-C themselves. After transplantation the stromal hepatic-derived cell lines still prominently expressed tenascin-C	Sakai et al. ²⁴²

metastasis, invasion or survival in gastric carcinoma;^{107,108} survival in oral and pharyngeal SCC;¹⁰⁹ survival, clinical stage or metastasis in pancreatic carcinoma.¹¹⁰ There are also cases where tenascin-C expression is absent in poor prognosis tumors, such as in cases of cervical carcinoma.¹¹¹ Other studies found no associations between tenascin-C expression pattern and a number of clinical features such as nodal metastasis, tumor necrosis or blood vessel invasion in 32 invasive breast carcinomas.¹¹² Interestingly, one study found that in colorectal carcinoma (CRC) low expression of tenascin-C is associated with lymph node metastasis, perhaps indicating that in some cancers tenascin-C is capable of enforcing some anti-metastasis mechanisms.¹¹³

Specific FNIII repeats and their association with tumors

Technological advances in the field now permit the identification of specific spliced tenascin-C variants in cancer, and many studies have used these tools to identify subsets of tenascin-C isoforms, or single alternatively spliced FNIII repeats in tumors. While a systematic analysis of every splice variant in specific tumors is for the most part still lacking, these studies illustrate the distribution of specific FNIII repeats in malignancy, and shown that in some cases distinct FNIII repeats are associated with increased tumor grade, invasion, migration and metastases (Table 6).

For example, many IHC studies have utilized mAbs specific to regions FNIII-A1/A4, such as BC-2; showing that FNIII A1/A4 containing variants are not detected in normal cells, but are found at the tumor invasion front with apparent associations with migrating tumor cells. In oral SCC FNIII-A1, A4 containing variants are localized to the tumor stroma at the interface near invading cells, with greater expression correlating with higher disease stage.¹¹⁴ Similarly, FNIII-A1,A4 and A3,A4,B are absent in normal prostate, yet strongly localized to neoplastic

glands and the tumor invasion front in prostatic adenocarcinoma by IHC and ISH respectively.¹¹⁵ In addition, FNIII-C containing tenascin-C isoforms were identified in brain cancer tissues via IHC using the mAb TN-11,¹¹⁶ and subsequently using a catalog ISH probes and RT-PCR techniques. This study found strong associations of FNIII-C with high grade astrocytoma and glioblastoma, cerebral cavernomas and lung cancers where the distribution of FNIII-C was consistently associated with tumor stroma, tumor blood vessels and proliferating cells in these tissues.¹¹⁶⁻¹¹⁸ Likewise, FNIII-B containing tenascin-C isoforms co-localize to laminin-5/ γ 2 in the basement membrane region of oral SCC, where increased expression positively correlates with malignancy grade.¹¹⁹ Another study revealed by IHC and RT-PCR that invasive urothelial cell carcinoma (UCC) exhibits restricted expression of FNIII-A1/ B or D which is closely associated with tumor blood vessels, invasive tumor stroma and damaged muscle. Moreover, the same study identified an association between AD1 and a compact invasion pattern.¹²⁰

Similarly, the preferential expression of tenascin-C isoforms containing FNIII repeats B/ D/ AD1/ AD2/ B,D and B,AD1,D is well characterized in breast cancers including ductal carcinoma in situ (DCIS) and is known to occur at the tumor invasion front, and in cancers with elevated risk of metastasis.^{49,121-124} These isoforms were originally thought to be tumor specific; although a more recent study identified FNIII-AD1 and AD2 expression in the myoepithelium of larger normal ducts in the breast, while also detecting their presence in 34.9% and 23.1% of invasive breast carcinomas respectively.¹²³ There is also a clear correlation between expression of AD1 and AD2 containing tenascin-C isoforms and invasive estrogen receptor negative breast cancer in younger women (≤ 40 y of age), however no such correlation was observed between FNIII-AD1 and AD2 prevalence with human epidermal growth factor receptor 2

Table 7. Associations of specific tenascin-C splice variants, or individual alternatively spliced FNIII repeats in cancer. Individual splice variants separated by (/) are included in the same transcript, while those separated by (,) are not

Species	Alternatively Spliced FNIII Repeats (or size of splice variant if known)	Cell or Tissue Type	Features of Study	Reference
Human	None/ D/ A4,B,D/ A1,A2,A3, A4/ A1,A2,A3,A4,B,D/ A1, A2,A3,A4,B, AD1/ A1,A2, A3,A4,B,C,D	U-251MG and U87-MG glioblastoma cell lines, MG-63 human osteosarcoma cell line, SK-N-SH neuroblastoma cell line, IMR-90 human lung fibroblast cell line, and HUVECs	Identified the novel FNIII-AD1 repeat located between FNIII-B and C, via RT-PCR and sequencing. Identified other large splice variants also by RT-PCR	Sriramarao and Bourdon et al. ²⁷
	A1,A2,A3,A4,B,C,D	U-251MG Glioblastoma cell line	Utilized radio-ligand binding assay to identify cell surface annexin II as a high affinity receptor for the whole alternatively spliced FNIII A-D region of tenascin-C (at the time this excluded FNIII AD2 and AD1)	Chung and Erickson. ⁸⁸
Human	AD1	Hs578T breast ductal carcinoma, SK-MEL-24 melanoma, WERI retinoblastoma and BCC basal cell carcinoma, A431 epidermoid carcinoma cell line	Identified the presence of AD1 in human cell lines Hs578T, SK-MEL-24, WERI, BCC but not in A431 epidermoid carcinoma cells	Derr et al. ⁴⁹
Chicken	Small (190 kDa) and large (200 kDa, 230 kDa) QT6 contained AD1,D/ A,AD1, C/ A,AD1,D/ AD1,C,D/ A, AD1,C,D/ A,B,AD1,D/ A,B, AD2,AD1,D	QT6 quail fibrosarcoma, SL-29 chick embryo fibroblast	SL-29 express 190, 200 and 230 kDa isoforms encoding 0, 1 and 3 alternatively spliced FNIII in equal ratios. QT6 fibrosarcoma expressed predominantly 230 kDa isoform containing 3 spliced FNIII but has minor bands containing 0, 1, 2 and 4 alternatively spliced FNIII. In QT6 cells splices containing more than 3 alternatively spliced FNIII are rare in protein form	Derr et al. ⁴⁹
	AD2	Malignant oral mucosae	Discovered human xFNIII-AD2 repeat. Possesses 70% amino acid and 55% amino acid sequence similarity with chicken-AD2. Identified in 2/10 oral cancers, but was absent in 40/40 normal, reactive, pre-malignant and other oral mucosae specimens	Mighell et al. ²⁸
	Large A1,A2,A3,A4,B,AD1,C,D	Melanoma SK-MEL-28	Large isoform is predominant isoform expressed by SK-MEL-28 although others are expressed in lesser abundance. Identified via sequencing, SB, RT-PCR	Bell et al. ⁹⁶
	C	Astrocytoma and glioblastoma	FNIII-C associated with high grade (III) astrocytoma and glioblastoma tumors, blood vessels and proliferating cells. Absent in healthy tissues and barely detected in meningioma, low grade astrocytoma, breast, lung and gastric carcinomas. Used TN-11 (mAb for FNIII-C)	Carnemolla et al. ¹¹⁶
	D/ A1,A2,A4 containing large isoforms.	CRC, ulcerative colitis and liver metastases Oral SCC	IHC with mAbs K8 (small isoform), 19H12, 201A, J1/tn1 (A1,A2,A4 region) and J1/tn2 (FNIII-D region). J1/tn2 stained strongly in stage I/II CRC but less so in stage III/IV tumors and liver metastases. The 19H12 staining was much stronger in metastases than early stage tumors	Dueck et al. ¹²⁵

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Species	Alternatively Spliced FNIII Repeats (or size of splice variant if known)	Cell or Tissue Type	Features of Study	Reference
	Small and large (A1,A4 containing variants)		Large tenascin-C variant stained strongly in tumor stroma, and expressed in single positive layer of cells at the tumor-stroma interface near invading cells, illustrated by ISH and IHC with mAb BC-2. Higher expression of large variant associated with increasing tumor stage	Hindermann et al. ¹¹⁴
	D, B/D	Breast Cancer cells MDA-MB 231, MDA-MB 468, MCF-7, T47D	Associated with metastasis and elevated invasion risk. Via RT-PCR, SB, sequencing. These variants are synthesized by stromal fibroblasts in malignant tissue, and by periductal fibroblasts, and myoepithelial cells in DCIS	Adams et al. ¹²¹
	C containing large isoforms	Cerebral cavernomas	Total tenascin-C localized to vascular walls and in interspace between blood cavities in cavernomas. FNIII-C localized to sub-endothelium of blood vessels in cavernomas and white matter surrounding the lesion sites. No FNIII-C was found in normal brain tissue. FNIII-C associated to tumor blood vessels in brain cancer	Viale et al. ¹¹⁷
	B containing large isoforms	Breast cancer (intraductal cancers)	Recombinant full length FNIII region of tenascin-C promoted in vitro migration and mitotic activity, effects perturbed by adding mAb 4C8MS (against FNIII-B). Large tenascin-C variants localized to invasion front of intraductal and ductal cancers. Positive correlation between large tenascin-C isoform expression and proliferation rate	Tsunoda et al. ¹²²
	A1,A4/ A3, A4, B	Prostatic Adenocarcinoma	IHC revealed normal prostate tissue devoid of FNIII-A1,A4 containing tenascin-C, but staining is observed in tumor stroma with strong deposition around neoplastic glands. ISH revealed FNIII-A3,A4,B abundant in tumor cytosol and is associated with tumor invasion front and loss of cell adhesion in all adenocarcinomas tested	Katenkamp et al. ¹¹⁵
	A1, B, D, AD1	UCC	A1, B, D Restricted to invasive tumors, tumor blood vessels and destructed muscle. AD1 associated with compact invasion pattern. Studied via Semi-quantitative IHC and RT-PCR correlated to tumor stage	Berndt et al. ¹²⁰
	A1/ D	U87 Glioblastoma	Generated recombinant human mAbs to FNIII A1 and D (F16 and P12 respectively). F16 selectively localized at tumor site in U87 glioblastoma, and was rapidly cleared from other organs. F16 identified for potential antibody-based pharmaceutical development	Brack et al. ¹⁵⁴
	Small (322bp fragment) and large (2243bp fragment A1,A2,A3,A4,B,C,D)	Cutaneous SCC and actinic keratosis (AK)	Large isoform found in 5%, 63% and 88% of normal, AK and SCC samples respectively. In SCC, tenascin-C associated with basal cells at invasion front and papillary/reticular dermis	Dang et al. ²⁴³

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Species	Alternatively Spliced FNIII Repeats (or size of splice variant if known)	Cell or Tissue Type	Features of Study	Reference
	A1 and A4 containing large isoforms	Immortalized human corneal epithelial cells (HCEs)	Co-deposited laminin-332 and large tenascin-C variant in plaque beneath adhering cells and was Golgi dependent. HCE adhesion to laminin and large variant tenascin-C was dependent on $\alpha 3\beta 1$ integrin	Katz et al. ²⁴⁴
	C	Lung cancer	Generated mAb G11 specific to FNIII-C and via IHC observed expression in majority of lung cancers, in vascular and stromal pattern. G11 demonstrated preferential localization to tumor site in rats grafted with U87 gliomas, so may have application in delivery of imaging or therapeutics to glioma or lung tumors	Silacci et al. ¹¹⁸
	None and A1,A2,A3,A4,B, AD2,AD1,C,D	Thyroid carcinoma cell lines. TT medullary carcinoma, ARO/ FRO anaplastic carcinoma, WRO follicular carcinoma, BHP 2/ BHP 5/ BHP 7/ BHP 10/ BHP 14/ BHP 17/ BHP 18, BHP 19 and SW579 papillary carcinoma	RT-PCR illustrated large transcript fragment containing full alternatively spliced cassette (variable splice region 1 - VS1) was more prevalent than the small variant in all cancers tested, except for medullary carcinoma cell line TT	Tseleni-Balafouta et al. ²⁴⁵
	A1 and A4 containing large isoforms	Atypical oral brush epithelium	Specificity and sensitivity of conventional HE staining for atypical oral brush biopsies increased from 96–99% and 78–95% respectively when combined with BC-2 immunostaining for large FNIII-A1/A4 containing tenascin-C isoforms	Driemel et al. ¹²⁶
	B containing large variants	Oral SCC	IF with BC-3 mAb (for FNIII-B). No large tenascin-C variant identified in normal oral basement membrane. In dysplastic and neoplastic oral mucosa long tenascin-C variant containing FNIII-B was co-localized to laminin-5/ γ -2 in the basement membrane region. Extent of reorganization of large variant in basement membrane correlated with malignancy grade	Franz et al. ¹¹⁹
	C	CRC	Serum levels of large-tenascin-C variants containing FNIII-C quantified by ELISA. Primary CRC patients 5260 $\pm$ 3243.3 pg/ml, recurrent CRC patients 4106 $\pm$ 2261.1 pg/ml and healthy donors 2364 $\pm$ 749.6 pg/ml. Sensitivity for detecting CRC via serum levels was 56.6%, exceeding conventional tumor markers CEA at 40.1% and CA19–9 at 23.6%	Takeda et al. ¹⁴⁶
	C	Hepatic recurrence of CRC	High serum levels of large tenascin-C splice variants in 2 patients; as detected via ELISA sensitive to FNIII-C domain, were associated with hepatic recurrence of CRC	Takeda et al. ¹⁴⁷
	B, C	UCC	Measured urine concentration of FNIII-B, C in 104 UBC patients, 11 patients with cystitis and 15 healthy donors. Increased urinary FNIII-B concentration correlates with tumor progression in UBC. Proteolytic fragmentation of tenascin-C was also observed in urine from invasive tumor patients	Richter et al. ¹⁴⁸

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Table 7. Associations of specific tenascin-C splice variants, or individual alternatively spliced FNIII repeats in cancer. Individual splice variants separated by (/) are included in the same transcript, while those separated by (/) are not (*Continued*)

Species	Alternatively Spliced FNIII Repeats (or size of splice variant if known)	Cell or Tissue Type	Features of Study	Reference
	AD1, AD2	Breast cancer DCIS, ductal carcinomas, lobular carcinomas, fibroadenoma	AD1 and AD2 present in 34.9% and 23.1% of invasive breast carcinomas respectively. AD1 and AD2 not tumor specific but expression is increased in carcinomas from younger women. AD1 localized to tumor cells and myoepithelium of normal breast ducts. AD1 associated with ER negative and grade III tumors	Guttery et al. ¹²³
	A1	Melanoma	24 primary and 29 metastatic melanoma lesions stained with F8, L19 and F16 mAbs for extra domain-A containing FN (EDA-FN), EDB-FN and FNIII-A1 respectively. F16 strongly stained basal lamina and deeper layers of tissue compared to others. F16-IL2 could therefore be useful as therapeutic for malignant melanoma	Frey et al. ²⁴⁶
	B	UBC	Determined urine levels of FNIII-B/ C containing tenascin-C in 35 patients via ELISA. FNIII-B could predict cases without tumor recurrence, or with tumor existence. Could also predict whether UBC was muscle/or non-muscle invasive	Gecks et al. ¹⁴⁹
	A1/ C	Clear cell RCC (ccRCC), papillary (pRCC), chromophobe-primary RCC (chRCC)	Detected FNIII A1/C in RCC samples. FNIII A1 in addition to FN ED-A/B was associated with vascular structures. By contrast FNIII-C was absent in ccRCC, strongly expressed in 80% of pRCC and was widely expressed in chRCC	Galler et al. ²⁴⁷
Mouse, human	Large isoforms	NIH-3T3, human melanoma cells	Overexpression of SRSF6 transcription factor in mice induces hyperplasia of sensitized skin, and in melanoma is associated with increases in expression of full length tenascin-C isoforms found in invasive carcinomas; Knockdown of SRSF6 in NIH-3T3 down-regulated large tenascin-C variant. SRSF6 associates with exons 10–14 (encoding FNIII-5,A1,A2,A3,A4)	Jensen et al. ¹⁶⁸

expression. Since one of the primary chemotherapeutics for the treatment of breast cancer is the ER antagonist tamoxifen, the prevalence of FNIII AD1 and AD2 may be suitable as predictor for tamoxifen-resistant breast cancer, or breast cancer which is not yet tamoxifen resistant; yet may become so. In this way, surveillance of the tenascin-C isoform expression pattern could positively impact patient treatment.¹²³

Other IHC studies illustrated how the pattern of tenascin-C splicing changes dynamically during disease progression. For example, anti-FNIII-D mAbs stained strongly in early stage I/II CRC, but less so in later stage III/IV tumors and liver metastases. However, FNIII-A1,A2,A4 containing isoforms were much more abundant in metastases than early stage tumors demonstrating a shift in expression from FNIII-D to A1,A2,A4 during the development of CRC.¹²⁵

These observations may prove useful in cancer patient diagnosis or stratification. For example, Driemel et al. demonstrated the diagnostic potential of combining hematoxylin and eosin

staining with BC-2 IHC, in atypical oral brush biopsies, which would allow for the additional detection of FNIII-A1/ A4 containing isoforms. This combination protocol increased the sensitivity and specificity of conventional HE staining from 96 to 99% and 78 to 95% respectively.¹²⁶

Consequences of FNIII repeat expression in cancer

Tenascin-C possesses the ability to modulate cell behavior in normal, tumor and tumor-associated cells in a manner which is cell-type specific, and which may either directly or indirectly affects cancer initiation and progression. Functional studies have identified roles for large tenascin-C variants in promoting a tumor supportive tissue microenvironment, with early studies identifying roles in the down-regulation of focal adhesion activity,¹²⁷ promotion of cell migration,⁵⁰ up-regulation of ECM degrading enzymes¹²⁸ and prevention of binding to FN;^{39,61,129} processes which would favor the development of malignancy by reducing adhesion and facilitating cell migration (Table 8).

Table 8. Functional consequences of tenascin-C alternative splicing in cancer. Individual splice variants separated by (,) are included in the same transcript, while those separated by (/) are not

Species	Alternatively Spliced FNIII Repeats (or size of splice variant if known)	Cell or Tissue Type	Features of Study	Reference
Human	none and A1,A2,A3,A4,B,C,D	U-251MG cell line, and BHKs transfected with human tenascin-C	Small isoform tenascin-C binds purified and mixed FN substrate via FNIII-3 and a region within FNIII-6,7,8. Solid phase binding assay demonstrated stronger binding of small rather than full length tenascin-C to FN. FNIII-A-D fusion protein showed minimal displacement of bound FN-Tenascin-C complexes compared to FNIII-3/3-5/1-5	Chung et al. ¹²⁹
Human	Small and large (220 and 320 kDa isoforms respectively)	Chondrosarcoma Cell Line JJ012	Small isoform of tenascin-C binds FN and promotes adhesion when bound to plastic. Large isoform does not bind FN and fails to promote cell attachment. Determined via ELISA, cell attachment assays, antibody blocking experiments	Ghert et al. ⁶¹
Human	D (~250 kDa)	Non-Small Cell Lung Carcinoma	Inhibits CD3-dependent lymphocyte proliferation and INF- γ secretion in tumor-invasive lymphocytes. Observed via Lymphocyte proliferation assay, ELISPOT assay and antibody perturbation. Eighteen-fold increase in large isoform expression	Parekh et al. ¹⁰⁴
Human	A2	SV40-transformed human embryonic lung fibroblasts (WI-38-VA-13 cell line)	FNIII-A2 domain contains syndecan-4 binding cryptic site exposed by MMP-2 digestion. Stimulates β 1 integrin mediated cell adhesion to FN. Suggests the extracellular portion of syndecan-4 binds FNIII-A2 and subsequently promotes β 1 integrin clustering and activation	Saito et al. ¹³²
Human	D/ B,D	Breast cancer (MCF-7, T47D, MDA-MD-231, MDA-MB-468, GI101) and fibroblasts	Overexpression of full length tenascin-C increased mean invasion index (MI) in MDA-MD-231 and T47D cells, but overexpression of FNIII- D and B,D by tumor cells enhanced proliferation and invasion significantly. Fibroblasts overexpressing full length, or FNIII-B/ B,D also promoted invasion of tumor cells. Tenascin-C upregulated expression of MMP-13 and TIMP-3	Hancox et al. ¹³⁰
Human	A1,A2,A3,A4,B,AD2,AD1,C,D	Pancreatic cancer	Recombinant FNIII-A-D bound cell surface annexin II, and suppressed gemcitabine mediated cytotoxicity in pancreatic cancer cells in dose dependent manner. This interaction increased intracellular phosphorylation status of PI3K, Akt, IKK α/β and NF-kB. NF-kB inhibition by siRNA restored gemcitabine cytotoxicity	Gong et al. ¹³⁵
Human	B,AD1,D	Breast cancer cell lines (MCF-7, T-47 D, ZR-75-1, MDA-MB-231 and GI-101)	Overexpression of FNIII B,AD1,D enhanced tumor cell invasion and growth relative to baseline levels	Guttery et al. ¹²³
Human	Small (220 kDa) and large (320 kDa)	Chondrosarcoma	Exogenous addition of 320 kDa isoform stimulates twice the levels of MMP-1 expression observed when adding 220 kDa isoform. Thirty-fold activation of MMP-1 promoter by 320 kDa isoform, compared to 220 kDa isoform. Furthermore, collagenase and invasion activity of chondrosarcoma increased 3-fold in 320 kDa variant treated cells	Galoian et al. ¹²⁸

Large tenascin-C isoforms may promote the destruction of the local tissue environment during tumorigenesis and metastasis by inducing the expression of ECM degrading enzymes such as MMP-1 in chondrosarcoma¹²⁸ and MMP-13 in breast cancer. Although here MMP-13 expression is accompanied by increased levels of TIMP-3, and was not tenascin-C isoform specific.¹³⁰ The degradation of the ECM and the driving of an epithelial-mesenchymal transition is crucial in the process of tissue invasion as observed in metastasis. In chondrosarcoma, addition of the large 320 kDa tenascin-C isoform conferred a 3-fold increase in collagenase and invasion activity via modulation of MMP-1 expression.¹²⁸

Functional studies in human cells have also served to elucidate the role of specific tenascin-C isoforms in malignancy, and these have been best characterized in breast cancer (reviewed in¹²⁴). AD1 containing isoforms promoted cell growth and invasion above control levels in breast cancer cells.¹²³ These data are supported by previous findings of FNIII-AD1 expression in breast ductal carcinoma, melanoma, retinoblastoma, basal cell carcinoma cell lines and in highly invasive Japanese quail fibrosarcoma cells (QT6 cell line).⁴⁹ QT6 predominantly express a major 230 kDa isoform containing 3 alternatively spliced FNIII, although minor isoforms containing 0, 1, 2 and 4 spliced FNIII are detected in low abundance. FNIII-AD1 is abundantly expressed in QT6 cells, and correlates with these cells exhibiting invasive capabilities as demonstrated in a Boyden chamber assay where they passed through a FN coated filter.⁴⁹ Other studies have also identified AD1 containing tenascin-C isoforms at sites of high cell motility.¹³¹ Together, these findings may suggest that AD1 containing tenascin-C isoforms modulate cell-FN interactions and contribute to a tissue microenvironment that facilitates changes in cell morphology, proliferation and migration in cancer.^{49,123,131}

Large tenascin-C isoforms have been directly implicated in the induction of proliferation, migration and control of cell spreading in vitro. MMP-2 mediated proteolytic cleavage of large tenascin-C variants reveals a cryptic syndecan-4 binding site within the FNIII-A2 domain. Here, a 22mer fusion peptide containing an adhesive YTTIRG sequence bound to the heparin sulfated side chains of the β 1 integrin co-receptor syndecan-4, hence inducing cell adhesion and spreading on FN.¹³² This study found that mutation of the cytosolic region of syndecan-4 had no effect on promoting the inhibition of β 1 integrin action, suggesting the extracellular portion of syndecan-4 binds FNIII-A2 and hence promotes β 1 integrin clustering and activation, rather than mediating this effect via downstream targets of syndecan-4. Elevated levels of both activated MMP-2 and degraded tenascin-C were identified in NSCLC, with proteolytically degraded tenascin-C serving as a good indicator of stage-1 NSCLC recurrence.¹³³ It is therefore possible to speculate a role for MMP-2 in degrading tenascin-C in lung cancer, and that FNIII-A2 cryptic site interaction with syndecan-4 in lung tissues play a role in mediating early stage recurrence of the disease.

A follow up study identified that sustained activation of α 5 β 1 by the FNIII-A2 cryptic site caused non-transformed mouse embryonic fibroblasts NIH-3T3 to become resistant to serum

deprivation-induced anoikis by activating the pro-survival Akt/Bcl-2 pathway, and enhanced PDGF-dependent proliferation of NIH-3T3 cells via promoting the association between PDGF receptor β (PDGF-R β) and the activated molecular complex that comprises of α 5 β 1 and syndecan-4.¹³⁴ This extended the activation of the PDGF-R β and its downstream Ras/MAPK pathways. Furthermore, the study suggested that tenascin-C may de-regulate cell growth processes by triggering the continual activation of α 5 β 1 by the FNIII-A2 cryptic site; this mechanism was implied by the observation that confluent monolayers of fibroblasts continued to proliferate in an α 5 β 1 dependent manner, and had apparently overridden their innate cell-density mediated cell cycle arrest mechanisms.¹³⁴ This demonstrates a highly significant role for MMP-2 cleaved tenascin-C in the promotion of cancer.

Chung and Erickson,⁸⁸ first showed that the large tenascin-C isoform containing the full alternatively spliced FNIII cassette is a high affinity binding partner of the cell surface located calcium-dependent phospholipid-binding protein annexin II, binding of which reduces focal adhesions, enhances mitogenesis and promotes cell migration.^{89,127} Similarly, addition of full length tenascin-C to pancreatic cancer cells ameliorated the cytotoxic response to chemotherapeutic agent gemcitabine, in a manner which was dependent upon binding between FNIII A1-D and cell surface annexin II.¹³⁵ Addition of recombinant FNIII A1-D increased the intracellular phosphorylation status of protein kinase B signal cascade constituents including PI3k, Akt, IKK α and NF-kB.¹³⁵ Although in this study the effects of FNIII A1-D were not assessed in the context of promoting cell survival, proliferation or growth, such phosphoproteins are central mediators of these processes; for example, phospho-Akt is a major promoter of cell survival and effects this via phosphorylating and hence inactivating a number of pro-apoptotic target proteins including Bad, Bax, Bim and Forkhead transcription factors (FOXOs).¹³⁶

The anti-FNIII-B mAb 4C8MS attenuated in vitro migration and mitotic activity in intraductal breast cancers.¹²² Functional overexpression of FNIII-D/ B,D and B,AD1,D in human breast cancer cell lines or FNIII B and B,D in fibroblasts significantly increased proliferative and invasive capabilities of breast cancer cells compared to vector controls in vitro; in addition to inducing a 2- and 4-fold increase in expression of MMP-13 and TIMP-3 respectively.^{123,130} Interestingly, during embryonic development, FNIII B and D promote neurite outgrowth in a manner dependent upon interactions with the F3/contactin and α 7 β 1 integrin,^{78,91} permitting speculation that conserved mechanisms promoting cell migration in embryogenesis are being re-activated in tumorigenesis.

A further antibody perturbation study determined that specific splice variants of tenascin-C containing FNIII-D reduced immune surveillance in solid tumors, evidenced by the large tenascin-C variant inhibiting CD3-FN mediated lymphocyte proliferation and interferon- γ (IFN γ) production in tumor invading lymphocytes.¹⁰⁴ The presence of FNIII-D would therefore be expected to be accompanied by increased tumor recurrence rates on account of an impaired immune clearance mechanism. Interestingly, Parekh et al.¹⁰⁴ found that large 250 kDa isoform expression is increased 18-fold in recurrent

NSCLC compared to non-recurrent NSCLC; which if presuming FNIII-D expression was increased also, would be consistent with this hypothesis.

Serum levels of tenascin-C

Without taking into account individual splice isoforms, global serum levels of tenascin-C have been shown to have either diagnostic or prognostic functions in pancreatic adenocarcinoma,¹³⁷ CRC (healthy control 3.2 ± 1.7 $\mu\text{g/ml}$, CRC mean 5.0 ± 4.2 $\mu\text{g/ml}$, CRC without metastases 4.3 ± 3 $\mu\text{g/ml}$

CRC with metastases 6.8 ± 6 $\mu\text{g/ml}$),¹³⁸ inflammatory bowel disease (healthy control 3.2 ± 1.7 $\mu\text{g/ml}$, prior to surgery for ulcerative colitis (UCC) 17.2 ± 4.6 $\mu\text{g/ml}$, ulcerative colitis + ongoing medical treatment, median 5.3 $\mu\text{g/ml}$, Crohn's disease + ongoing medical treatment, median 4.9 $\mu\text{g/ml}$),¹³⁹ melanoma (healthy control 444 ± 171 ng/ml, stage I/II melanoma 419 ± 103.3 , stage IV melanoma 782.6 ± 629.5),¹⁴⁰ and auto-immune bullous diseases of the skin.¹⁴¹ In NSCLC patients tenascin-C is cited as a predictive biomarker of angiogenesis, and levels above 96 ng/ml in patient serum inversely correlate with patient survival.¹⁴² However in diseases like breast cancer and ovarian cancer where serum levels of tenascin-C are elevated from 137 ± 26.8 pg/ml and 90.1 pg/ml to 344.1 ± 42.4 pg/ml and 130.5 pg/ml respectively, there is no predictive or prognostic function in relation to patient survival,^{143,144} with another study identifying elevated serum tenascin-C levels as a dubious tumor biomarker as serum levels also increased in during infection and as a consequence of inflammation.¹⁴⁵

Two studies identified elevated serum levels of FNIII-C as a suitable tumor biomarker for CRC (healthy control 2364.3 ± 749.6 pg/ml, primary CRC 5260 ± 3243.3 pg/ml, recurrent CRC 4160 ± 2261.1 pg/ml), where it exceeded the sensitivity of conventional CRC diagnostic tests for serum markers carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) at 40.1% and 23.6% respectively, and diagnosed CRC with 56.6% sensitivity,¹⁴⁶ furthermore elevated serum levels of FNIII-C were associated with hepatic recurrence of CRC metastases.¹⁴⁷

Urine levels of FNIII-B from patients with UCC and urothelial carcinoma of the urinary bladder (UBC) have been quantified in several studies using commercially available ELISA kits (FNIII-B containing isoforms: healthy control 200 ng/ml, cystitis 650 ng/ml, pTa grade UBC ~ 1000 ng/ml, pT3-4 grade UBC ~ 8500 ng/ml, FNIII-C containing isoforms: healthy control ~ 60 ng/ml, cystitis ~ 200 ng/ml, pTa grade UBC ~ 200 ng/ml, pT3-4 grade UBC ~ 990 ng/ml) (FNIII-B containing isoforms: non-muscle invasive UBC ~ 2.4 ng/ml, non-muscle invasive relapse ~ 2.9 ng/ml, muscle invasive relapse ~ 8 ng/ml).^{148,149} While proteolytic fragmentation of tenascin-C was observed in the urine from invasive UCC patients, and was indicative of enhanced protease activity and tissue destructive disease,¹⁴⁸ FNIII-B concentration in UBC patient urine could predict existence or recurrence of a tumor, and also whether the disease was muscle invasive.¹⁴⁹ The first study also quantified

urine levels of FNIII-C containing isoforms, although these were found not to be indicative of disease stage in UCC patients.¹⁴⁸

However, the number of cancers where serum levels of tenascin-C sufficiently correlate with disease progression is low and tenascin-C would appear an unsuitable universal cancer biomarker as had perhaps once been hoped. Moreover, the serum levels of tenascin-C detected in healthy controls vary vastly from one study to another, which may partly be explained by use of different ELISA kits and sample preparation, but not wholly, as many of these studies use comparable methodology. Therefore at the current time, in some specific disease types, serum or urine tenascin-C surveillance may improve diagnosis when combined with already existing techniques. Interestingly, tenascin-W exhibits an even more restricted expression pattern in normal tissues than tenascin-C and has been proposed as a superior biomarker for malignancy.¹⁵⁰

Therapeutic exploitation of tenascin-C splice variants

The strong association between the expression of alternatively spliced FNIII repeats of tenascin-C with tumors, and in many cases very aggressive tumors, combined with their reduced expression in normal healthy tissues has raised interest in pharmacological targeting of these modules. These studies are extensively reviewed in,³ and are therefore only summarized below.

Most studies to date have focused on using antibodies to alternatively spliced FNIII domains to deliver cytotoxic or anti-inflammatory factors direct to the tumor, but given the active role these domains play in driving tumor cell biology, there also exists the potential for developing antagonists of these domains for use in cancer treatment. Antibodies specific for FNIII-A1 (F16) and FNIII-C/D (81C6) have been best characterized, although others are in development. ¹³¹I-labeled 81C6 has been trialled as a therapeutic in malignant glioma where it targets residual cancer cells following brain surgery.¹⁵¹ ¹³¹I-81C6 increased median survival of glioblastoma multiforme patients, although correlations were observed between irreversible neurotoxicity and decreased survival. Nevertheless, ¹³¹I-labeled 81C6 delivers highly targeted radio-isotope therapy and is now undergoing phase III trials with the trade name Neuradiab.^{152,153} Three other anti-tenascin-C antibody conjugates are currently under development, all of which use the FNIII A1 targeting antibody F16.¹⁵⁴ F16 is conjugated with interleukin (IL) -2, ¹³¹I, and ¹²⁴I for the therapy of lung and breast cancer, Hodgkin's lymphoma and various tumors respectively.¹⁵³ F16-IL-2 is reportedly a highly efficacious and safe immunotherapeutic when administered in combination with the chemotherapeutic temozolomide to BALB/c nude mice with sub-cutaneous and intracranial human glioblastoma tumors, F16-IL-2 induced a complete remission in these animals, and promoted leukocyte infiltration of the tumors. Following complete remission, the mice remained tumor free for 160 d.¹⁵⁵ The latest instalment in this exciting story is published in this issue in the article by Catania et al, who report data from a phase Ib/II trial administering F16-IL2 cytokine fusion proteins in combination with doxorubicin to patients with solid tumors and metastatic breast cancer.

Alternative splicing beyond development and cancer

Alternative splicing of tenascin-C is not restricted to developing tissues and tumors, but is more likely a universal feature of tenascin-C expression during tissue remodeling. Indeed 22 different tenascin-C splice isoforms were identified in pseudo-phakic and aphakic bullous keratopathy corneas, with the predominant isoforms containing one, 2, both or neither of the FNIII A1/D modules.^{156,157} These data suggest that a detailed and systematic examination of tenascin-C splicing will reveal important information about the role of this molecule in different locations. Using well-established PCR techniques requiring the generation of primers to flank the alternatively spliced region and subsequent ISH, cloning and sequencing of the expanded PCR products,^{31,69} or exploiting the ability of deep sequencing technology to identify all mRNA species present within cells of different tissues and lineages, will help to reveal the full picture of this post transcriptional modification of tenascin-C at the mRNA level.

Regulation of tenascin-C splicing

As described above, tenascin-C splicing is regulated in a very distinct spatio-temporal manner, which is clearly observed during embryogenesis and in numerous pathological conditions. The mechanisms by which this complex process is controlled in such a highly orchestrated time and location dependent manner are beginning to emerge.

The regulation of tenascin-C expression is mediated by growth factors and cytokines in a situation-specific response. During embryogenesis in humans and mice, tenascin-C expression is upregulated by TGF β .^{47,158} TGF β -1 provides the most significant upregulation of tenascin-C although expression is also responsive to TGF β -2, -3 and -5.¹⁵⁹ TGF β -1 is also observed to induce preferential expression of the small tenascin-C isoform lacking FNIII A1-D in endometrial adenocarcinoma and NIH-3T3 cell lines.¹⁶⁰ In vivo, tenascin-C expression closely correlates with expression of TGF β in the heart, bone marrow megakaryocytes, breast cancer, ovarian cancer and inflamed joints.^{161,162} FGF is also a potent inducer of tenascin-C expression, with the expression pattern of FGF2 in the mouse CNS corresponding with that of tenascin-C.⁴⁷ In addition to FGF up-regulating tenascin-C expression on the whole, FGF1 and FGF2 has been shown to preferentially induce the expression of large tenascin-C splice variants in the lateral ventricles of the rat brain, and the NIH-3T3 fibroblast cell line respectively.^{47,160,163}

The expression of tenascin-C is influenced by the presence of pro-inflammatory cytokines, which also can either up-regulate the expression of all tenascin-C isoforms, or induce the preferential expression of long (tenascin-C isoforms containing one or more alternatively spliced FNIII), or short isoforms (containing no alternatively spliced FNIII). This effect has been investigated in keratinocytes, where IL-4 has been shown to induce the expression of short and long mRNA transcripts equally, with a relative abundance of 1:1.5. In contrast, the potent immune-activator tumor necrosis factor- α (TNF α) preferentially induces the expression of short tenascin-C transcripts, while IFN γ favors the expression of long transcripts.¹⁶⁴

Another important regulator of tenascin-C splicing is intracellular pH (pHi). In cell cultures of lung and dermal fibroblast cell-lines, a physiological pH $\sim$ 7.0 results in the expression of small tenascin-C splice isoforms lacking the alternatively spliced FNIII domains A1-D. However when cells are exposed to more basic pH $\sim$ 7.30–7.50 as is the case in artificial culture experiments, fetal tissues and aggressive tumors, tenascin-C expression switches to the longer-isoform with one or more alternatively spliced FNIII repeats included in the transcript.^{153,165,166} pH regulation of tenascin-C splicing may therefore contribute to the diverse range of tenascin-C splice variants which are found in human fetal membranes.⁹⁶ Similarly, malignantly transformed fibroblasts principally express large tenascin-C isoforms regardless of the extracellular pH in vitro (pHo); interestingly, this is because these cells maintain a basic pHi throughout a wide range of pHo.¹⁶⁶ Bumke et al.,¹⁶⁷ confirmed that the large isoform is preferentially expressed at basic pHo, but that serum starvation in fibroblasts reduces expression of the large tenascin-C isoform; independent of culture media pH.

Most recently, the splicing factor protein SRSF6 has been identified as a regulator of tenascin-C alternative splicing. SRSF6 induction in transgenic mice resulted in expression of full-length tenascin-C isoforms, which were absent in normal uninduced skin. The presence of the full-length tenascin-C isoform accompanied hyperplasia of keratinocytes, although this effect and the abundance of full-length tenascin-C could be reverted following removal of SRSF6 induction.¹⁶⁸ SRSF6 mediated alternative splicing regulation of tenascin-C results in the excessive proliferation of keratinocytes in response to tissue injury, and therefore SRSF6 can modulate tissue homeostasis in vivo by promoting the expression of long tenascin-C isoforms in preference to shorter ones.

Post Translational Regulation of Tenascin-C

In addition to control of tenascin-C function at the transcriptional and post transcriptional level, the impact of tenascin-C on cell phenotype is profoundly affected by modifications to the protein after translation.

Tenascin-C biosynthesis

Following translation, tenascin-C assembles intracellularly into a 1.1–2.0 MDa 6-armed hexabrachion structure comprised of 6 monomers.¹⁶⁹ This process is largely determined by N-terminal residues which are arranged into 3 cysteine-rich heptad repeats which form a coiled-coil region (amino acids 118–145 in human tenascin-C);^{38,170} a feature common to other oligomeric matricellular proteins such as thrombospondin and cartilage oligomeric matrix protein-1.¹⁷¹ Coiled-coils have a repeat pattern of hydrophobic and charged amino acids arranged as *hxxhxcx* (denoted *a-g*) called a heptad. In tenascin-C, these repeats produce short α -helices in which residues *a* and *d* form a 3,4-hydrophobic repeat, creating a stable non-polar core buried at the center of the helix. This stable arrangement projects the charged Cysteine side-chains at positions *e* and *g* out from the

helix, where they can promote the oligomerization of tenascin-C subunits by forming inter-chain di-sulfide bonds.¹⁷⁰

Two models for tenascin-C oligomerization have been proposed. In one model this event is a rapid co-translational process where 6 monomers are simultaneously assembled into a single hexabrachion in as little as 60 minutes following translation initiation. These data are based on pulse-chase analysis which revealed an absence of any intermediate oligomers suggesting that assembly is initiated at the N-terminus and completed in a single step, before translation is completed.¹⁷¹ This contrasts with models of assembly described for other large ECM proteins such as collagen, FN and vonWillebrand factor which are all assembled post-translationally from C-terminal assembly start points. In this model, 6 ribosome-associated polypeptide chains would have to multimerize simultaneously during translation. It has been suggested that the conformation of poly-ribosomes on the surface of the endoplasmic reticulum may be conducive to this: these ribosomes have been observed by electron microscopy to be 35 nm apart. Taking in to account the length of the N-terminal region of the TA domain and the ER membrane-spanning region in the newly translated peptide, any newly synthesized peptide must be 55 nm in length to permit N-terminal binding with other ribosome associated transcripts. As human tenascin-C transcripts have been recorded at 86.6 nm in length when folded,¹⁷² it is argued that the N-terminal heptad repeats on newly synthesized tenascin-C transcripts could engage each other and form the hexabrachion in a co-translational manner.¹⁷¹ In concordance with this, tenascin-R is shorter than tenascin-C and has previously been observed to form only dimers and trimers, but not hexamers; perhaps because the polypeptide chain is too short to allow interactions with 6 other transcripts simultaneously.

In the second model, the hexabrachion has been described as a composite of 2 trimers; each of which are stabilized by coiled-coil interactions prior to dimerization of these 2 subunits into the hexabrachion.¹⁷⁰ This description concurs with preliminary electron micrograph images of the hexabrachion by Erickson and Inglesias, which revealed the presence of a single central globular domain tethered by 2 T-junctions, each in turn bound to 3-arms. In this model, hexabrachion assembly is described as a 2-step process dependent upon key Cysteine residues and a conserved glutamic acid residue. Site-specific mutagenesis was utilized to create numerous N-terminal tenascin-C polypeptide fragments which were then analyzed by far-UV circular dichroism for signs of secondary structure development. In this model the tenascin-C monomers form homotrimer intermediates stabilized at the N-terminal heptad repeats by a parallel 3-stranded α -helical coiled-coil (Fig. 3). This conformation clusters the 3 TA domains from each homotrimer together (Fig. 3), allowing 2 homotrimers to bind via their respective TA domains by a high concentration of weak homophilic interactions.¹⁷⁰

The substitution of the amino acid residues present within or nearby the N-terminal heptad repeats can significantly alter the hexabrachion assembly process. Substitution of the N-terminal C-64 residue with glycine results in the formation of trimeric tenascin-C intermediates suggesting a functional role for C-64 in

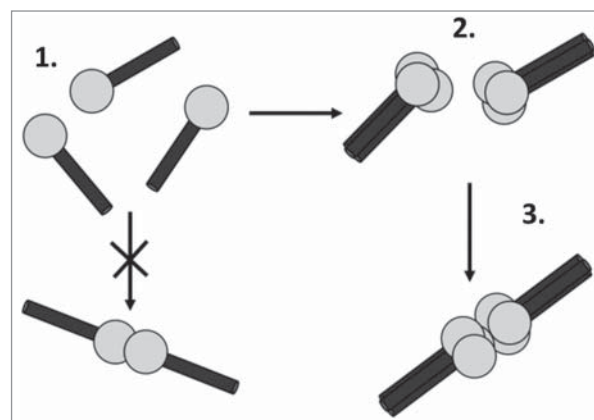


Figure 3. Hexabrachion assembly is a 2-step process. Multimerization of the N-terminal region of tenascin-C during hexabrachion assembly. 1. The N-terminal region of 3 tenascin-C monomers. Black cylinders represent the N-terminal heptad repeat residues 118–145, and gray circles represent TA domains. 2. The N-terminal heptads contain 3 cysteine-rich heptad repeats with hydrophobic (h) and charged (c) amino acid residues arranged in the conformation *hxxhxc*. These monomers form an intermediary trimer which is stabilized by α -helical coiled-coil interactions between the N-terminal domains of the monomers. Three. The oligomerization of the adjacent TA domains increases homophilic binding affinity between the 2 trimers, which bind to form the hexabrachion. Disulfide bonds stabilize the hexabrachion but are not required for its formation (Adapted from Kammerer et al.¹⁷⁰).

joining trimeric intermediates into hexabrachions, perhaps by disulfide bonding. No other N-terminal Cysteine residue is considered essential for hexabrachion assembly, because site directed mutagenesis of C other than C-64 within the N-terminal heptad repeat region (C-111, 113, 146, 147) still results in the secretion of hexamers from the cell regardless of whether conducted on individual Cysteine or in pairwise combinations.¹⁷³ Substitution of all Cysteine residues simultaneously at positions 111, 113, 140, 146 and 147 did not prevent hexabrachion formation, with the majority of oligomers containing over 4 subunits. However subsequent mutation of C-64 resulted in tenascin-C monomers, compounding the importance of C-64 in tenascin-C oligomerization via di-sulfide bonds.¹⁷³

Substitution of a conserved glutamic acid-130 with alanine or leucine at heptad position *e*, abolishes the interaction between the residue at this position and hydrophobic residues at positions *a* and *d*. This allows for more efficient packing of hydrophobic residues within the heptad to increase the number of α -helices which can join the coiled-coil from 3 to 4; forming a 4 stranded α -helical coiled-coil.¹⁷⁰ Normally, E130 forms a salt-bridge linkage with arginine-125 in a preceding heptad on an adjacent tenascin-C polypeptide chain.

While hexabrachions are often formed as homotypic molecules comprised of identical subunits, there are instances where heterotypic molecules can be formed. Kammerer et al.¹⁷⁰ demonstrated that 2 triplets of tenascin-C trimers; one missing both the FNIII and FBG domain, were able to heterodimerize forming a heterotypic hexabrachion. Furthermore, smaller oligomers comprising of 2–5 tenascin-C molecules have also been

identified, although it is not known whether these smaller isoforms are deliberately assembled in this manner, or if they are merely remnants of hexabrachions degraded by proteases.¹⁷²

Post-translational modification of tenascin-C

The assembly of the hexabrachion from tenascin-C monomers reportedly takes only 5 minutes, with a rate limiting step in tenascin-C biosynthesis coming at the point of transport from the endoplasmic reticulum to the golgi apparatus.¹⁷¹ This is indicative of substantial post-translational modification of tenascin-C and indeed this molecule has been shown to be glycosylated.

Human tenascin-C possesses 26 potential *N*-glycosylation sites, of which 2 are in the TA domain, 2 are in the EGF-L repeat region, 21 are in the FNIII domain, and 1 is in the FBG domain (Fig. 4).^{23,174} Analysis of the human tenascin-C cDNA sequence revealed a cluster of 10 potential N-linked glycosylation sites within the alternatively spliced FNIII repeats A1-A4, within 2 clusters given as NLT-X₁₁-NWT-X₂₃-NLT-X₆₃-NWT-X₂₃-NLT. Three further putative *N*-glycosylation sites were identified in human FNIII B,^{19,23} and further analysis with the NetNGlyc 1.0 Server revealed 4 more predicted *N*-glycosylation sites in the FNIII AD1 and AD2 domains.¹⁷⁴ In chicken tenascin-C, 17 potential *N*-glycosylation sites were identified by gene analysis, 6 of which were located in the FNIII AD1 and AD2 domains.³⁸

Analysis of the human tenascin-C peptide sequence with the NetOGlyc 4.0 Server tool; which predicts mucin type GalNAc *O*-glycosylation sites in mammalian proteins, predicts 34 putative *O*-glycosylation sites within tenascin-C.¹⁷⁵ Of these, 8 were located in the TA domain, 2 within the EGF-L repeat region and 24 within the FNIII domain, while none were located in the FBG domain (Fig. 4).¹⁷⁵ At first glance, the distribution of potential *O*-glycosylation sites within tenascin-C clearly contrasts with that of *N*-glycosylation sites. *O*-glycosylation sites are densely arranged within the TA domain and FNIII 1–5, perhaps suggestive of a role in modulating macromolecular assembly and cell adhesion processes. By contrast, *N*-glycosylation sites are predominantly found within the alternatively spliced FNIII and may therefore be implicated in mechanisms related to development and tumorigenesis.

Confirmation of tenascin-C glycosylation derived from studies where purified human or chicken tenascin-C was treated with

glycoside hydrolase enzymes and revealed changes in apparent *M_r* when subject to reducing-PAGE. Treatment of affinity purified chick embryonic fibroblast tenascin-C with endoglycosidase-F, was shown in 2 studies to result in a ~20 kDa,¹⁷⁶ and ~10 kDa,⁵⁰ reduction in apparent *M_r* of all 3 tenascin-C splice variants detected. Additionally, treatment with neuraminidase resulted in a 10 kDa reduction in *M_r* of all gradient purified human glioma and chicken embryonic fibroblast tenascin-C variants detected.¹⁶⁹ All together, these data suggest that carbohydrates contribute significantly to the mass of tenascin-C in humans and chickens, and that the most active glycosylation sites must exist within the constitutively expressed portion of the molecule, as in each study the *M_r* of all tenascin-C splice variants was affected equally by endoglycosidase-F and neuraminidase treatment. Not much is known about the tissue specific expression patterns and the functional significance of tenascin-C glycosylation beyond studies performed on the HNK-1 epitope.⁷² The HNK-1 epitope, is presented on either an *N*- or *O*-linked oligosaccharide and believed to play a role in mediating cell adhesion and substrate binding processes. HNK-1 is distributed on a range of neural cell adhesion molecules,^{73–75} and also in some lymphocyte subsets. In tenascin-C this epitope is found in isoforms containing FNIII-A1,A2,A4,B,C,D and which promoted NSC proliferation via modulating the expression of the EGF-R.⁷¹ In this way the presence of glycosylation sites on alternatively spliced FNIII subunits confers greater control of protein function which can be regulated by alternative splicing.

Glycosylation of tenascin-C is likely to significantly impact its ability to modulate cell adhesive and protein binding functions. This may occur via the blocking of receptor and protein binding sites by large branched carbohydrate chains, which obscure tenascin-C from potential binding partners. It is perhaps via this mechanism that the collagen binding affinity of vitronectin is reduced upon glycosylation,¹⁷⁷ and that fibroblast cell adhesion and spreading is improved following a lack of FN glycosylation.¹⁷⁸ In contrast, the addition of new carbohydrate epitopes to tenascin-C may enhance protein and cell interactions, for example sialylation of vitronectin is required for rat hepatic stellate cell spreading,¹⁷⁹ and *O*-glycosylated oncofetal FN induces epithelial to mesenchymal transition in lung cancer cells in synergy with TGFβ.¹⁸⁰

Tenascin-C glycosylation may confer a strategy by which the protein is protected from proteolytic degradation. An early study on rat FN revealed that less glycosylated variants exhibit a greater rate of protease degradation,¹⁸¹ indicating early on that FN glycosylation protects the protein from fragmentation; therefore a similar protective mechanism may be conserved in tenascin-C. The abundance of putative *N*-glycosylation sites within the alternatively spliced FNIII A1-D closely associate with the FNIII domains which are susceptible to protease degradation by MMP-1, -2, -3 and -7;^{96,97} and may

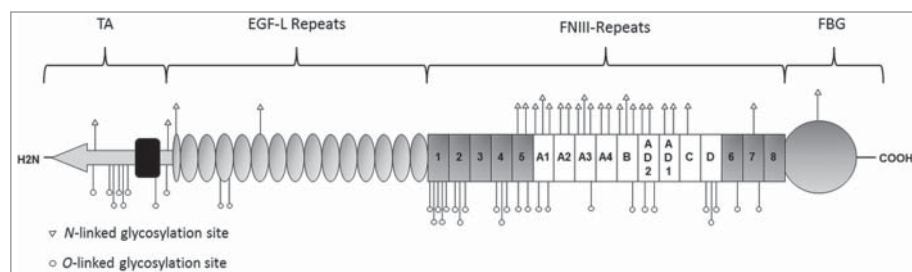


Figure 4. Predicted *N*- and *O*-glycosylation sites within tenascin-C. Tenascin-C possesses 26 putative *N*-glycosylation sites,^{19,23,174} and 34 putative *O*-linked glycosylation sites;¹⁷⁵ the locations of which are represented on the tenascin-C stick diagram by triangles and circles respectively.

therefore exist to improve the half-life of tenascin-C.

Glycosylation of tenascin-C may offer a mechanism by which macromolecular assembly of the hexabrachion can be regulated by the expression of glycosyltransferase enzymes. The impact of glycosylation on multimer assembly is observed in vitronectin, where a reduced glycosylation state is associated with increased multimer size.¹⁷⁷ It is therefore possible that the prevalence of *N*- and *O*-glycosylation sites within in the TA domain of tenascin-C is so high in order to function in a manner designed to interfere with macromolecular assembly of the hexabrachion, which may therefore indirectly affect the ability of the HXB to integrate into the ECM, although this is just speculation.

Despite a wealth of evidence revealing that tenascin-C is glycosylated, specific glycosylation sites have not yet been characterized in the protein, and as a result it is not known how many of the putative *N*- and *O*-glycosylation sites are active. Although glycosylation of tenascin-C is understood to modulate cell adhesion, substrate binding and cell proliferation processes, there are likely many more functions yet to be discovered. Further studies are required to advance our understanding of the exciting role played by tenascin-C glycosylation in modifying cell behavior.

Assembly of tenascin-C into a fibrillar matrix

Once suitably modified, the export of glycosylated tenascin-C from the golgi is rapid, with hexabrachions taking as little as 45 minutes to be detected in the conditioned culture medium of U-138 glioma cells after addition of a labeling medium.¹⁷¹

Tenascin-C is incorporated into the ECM in a manner which is dependent upon the presence of heparan sulfate proteoglycans (HSPGs). Chung and Erickson., generated Chinese hamster ovary cell mutants with a total block in heparin sulfate and chondroitin sulfate synthesis; this blockade resulted in the failure of the cells to assemble a FN matrix, and no tenascin-C was incorporated into the ECM as a result.¹⁸² The generation of a tenascin-C rich ECM has previously been shown to be dependent upon a template of FN and $\alpha 5$, αv and $\beta 1$ integrins.¹⁸³ It may be that once incorporated into the ECM, tenascin-C stabilizes FN as tenascin-C knockout mice generally exhibit reduced FN deposition.^{15,184}

Tenascin-C may bind to proteoglycan molecules which are themselves bound to FN fibrils; in support of this an analysis revealed a co-localization between perlecan, FN and tenascin-C. Perlecan binds the constitutive FNIII 3–5 of tenascin-C, and this binding is enhanced in the short tenascin-C isoform (~190 kDa). The insertion of alternatively spliced FNIII repeats after FNIII 5 results in an attenuation of perlecan-tenascin-C binding;¹⁸² and could possibly reduce adhesion in cells expressing longer isoforms of tenascin-C, as is observed in cancer. Another factor in matrix formation is the ECM molecule periostin, which has been shown to aid incorporation of tenascin-C into the ECM, albeit through a currently unknown mechanism.¹⁸⁵

It is not clear how tenascin-C may be affected by incorporation into a 3-dimensional matrix, a process very likely to change the conformation of this molecule, perhaps exposing or concealing specific domains and binding sites. Moreover, it is not known how tenascin-C signals as part of a multicomponent

ECM, where interaction with other matrix residents may affect the availability of binding sites and where molecules can synergistically regulate cell behavior. What is clear is that the conformation of tenascin-C does have profound effects on its function; this is evidenced by data showing that soluble tenascin-C binds readily to FN in glycerol gradients assays,¹²⁹ where the extended conformation of both proteins appear to favor interaction.¹⁸⁶ Conversely, FN does not bind to tenascin-C that has been immobilized onto ELISA plates, but tenascin-C can bind to immobilized FN.^{39,129,187,188} As such the context and conformation of tenascin-C should be considered major players in defining ECM function.

Proteolytic processing of tenascin-C

One other means of regulating tenascin-C function is proteolytic cleavage. Fragments of tenascin-C have been reported in swabs from individuals with gingivitis,¹⁸⁹ chronic venous leg ulcer exudates,¹⁶⁴ and atherosclerotic plaques¹⁹⁰ among other locations.

Mice lacking MMP-7¹⁹¹ or MMP-9¹⁹² exhibited lower levels of tenascin-C and tenascin-C fragments after myocardial infarction, suggesting that these metalloproteases can degrade tenascin-C in vivo. In contrast, MMP-19 null mice exhibited elevated tenascin-C protein during a model of asthma, indicating that MMP-19 may prevent the accumulation of tenascin-C.¹⁹³ Tenascin-C is also susceptible to proteolytic degradation by MMPs-1, 2, 3 and 7 in vitro with the majority of MMP cleavage sites located within the alternatively spliced region of the protein, making long tenascin-C isoforms more susceptible to fragmentation than shorter isoforms.⁹⁷ MMP-7 cleaves all tenascin-C isoforms by removing the 16 kDa N-terminal knob, but it also digests FNIII A3-D within the alternatively spliced region; its ability to digest AD1 and AD2 is not yet known.⁹⁷ However, in chronic wounds tenascin-C fragmentation was not inhibited by EDTA and E64 ruling out a role for MMPs and cysteine proteases. It was subsequently confirmed that tenascin-C was digested by leucocyte elastase and possibly other serine proteases.¹⁶⁴ Furthermore, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) -5 has demonstrated an ability to cleave tenascin-C into fragments of which some correspond to the N-terminal TA domain,¹⁹⁴ with specific tenascin-C fragments identified as inducers of cartilage matrix degradation.¹⁹⁵

Degradation of tenascin-C results in modulation of tissue protein levels, a process key to limiting tenascin-C expression in situations where transient expression is vital, for example in the developing embryo and in healing wounds. The reduction over time of longer variants compared to shorter variants fits well with data showing that longer isoforms are more readily cleaved. Proteolysis also releases smaller fragments from large multimodular proteins that exhibit quite different functions to the intact molecule. For example, fragmented EGF-L domains of tenascin-C induce apoptosis in cultured smooth muscle cells (SMCs) in contrast to full length tenascin-C,¹⁹⁰ and FN matrix assembly is unaffected by full-length tenascin-C, but is inhibited by the FNIII domains or the FBG domain of tenascin-C.¹⁹⁶ Similarly,

cleavage of tenascin-C within the FNIII A3 subunit by MMP-2 generates a cryptic binding site within FNIII A2 which permits interaction with heparan sulfated side-chains of syndecan-4 to promote $\beta 1$ integrin clustering and cell spreading.^{97,132} Finally, full-length tenascin-C is found to be anti-adhesive and to prevent FN-mediated cell spreading of T98G glioblastoma cells, but this effect was lost following cleavage with the metalloprotease meprin β .¹⁹⁷

Summary and Conclusions

Following tenascin-C through its life cycle, from the switching on of *TNC*, through transcription, translation, and protein modification, all the way through to its degradation, reveals a number of mechanisms by which different forms of this molecule are created. Moreover, it is clear that the spatial and temporal control of these events enables the expression of precise tenascin-C forms with distinct functions designed to carry out specific tasks at the right time and place. The molecular events that enable different stimuli to induce the expression of specific spliced variants of tenascin-C remain to be elucidated. However, the way in which the inclusion of alternatively spliced FNIII domains with unique interaction sites confers new binding capabilities or susceptibilities to proteolytic cleavage to tenascin-C is exemplified in many tissues during development and in tumors. Conversely, the inclusion of extra FNIII repeats that interrupt existing binding sites,

causing loss of a particular function, is also observed at these locations. Post translational modification of tenascin-C has not yet been extensively studied but already data suggest that glycosylation can have a profound effect on its function. Moreover, it is emerging that the architecture of tenascin-C matters; its assembly into a fibrillar matrix at the cell surface impacts the conformation of this molecule, potentially exposing cryptic binding sites or covering exposed binding sites, and it will be of great interest to determine precisely how tenascin-C acts within the context of complex, 3D tissue environments. Finally, even proteolytic destruction of tenascin-C creates collateral damage that confers novel functions to this molecule. The destruction of existing binding sites and generation of smaller fragments with new binding sites can drive entirely novel processes compared to the intact molecule. It seems that the more we learn about this extraordinary matrix glycoprotein, the more complex and intricate the story becomes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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C1855 How to detect and purify
tenascin-C

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2 Detecting tenascin-C expression

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Abstract

The extracellular matrix molecule tenascin-C (TNC) was discovered over 30 years ago, and its tightly regulated pattern of expression since sparked keen interest in the scientific community. In adult tissues, TNC expression is restricted to specific niches and areas of active remodeling or high mechanical strain. However, while most healthy tissues contain little TNC, its transient expression upon cellular stress or tissue injury helps to mediate repair and restore homeostasis. Persistent expression of TNC is associated with chronic inflammation, fibrosis, and cancer, where methods for its detection are emerging as a reliable means to predict disease onset, prognosis, and response to treatment. Because studying the expression of this large matrix molecule is not always straightforward, here we describe basic techniques to examine tissue levels of TNC mRNA and protein. We also describe methods for purifying recombinant TNC, knocking down its expression, and creating cell-derived matrices with or without TNC within.

1 INTRODUCTION

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Tenascin-C (TNC) is a highly conserved extracellular matrix protein, without obvious mutational hotspots, reflecting its importance for the maintenance of tissue homeostasis (Adams, Chiquet-Ehrismann, & Tucker, 2015). Its synthesis is tightly regulated (Chiovaro, Chiquet-Ehrismann, & Chiquet, 2015), with high levels expressed during development, and with expression restricted to remodeling tissues in the adult, for example, tendon, muscle, reticular fibers, and some stem cell niches (Chiquet-Ehrismann, Orend, Chiquet, Tucker, & Midwood, 2014; Midwood, Chiquet, Tucker, & Orend, 2016). TNC is transiently induced upon tissue trauma where it promotes repair; however, high levels of uncontrolled expression are associated with a wide range of pathological conditions including autoimmune diseases, fibrosis, and cancer. Data showing that TNC actively contributes to disease pathogenesis, and correlating expression levels with disease severity or prognosis, have raised interest in using TNC expression diagnostically, as well as in therapeutically targeting its activity (Berndt, Richter, Kosmehl, & Franz, 2015; Brosicke & Faissner, 2015; Franz, Jung, Lauten, Figulla, & Berndt, 2015; Kasprzycka, Hammarstrom, & Haraldsen, 2015; Lowy & Oskarsson, 2015; Spenle et al., 2015; Udalova, Ruhmann, Thomson, & Midwood, 2011). However, analysis of tenascin-C expression is a complex issue, colored by a myriad of transcriptional and translational modifications that confer tissue-specific functions to this modular matrix molecule. For example, alternative splicing within the fibronectin

type III-like repeats (FNIII) can generate hundreds of distinct isoforms, the structural organization of tenascin-C can define its binding capabilities to other matrix molecules and cell surface receptors, and citrullination of the fibrinogen-like globe (FBG) can alter the immunogenicity of this domain, creating variants able to break immune tolerance (Giblin & Midwood, 2015; Midwood et al., 2009; Raza et al., 2016; Schwenzer et al., 2016; Tucker & Chiquet-Ehrismann, 2015; Yoshida, Akatsuka, & Imanaka-Yoshida, 2015). Here, we describe methods to assess tissue and cellular levels of tenascin-C mRNA and protein, and to define the localization and structural organization of this molecule, as well as ways to profile splicing patterns and to detect autoantibodies generated to pathogenic forms of tenascin-C. We also describe how TNC expression can be downregulated, recombinant TNC can be purified, and cell-derived matrices with abundant and no TNC can be generated; in the companion chapter in this book, cellular assays in which these techniques can be applied to investigate tenascin-C function are described.

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2 ANALYSIS OF TENASCIN-C mRNA

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2.1 TENASCIN-C REAL-TIME qPCR

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Overview: Quantification of Tenascin-C mRNA copies/cell

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Duration: 3 h (1 h for sample and standard preparation, 2 h for PCR thermocycler run)

Equipment

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- Real-time PCR (RT-PCR) instrument Rotor-Gene 6000 (Corbett) or ViiA7 (Applied Biosystems)

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- 4-Strip tube or 384-well plate format compatible with respective RT-PCR thermocycler instruments

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- Micropipettes 1 mL, 200 μ L, and 10 μ L

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- 1 mL, 200 μ L, and 10 μ L pipette tips

u0030

- Autoclaved RNase- and DNase-free 1.5-mL Eppendorf tubes

u0035

- NanoDrop

Materials

u0040

- Pyrogen- and RNase-free, distilled water (dH₂O)

u0045

- 2 $\times$ TaqMan[®] Gene Expression Master Mix (ThermoFisher Scientific)

u0050

- 20 $\times$ TaqMan[®] Gene Expression Assay (ThermoFisher Scientific)

u0055

- cDNA samples

u0060

- DNA plasmid standard containing tenascin-C exon sequence/endogenous control gene sequences (I.M.A.G.E. consortium/Source Bioscience)

u0065

- Qiagen RNeasy Mini kit (Qiagen)

u0070

- AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies)

4 Detecting tenascin-C expression

p0090 **Method**

p0095 *Preparing cDNA samples and qPCR*

- o0010 **1.** Extract total RNA from cells as described using Qiagen RNA extraction kit. RNA should be stored at -80°C .
- o0015 **2.** Reverse-transcribe RNA to cDNA using Agilent Multi-temperature reverse-transcriptase kit according to the manufacturer's instructions (not detailed here). cDNA may be stored at -20°C for short periods, but should be stored long term at -80°C .
- o0020 **3.** On the day of the qPCR experiment, place cDNA samples on ice and defrost.
- o0025 **4.** Remove endogenous control gene/tenascin-C plasmids and TaqMan[®] gene expression assays from -20°C freezer and defrost on ice. Briefly microcentrifuge all tubes. See [Table 1](#)
- o0030 **5.** Do not freeze-thaw plasmid preparations. Prepare single use aliquots.
- o0035 **6.** Keep all TaqMan[®] reagents shielded from light, as this prevents deterioration of the fluorophore.

p0130 *Preparing standard curves*

- o0040 **1.** Quantify the concentration of tenascin-C and endogenous control gene plasmids by NanoDrop and calculate number of plasmid copies/ μL in the stock, using the known molecular weight of the plasmid.
- o0045 **2.** Create in triplicate an 8-point standard curve by preparing 10-fold serial dilutions of tenascin-C and endogenous control gene plasmids in dH_2O starting with a top concentration of 1×10^6 copies/ μL , decreasing to 1×10^{-1} copies/ μL . Prepare a minimum of 9 μL for each plasmid dilution, as 3 μL of diluted plasmid must be added per reaction tube.

t0010 **Table 1** TaqMan[®] Assays and DNA Plasmids for Tenascin-C and Housekeeper Genes

Gene	ThermoFisher TaqMan [®] Gene Expression Assay (Cat No)	Source Biosciences I.M.A.G.E. Fully Sequenced cDNA Plasmid (I.M.A.G.E. ID)	Use Plasmid DNA as a Standard in the Following Cell Types
Tenascin-C	Hs01115663_m1	IRCFp5008F0112D	All cells
RPLP0	Hs99999902_m1	IRAUUp969H0758D	U-87 MG, HDF, RA-SF, OA-SF, NSF
HPRT1	Hs02800695_m1	IRAUUp969B109D	GM-CSF and M-CSF macrophages, neutrophils
GNB2L1	Hs00272002_m1	IRAUUp969A041D	MDDCs
GAPDH	Hs02758991_g1	IRAUUp969E0824D	Monocytes
YWHAZ	Hs03044281_g1	IRATp970D09108D	CD4+, CD8+ T cells

Abbreviations for cell types: HDF, human dermal fibroblast; MDDC, monocyte-derived dendritic cell; NSF, normal synovial fibroblast; OASF, osteoarthritis synovial fibroblast; RASF, rheumatoid arthritis synovial fibroblast; U-87 MG, human glioma cell line.

- o0050 **3.** Ensuring that cDNA samples are within the dynamic range of the standard curve requires optimization, and depends upon the abundance of target mRNAs in specific cell types.
- o0055 **4.** Take care whilst preparing plasmid dilutions. Change gloves and thoroughly clean pipette after using, to prevent contamination of other reagents and samples.

p0155 *Preparing cDNA dilutions*

- o0060 **1.** Dilute cDNA samples appropriately in dH₂O to permit efficient amplification of the TaqMan[®] PCR product.
- o0065 **2.** 3 µL of diluted cDNA is required per reaction tube; samples should be assayed in triplicate (9 µL total).
- o0070 **3.** If necessary, conduct an optimization experiment to determine optimal cDNA sample dilutions, from 1:3 to 1:10 dilutions in dH₂O. We recommend:
- u0075 o 1:3 diluted cDNA for human GM-CSF and M-CSF macrophages, monocyte-derived dendritic cells (MDDCs), CD4+ and CD8+ T cells.
- u0080 o 1:10 diluted cDNA for human dermal fibroblasts, synovial fibroblasts, and U87-MG glioblastoma.

p0185 *Preparing reaction mixture*

p0190 All reactions should be performed in triplicate. Calculate the total number of reaction tubes required. Usually quantification assays contain three elements:

- u0085 • Standards: Two 10-fold serial diluted plasmid standard curves (tenascin-C and endogenous control gene)
- u0090 • Samples: Diluted cDNA samples for quantification
- u0095 • No template controls: Negative control to confirm no contaminants in dH₂O, TaqMan[®] gene expression assay, or TaqMan[®] MasterMix

- o0075 **1.** Prepare the following mixture immediately before loading into RT-PCR tube/well. Prepare 5%–10% extra total volume.

t0035	• 5 µL of TaqMan[®] Gene Expression MasterMix • 0.5 µL of TaqMan[®] Gene Expression Assay • 1.5 µL of dH₂O	Per reaction tube (=7 µL)

- o0080 **2.** Mix thoroughly and centrifuge.
- o0085 **3.** Carefully pipette 7 µL of the mixture into each reaction tube, and then pipette 3 µL diluted cDNA into the appropriate reaction tubes, avoiding cross-contamination.
- o0090 **4.** Always include a negative control (without cDNA) by adding 3 µL dH₂O to the 7 µL reaction mixture, instead of diluted cDNA.

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- o0095 5. Commence the RT-PCR thermocycler run according to the TaqMan[®] Gene Expression MasterMix manufacturer's instructions.
- o0100 6. Optimal threshold cycle (C_t) values should be in the 15–30 range, and caution should be taken when analyzing samples with C_t values over 30.
- o0105 7. Primary analysis of data can be performed using the Corbett Rotor-Gene 6000 software, or Applied Biosystems ViiA7 software.
- o0110 8. Calculating the number of mRNA copies/cell requires knowledge of the total number of cells from which the RNA was extracted, the total RNA yield, the proportion of the RNA that was reverse transcribed into cDNA, and it must take into account the dilutions of the cDNA made in the RT-PCR setup. This analysis can be performed in Microsoft Excel.

s0025 2.2 RELATIVE QUANTIFICATION OF ALTERNATIVELY SPLICED FNIII mRNA BY SYBR GREEN qPCR

p0265 *Overview:* Quantification of the alternatively spliced domains of the fibronectin type III repeats of human tenascin-C

p0270 *Duration:* 4h (2h setup, 2h RT-PCR thermocycler run)

Equipment

- u0115 • 384-Well ViiA7 compatible plate
- u0120 • ViiA7 instrument (Applied Biosystems)
- u0125 • Micropipettes 1 mL, 200 μ L, and 10 μ L
- u0130 • 1 mL, 200 μ L, and 10 μ L pipette tips
- u0135 • Autoclaved RNase- and DNase-free 1.5-mL Eppendorf tubes
- u0140 • Agarose gel electrophoresis tank
- u0145 • Gel imaging system (GelDoc)
- u0150 • NanoDrop

Materials

- u0155 • Pyrogen and RNase-free, distilled water (dH_2O)
- u0160 • PowerUp[™] SYBR[®] Green Master Mix (2 $\times$) (ThermoFisher Scientific)
- u0165 • Custom DNA Oligos (Primers) (custom-made ThermoFisher Scientific)
- u0170 • cDNA samples
- u0175 • DNA plasmid containing tenascin-C exon sequence with all alternatively spliced FNIII exons (pBS KS II human tenascin-C + AD2 + AD1 plasmid; [Huang, Chiquet-Ehrismann, Moyano, Garcia-Pardo, & Orend, 2001](#))/DNA plasmid containing endogenous control gene sequences (I.M.A.G.E. consortium/Source Bioscience)
- u0180 • TopVision[™] Agarose (ThermoFisher Scientific)
- u0185 • GelRed (Biotium)

p0350 **Method**

p0355 *Designing primers*

p0360 Primer sequences were designed to anneal within exons corresponding to human tenascin-C FNIII A1, A2, A3, A4, B, AD2, AD1, C, D, FN7–8, and to control genes GAPDH, RPLP0, HPRT1, GNB2L1, and YWHAZ; using the NCBI exon sequences for full-length tenascin-C (NCBI code: [X78565.1](#)), tenascin-C FNIII-AD2 (NCBI code: [U88892.1](#)), tenascin-C FNIII-AD1 (NCBI code: [EU295718.1](#)), GAPDH (NCBI code: [BC023632.2](#)), RPLP0 (NCBI code: [BC107717.1](#)), HPRT1 (NCBI code: [BC000578.2](#)), GNB2L1 (NCBI code: [BC017287.2](#)), and YWHAZ (NCBI code: [BC073141.1](#)). Amplicons for all primers were between 72 and 139 bp in length and compatible with SYBR Green qPCR methods. The efficiency and specificity of the primers was evaluated by performing SYBR Green qPCR on four-fold serial dilutions of template plasmid specific for each primer set, and pooled cDNA taken from human dermal fibroblasts and glioma cells where tenascin-C mRNA is abundant ([Table 2](#)). Primers were ordered using Invitrogen custom DNA oligos from ThermoFisher and the sequences listed in [Table 2](#).

p0365 *Preparing standard curves*

- o0115 1. For the quantification of individual alternatively spliced FNIII mRNA, a plasmid template containing all of the TNC alternatively spliced FNIII exons is required.
- o0120 2. For this purpose, we recommend the use of a noncommercially available pBS KS II hu tenascin-C+AD2+AD1 plasmid ([Huang et al., 2001](#)).
- o0125 3. Identify a suitable endogenous control gene to use in your assay. The optimal choice will depend on the nature of your experiment and the cell type. Once identified, obtain a plasmid containing the exon sequence of the control gene (see [Table 1](#) for endogenous control gene plasmids).
- o0130 4. Prepare plasmid aliquots and store at -80°C . Do not freeze–thaw aliquots prior to using as a standard. Quantify the concentration of the plasmid aliquots using a NanoDrop.
- o0135 5. Create an 8-point standard curve by preparing 10-fold serial dilutions of pBS KS III hu tenascin-C+AD2+AD1 plasmid, and the endogenous control gene plasmids in dH_2O . Start with a top concentration of 1×10^6 copies/ μL , and decrease to 1×10^{-1} copies/ μL . A minimum of $3 \mu\text{L}$ diluted plasmid must be added per reaction well.
- o0140 6. Ensuring that cDNA samples are within the dynamic range of the standard curve requires optimization and depends upon the abundance of mRNAs in specific cell types.

p0400 *Preparing cDNA dilutions*

- o0145 1. Dilute cDNA samples appropriately in dH_2O to permit efficient amplification of the PCR amplicons.
- o0150 2. $3 \mu\text{L}$ of diluted cDNA is required per reaction well; samples should be assayed in duplicate ($6 \mu\text{L}$ total).

10015 **Table 2** DNA Primer Sequences for the Quantification of Tenascin-C Alternatively Spliced FNIII mRNAs and Endogenous Control Genes by SYBR-Green qPCR

cDNA Target	Primer Name	Primer Sequence 5'-3'	T _m (°C)	Amplicon Size (bp)	Primer Efficiency (%)
FNIII A1	SYBR-A1F	GCCGAGAAAGGCAGACACAAG	61.8	92	92.58
	SYBR-A1R	ACGGTGAGGTTTTCCAGCTCAG	62.76		
FNIII A2	SYBR-A2F	CCCAGAACCTCACCGTCCCA	63.66	105	91.1
	SYBR-A2R	TGAAGTCCTGAGTGACCCCGC	64.09		
FNIII A3	SYBR-A3F	GAGGCTCACAAATCTCACGGT	59.75	80	87.08
	SYBR-A3R	ACTGTGTAAGGAGTGCCAGC	59.96		
FNIII A4	SYBR-A4F	ACAGCTGGGAGATTTAGCCG	61.65	108	90.86
	SYBR-A4R	ACCTCCTGCACCTGAATGAC	60.12		
FNIII B	SYBR-BF	TGCTGAACGAACCTGCCATA	59.68	96	86.67
	SYBR-BR	ATGGTTTGGTCCGGATGCT	59.96		
FNIII AD2	SYBR-AD2F	GTCCATCTCGTGGGAAGCTC	60.18	85	88.29
	SYBR-AD2R	CATGGTCTCATGGGGTGAT	59.15		
FNIII AD1	SYBR-AD1F	CACAGTTGGGCACGCTAATC	59.55	73	93.45
	SYBR-AD1R	CCAGCTTGAGTGGTCCATGA	59.67		
FNIII C	SYBR-CF	AGTTTCTGGATGGCATGGG	60.11	72	85.51
	SYBR-CR	AGCAGCTTCCAGAAATCCAC	60.03		
FNIII D	SYBR-DF	AGCTGATGAAGGGGTCTTCG	59.46	94	87.54
	SYBR-DR	TTCGGGGGGAAGTAGGGTTA	60.55		
FNIII 7-8	SYBR-FN7-8F	CCCCAGAAAGAGCTCAACCAT	59.38	99	94.33
	SYBR-FN7-8R	AAGGAGGGCAGTTTCCGACT	61.13		
RPLP0	SYBR RPLP0-F	GCGTCCTCGTGGAAAGTGAC	60.73	102	92.43
	SYBR RPLP0-R	GTAGTTGGACTTCCAGGTCCG	60.67		
HPRT1	HPRT1-F1	GGCGAACCTCTCGGCTTTCC	63.46	132	93.21
	HPRT1-R1	TGCGGGTCCGCATAACGGA	64.24		
GNB2L1	GNB2L1-F	CTCTGGGATCTCACACGGG	59.82	84	91.68
	GNB2L1-R	GTCAAGAGGAGAAGGCCACAC	60.04		
YWHAZ	YWHAZ-F1	CGAGCAGGCTGAGCGGATATG	60.2	139	92.67
	YWHAZ-R1	CCAAAGATGACCTACGGGGCTC	59.7		

- o0155 **3.** If necessary, conduct an optimization experiment to determine optimal cDNA
sample dilutions, from 1:3 to 1:10 dilutions in dH₂O. For quantifying the
abundance of TNC alternatively spliced FNIII mRNA, we recommend:
- u0190 o Neat cDNA for CD4+ and CD8+ T cells,
- u0195 o 1:3 diluted cDNA for human GM-CSF and M-CSF macrophages, MDDCs,
osteoarthritis synovial fibroblasts, rheumatoid arthritis synovial fibroblasts,
- u0200 o 1:10 diluted cDNA for human dermal fibroblasts, nondiseased synovial
fibroblasts, and U87-MG glioblastoma.

p0435 *Preparing reaction mixture*

- o0160 **1.** Defrost the 100 μ M stock of custom DNA oligos (primers) on ice, and dilute them
1:10 in dH₂O to generate a 10 μ M working solution for each forward and reverse
primer.
- o0165 **2.** Shield the PowerUp™ SYBR® Green Master Mix from light, and place on ice.
- o0170 **3.** All reactions should be performed in duplicate. Therefore, calculate the
number of reaction required for the assay, taking into account plasmid standards,
cDNA samples, and nontemplate controls (prepare 5%–10% extra).
- o0175 **4.** Prepare the following mixture immediately before loading into the 384-well
plate.

t0040	a. 5 μ L PowerUp™ SYBR® Green Master Mix (2 $\times$)	Per reaction tube (= 7 μ L)
	b. 1 μ L forward primer (10 μ M)	
	c. 1 μ L forward primer (10 μ M)	

- o0195 **5.** Carefully pipette 7 μ L of the reaction mixture per well in the 384-well plate. Then
pipette 3 μ L of the diluted plasmid standard, or cDNA mixture into the
appropriate wells.
- o0200 **6.** Always include a negative control (without cDNA) by adding 3 μ L dH₂O to the
7 μ L reaction mixture.
- o0205 **7.** Run RT-PCR on a ViiA™ instrument (Applied Biosystems) using the
PowerUp™ SYBR® Green Master Mix manufacturer-recommended
thermocycler conditions (see [Tables 3 and 4](#)).

t0020 **Table 3** Standard SYBR Green Thermocycler Conditions as Described
in PowerUp™ SYBR™ Green Master Mix Instruction Manual

Step	Temperature (°C)	Duration (s)	Cycles
UDG activation	50	120	Hold
Dual-Lock™ DNA polymerase	95	120	Hold
Denature	95	15	40 Cycles
Anneal/extend	60	60	

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Table 4 Melt Curve/Dissociation Curve SYBR Green Thermocycler Conditions as Described in PowerUp™ SYBR™ Green Master Mix Instruction Manual

Step	Ramp Rate (°C/s)	Temperature (°C)	Cycles
1	1.6	95	Hold
2	1.6	60	Hold
3	0.15	95	40 Cycles

8. Always perform dissociation (melt) curves immediately after the RT-PCR thermocycler run.
9. Confirm that the size of the PCR amplicon is correct by running 5 µL of the finished RT-PCR product on a 0.8% w/v agarose gel containing 1:10,000 GelRed (Biotium) for 30 min at 135 V. The dissociation curve and agarose gel electrophoresis of the RT-PCR product will confirm if the primers are specific and that only one amplicon is amplified.
10. Optimal threshold cycle (C_t) values should be in the 15–30 range, and caution should be taken when analyzing samples with C_t values over 30 due to lower accuracy as a result of possible background interference.
11. Primary analysis of data can be performed using the Applied Biosystems ViiA7 software.
12. Use the delta–delta C_t ($\Delta\Delta C_t$) method to compare the fold changes in TNC alternatively spliced FNIII mRNA levels, relative to endogenous control genes.
13. Quantify the absolute levels of TNC alternatively spliced FNIII mRNA using standard curve qPCR. This analysis requires accurate knowledge of the number of mRNA copies/cell and requires knowledge of the total number of cells from which the RNA was extracted, the total RNA yield, the proportion of the RNA that was reverse transcribed into cDNA, and it must take into account the dilutions of the cDNA made in the RT-PCR setup. This analysis can be performed in Microsoft Excel.

3 ANALYSIS OF TENASCIN-C PROTEIN—WESTERN BLOTTING

s0030

p0520 *Overview:* Western blot detection of human and mouse tenascin-C

p0525 *Duration:* 1 day and a half

Equipment

- u0205 • Micropipettes 1 mL, 200 µL, and 10 µL
- u0210 • 15/50-mL Falcon tubes
- u0215 • 1.5-mL Eppendorf tubes
- u0220 • Nitrocellulose membranes

- u0225 • Transfer stacks
- u0230 • Trans-turbo transfer machine (Bio-Rad)
- u0235 • Gel tank and power pack
- u0240 • Pipet-Aid
- u0245 • 5- and 10-mL pipettes
- u0250 • Glass plates for acrylamide gel casting
- u0255 • 10–15-well combs
- u0260 • X-ray film (Amersham)

Materials

- u0265 • Autoclaved PBS
- u0270 • Bovine serum albumin
- u0275 • PBS 0.05%/0.1% Tween-20
- u0280 • Amersham ECL western blotting reagent
- u0285 • dH₂O-saturated butanol
- u0290 • ProteinPlus high molecular weight ladder 250kDa (ThermoFisher)
- u0295 • Transfer buffer
 - o0240 40mL of 5 × transfer buffer (Bio-Rad)
 - o0245 40mL Pure ethanol
 - o0250 120mL dH₂O
- u0300 • SDS running buffer (10 ×) 1L
 - o0255 29 g Tris–base
 - o0260 144 g Glycine
 - o0265 10 g SDS
 - o0270 Up to 1 L dH₂O
- u0305 • Tris–HCl pH 6.8 (100mL)
 - o0275 6.05 g Tris–base in 40mL H₂O. Adjust pH with HCl
 - o0280 Up to 100mL H₂O
 - o0285 Filter through 0.45 µm syringe filter
 - o0290 Add 0.4 g SDS
- u0310 • Tris–HCl pH 8.8 (100mL)
 - o0295 18.2 g Tris–base in 60mL dH₂O. Adjust pH with HCl
 - o0300 Up to 100mL H₂O
 - o0305 Filter through 0.45 µm syringe filter
 - o0310 Add 0.4 g SDS
- u0315 • Ammonium persulfate 10%
 - o0315 1 g in 10mL dH₂O. Aliquot in 1 mL
- u0320 • 6 × SDS loading buffer
 - o0320 2.5mL of 0.5 M Tris–HCl pH 6.8
 - o0325 1.2 g SDS
 - o0330 2.5mL β-mercaptoethanol
 - o0335 5mL Glycerol 100%
 - o0340 5mg Bromophenol blue

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- u0325 • Stacking gel 4% acrylamide
- o0345 650 μ L of 30% acrylamide
- o0350 1.25 mL of Tris/HCl pH 6.8
- o0355 3.05 mL dH₂O
- o0360 25 μ L Ammonium persulfate 10%
- o0365 5 μ L TEMED
- u0330 • Separating gel 5% acrylamide
- o0370 2.5 mL of 30% acrylamide
- o0375 3.75 mL of Tris/HCl pH 8.8
- o0380 8.75 mL dH₂O
- o0385 50 μ L Ammonium persulfate 10%
- o0390 10 μ L TEMED

- o0395 1. Mix 5 volumes of 200–500 ng of protein + 1 volume of loading buffer in an 1.5-mL Eppendorf tube (volume dependent on the wells size).
- o0400 2. Place the tubes on a heating block at 90°C for 10 min. Sonicate for 10 minutes. Repeat the boiling for 10 min. Briefly spin down.
- o0405 3. Place the gel in the gel tank, add 1 $\times$ running buffer to fill the tank. Run $\sim$ 1 h and 15 min at 125 V (or $\sim$ 2 h at 20 mA/gel).
- o0410 4. Soak 2 transfer stacks + 1 nitrocellulose membrane per gel with the transfer buffer.
- o0415 5. Place one stack, the membrane, the gel, and the second stack in the transfer cassette.
- o0420 6. Transfer for 10 min using “high molecular weight” program.
- o0425 7. Block the membrane in 5% BSA/0.05% PBS–Tween-20 for 1 h at room temperature.

s0035 3.1 HUMAN TENASCIN-C WESTERN BLOT

- o0430 1. Prepare primary antibodies in 1 $\times$ PBS–0.05% Tween-20 + 3% BSA and stain at 4°C overnight, or 2 h at room temperature:
 - u0335 • mAb1908 (dilute 1:1000) [Merck Millipore]
 - u0340 • mAb1923 (dilute 1:1000) [Merck Millipore]
 - u0345 • mAb1916 (dilute 1:1000) [Merck Millipore]
 - u0355 • BC24 (dilute 1:1000) [Sigma Aldrich]
- o0435 2. Wash the membranes 3 $\times$ 10 min in 1 $\times$ PBS–0.05% Tween-20
- o0440 3. Add secondary antibody diluted in 1 $\times$ PBS–0.05% Tween 20 + 3% BSA and incubate at room temperature for 1 h
 - u0360 • Rabbit f(ab')₂ anti-mouse IgG:HRP (STAR13B) (dilute 1:20,000) [Bio-Rad Antibodies]

- o0445 4. Wash the membranes 3×10 min in $1 \times$ PBS–0.05% Tween-20
 o0450 5. Incubate the membrane for 2 min in 2 mL of Amersham ECL western blotting reagent and develop

s0040 3.2 MOUSE TENASCIN-C WESTERN BLOT

- o0455 1. Prepare primary antibody in $1 \times$ PBS–0.05% Tween 20+5% BSA and stain at 4°C overnight.
 u0365 • MTn-12 (dilute 1:4000) [Sigma Aldrich]
 o0460 2. Wash the membranes 3×10 min in $1 \times$ PBS–0.1% Tween-20
 o0465 3. Add secondary antibody diluted in $1 \times$ PBS–0.05% Tween-20+5% BSA and incubate at room temperature for 1 h
 u0370 • Rabbit anti-rat HRP (A5795—1 mL) (dilute 1:20,000) [Sigma Aldrich]
 o0470 4. Wash the membranes 3×10 min in $1 \times$ PBS–0.1% Tween-20
 o0475 5. Incubate the membrane for 2 minutes in 2 mL of Amersham ECL western blotting reagent and develop

s0045 4 ANALYSIS OF TENASCIN-C PROTEIN—TISSUE STAINING

s0050 4.1 IMMUNOHISTOCHEMICAL ANALYSIS

p0950 *Overview:* Immunohistochemical detection of tenascin-C in mouse and human tissue

p0955 *Duration:* 1 day and a half

p0960 *Deparaffinization*

Materials

- u0375 • Paraffin sections, 7 µm thick
 u0380 • 100% Toluene
 u0385 • 100% Ethanol
 u0390 • 95% Ethanol
 u0395 • 70% Ethanol
 u0400 • Distilled water

p0995 Method

p1000 The slides with the tissue sections are placed on a rack and undergo the following sequential incubation: toluene for 15 min, 100% ethanol for 5 min, 95% ethanol for 5 min, 70% ethanol for 3 min, and distilled water for 3 min.

p1005 *Antigen retrieval*

p1010 Equipment

- u0405 • Scientific Microwave

p1020 Materials

- u0410 • Citrate buffer: sodium citrate pH 6.0
 u0415 • Washing solution: PBS, 0.1% Triton X-100

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p1035 **Method**

- o0480 **1.** Slides in the holding rack are placed in a microwaveable vessel filled with citrate buffer.
- o0485 **2.** The vessel is put into the microwave together with a 500-mL beaker of distilled water. This will prevent the citrate buffer from evaporating. Avoid drying of slide.
- o0490 **3.** Set the microwave to 750 W and wait for the citrate buffer to boil. Allow the solution to boil for 10 min.
- o0495 **4.** Remove the vessel carefully from the microwave and allow the solution to cool down for at least 30 min.
- o0500 **5.** Remove slides from rack and wash with washing solution as follows: 1 quick wash and 3 washes for 5 min.

p1065 *Immunohistochemical staining*

p1070 **Materials**

- u0420 • Blocking solution: PBS, 5% NGS, 0.1% Triton
- u0425 • Primary antibodies: rabbit TNC 1.2 (Spenle et al., 2015) and mouse B28.13 (Schenk, Muser, Vollmer, & Chiquet-Ehrismann, 1995) for human TNC.
- u0430 • Washing solution: PBS, 0.1% Triton
- u0435 • Biotinylated anti-rabbit and anti-mouse secondary antibodies (Vector laboratories)
- u0440 • Peroxidase blocking solution: 0.5% H₂O₂ in methanol
- u0445 • ABC Elite Vectastain kit (Vector PK-6100)
- u0450 • DAB (3,3-diaminobenzidine) kit from Vector Laboratories (Burlingame, CA)

p1110 **Method**

- o0505 **1.** Incubate slides with tissue sections with blocking solution for 1 h.
Note: All incubations are carried out at room temperature unless stated otherwise.
- o0510 **2.** During the blocking step, prepare primary antibody solution (B28.13 at 1 µg/mL or TNC1.2 at 10 ng/mL in blocking solution). Around 150 µL of this solution is needed per section.
- o0515 **3.** Remove blocking solution and incubate with primary antibody at 4°C overnight.
- o0520 **4.** Wash sections. Note that the washing steps consist of 1 quick wash and 3 washes for 5 min with the washing solution.
- o0525 **5.** Incubate the sections with the secondary antibody at the recommended dilution for 90 min.
- o0530 **6.** Wash slides as described in step 4.
- o0535 **7.** Dry slides quickly and incubate sections with peroxidase blocking solution for exactly 30 min. This step allows to block endogenous peroxidase activity.
- o0540 **8.** Wash slides as described in step 4.
- o0545 **9.** Incubate for 30 min with Vectastain ABC reagent.
- o0550 **10.** Wash slides as described in step 4.
- o0555 **11.** Apply 100 µL of DAB solution per section to develop stain until desired intensity.
- o0560 **12.** Quickly wash slides with tap water then with distilled water for 5 min.

p1175 *Hematoxylin staining*

p1180 **Material**

- u0455 • Hematoxylin solution
- u0460 • 100% Toluene
- u0465 • 100% Ethanol
- u0470 • 95% Ethanol
- u0475 • 70% Ethanol
- u0480 • Vectashield mounting medium from Vector Laboratories (Burlingame, CA)

p1215 **Method**

- o0565 1. Stain slides for 10s with hematoxylin and wash with distilled water.
- o0570 2. Dehydration step: the slides are placed on a rack and undergo the following sequential quick bathing steps in 70% ethanol, 95% ethanol, 90% ethanol, 100% ethanol (twice), and 100% toluene (twice).
- o0575 3. Mounting medium is added and TNC staining can be observed under the microscope as shown in Fig. 1A.

s0055 4.2 IMMUNOFLUORESCENCE ANALYSIS

p1235 *Overview:* Immunofluorescence detection of tenascin-C in mouse and human tissue

p1240 *Duration:* 1 day and a half

Materials

- u0485 • Fresh frozen tissue in Tissue-Tek O.C.T. (7 μ m-thick sections)
- u0490 • Primary antibodies (rat MTn12 antibody (Sigma) for mouse TNC and rabbit TNC 1.2 (Spénle et al., 2015), mouse B28.13 (Schenk et al., 1995) or BC24 (Sigma, T2551) antibodies for human TNC)

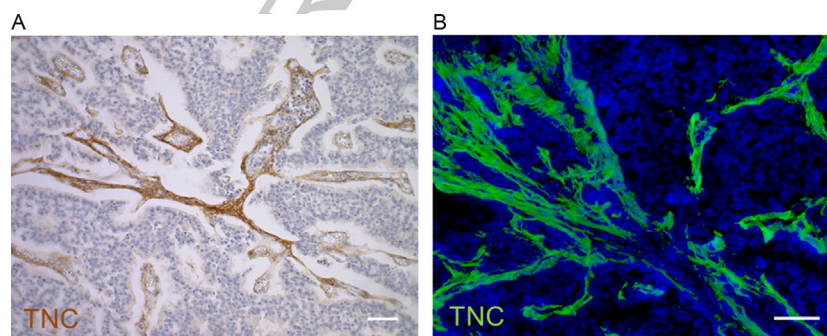


FIG. 1

Analysis of TNC expression by tissue staining. (A) Immunohistochemical staining of TNC in human insulinoma using the TNC 1.2 antibody. (B) Immunofluorescence staining of TNC in a RIP1-Tag2 tumor using the MTn12 antibody. Nuclei are stained by DAPI. Scale bar: 50 μ m. Pictures were obtained from Caroline Spénlé.

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- u0495 • Fluorochrome-coupled anti-rat, anti-rabbit, and anti-mouse secondary antibodies (Jackson ImmunoResearch) and DAPI
- u0500 • Blocking solution: PBS, 0.1% Tween-20, 5% normal goat serum (NGS)
- u0505 • Washing solution: PBS, 0.1% Tween-20
- u0510 • Antifading mounting medium (Millipore 345789)

Method

- o0580 1. Incubate slide with tissue section for 5 min with PBS. *Note:* All incubations are carried out at room temperature unless stated otherwise.
- o0585 2. Remove PBS and incubate with blocking solution for 1 h.
- o0590 3. During the blocking step, prepare primary antibody solution (MTn12 at 5 ng/mL and TNC1.2 at 10 ng/mL in blocking solution). Around 150 μ L of this solution is needed per section.
- o0595 4. Remove blocking solution and incubate with primary antibody at 4°C overnight.
- o0600 5. Wash slides. Note that the washing step consists of 1 quick wash and 3 5 min washing steps with washing solution.
- o0605 6. Incubate slide with secondary antibodies for 90 min in the dark. From this step onward, the slides should be protected from light.
- o0610 7. Wash slides as described in step 5.
- o0615 8. Incubate with DAPI for 10 min.
- o0620 9. Wash as described in step 5.
- o0625 10. Add antifading mounting medium and store slides at 4°C.
- o0630 11. TNC staining can then be observed by fluorescence microscopy as shown in [Fig. 1B](#).

5 ANALYSIS OF TENASCIN-C PROTEIN—ELISA

5.1 DETECTION OF TENASCIN-C

p1330 *Overview:* A method for quantifying TNC in sera, cell lysate, and conditioned medium (CM)

p1335 ELISAs for TNC detection are commercially available and have been tested in our lab, where we have determined sample dilutions for different cell lines ([Table 5](#)). ELISAs were carried out as described in the manufacturer's instructions. The ELISA from IBL (JP27767) detects the alternatively spliced fibronectin type III-like repeat B of TNC, and is recommended for use in detecting human, mouse, and rat TNC variants that contain this domain. The ELISA from USCN (SEB975Hu) detects a region within the constitutively expressed epidermal growth factor-like repeats of TNC. The ELISA is designed to recognize human TNC, but we found it also detects TNC in murine embryonic fibroblasts.

Table 5 Sample Dilutions for TNC ELISA Kits

ELISA Kit	Detection Range	Duration of Assay	Sample Dilutions		
				Cell Lysate	Supernatant
IBL (FNIII-B)	0.2–12.5 ng/mL	2.5 h	GM-CSF macrophage	1:1–2	1:1–2
			M-CSF macrophage	1:10	1:10
			MDDC	1:1	1:1
			HDF	1:30–50	1:50–100
			RASF	1:20	1:20
			OASF	1:2	1:20
			NSF	1:2	1:20
			U-87 MG	1:75	1:25
			GM-CSF macrophage	1:10	1:10–50
USCN (constitutive TNC)	31.2–2000 pg/mL	3 h	M-CSF macrophage	1:2	1:10
			MDDC	1:10	1:100
			HDF	1:30	1:40
			RASF	1:20	1:20–40
			OASF	1:20	1:20–40
			NSF	1:20	1:20–40
			U-87 MG	1:100	1:20–100

5.2 DETECTION OF ANTITENASCIN-C ANTIBODIES

Overview: A method for detection of antibodies against native and citrullinated tenascin-C peptides in human sera (Schwenzer et al., 2016)

Duration: Overnight coating + 6 h

Equipment

- Plate shaker
- Multichannel pipette
- Plate reader (OD₄₅₀)

Material

- RIA buffer
- 1% BSA
- 350 mM NaCl
- 10 mM Tris–HCl pH 7.6

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- o0650 1% Triton X-100
- o0655 0.5% Na-deoxycholate
- o0660 0.1% SDS
- o0665 Store at 4°C or freeze aliquots in 50 mL Falcon tubes at –20°C
- u0535 • Coating buffer
- o0670 15 mM Na₂CO₃
- o0675 35 mM NaHCO₃
- u0540 • Wash Buffer: 1 × PBS/0.05% Tween-20 (PBST)
- u0545 • Blocking Buffer: 2% BSA in PBS
- u0550 • Stopping Solution: 6% sulfuric acid
- u0555 • TMB Microwell Peroxidase Substrate Kit (2 Component), 50-76-03, Insight Biotechnology Limited
- u0560 • Anti-human IgG secondary antibody: Stratech, Human IgGFc Monoclonal Antibody (HRP), 6043HRP
- u0565 • NUNC Maxisorp ELISA plates
- u0570 • 96 deep-well plate
- u9000 • Peptide stock solution at 10 mg/mL (from Pepceuticals, >90% purity, 18–20 mers with N- and C-terminal cysteines, with antigenic epitope of interest in the middle of the peptide)

Method

- o0680 1. Dilute peptides to 10 µg/mL in coating buffer.
- o0685 2. Coat duplicate wells with 100 µL/well, also coat 2 wells that will serve as blank.
- o0690 3. Coat overnight at 4°C.
- o0695 4. Wash 4 × with PBST and dry plate on a paper.
- o0700 5. Block wells with 200 µL 2% BSA/PBS for 2 h at room temperature.
- o0705 6. After blocking do not wash with PBST, only tip out blocking buffer into sink and dry plate on paper.
- o0710 7. Sera are diluted 1:100 in RIA buffer in a deep-well 96-well plate.
- o0715 8. Add 100 µL of diluted sera per well in duplicates. Also add to wells with RIA buffer only (blank).
- o0720 9. Incubate for 2 h at room temperature.
- o0725 10. Wash 4 × with PBST and dry plate on a paper.
- o0730 11. Dilute anti-human IgG secondary antibody 1:3000 in RIA buffer.
- o0735 12. Add 100 µL of secondary antibody per well.
- o0740 13. Incubate for 1.5 h at room temperature.
- o0745 14. Wash 4 × with PBST.
- o0750 15. Add 100 µL of TMB per well.
- o0755 16. Incubate for 5 min at room temperature.
- o0760 17. Stop with 100 µL stopping solution.
- o0765 18. Read the assay on a plate reader at 450 nm within 60 min of completion of the test.

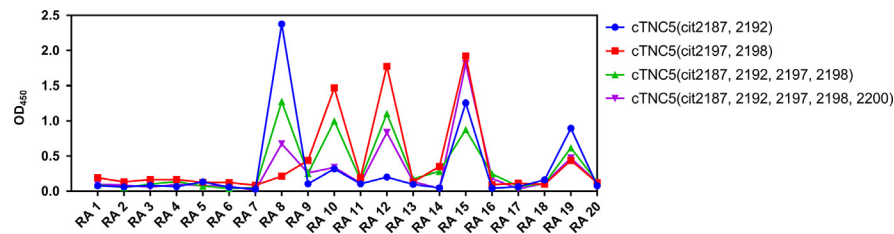


FIG. 2

Antibody response toward citrullinated peptides from TNC. Sera from 20 patients with rheumatoid arthritis (RA) were analyzed for the presence of antibodies against different citrullinated peptides derived from the C-terminal region of the FBG domain of TNC.

Data analysis: For data analysis subtract the OD₄₅₀ from the blank from every sample. Sera with an OD₄₅₀ higher than the 98% percentile of control sera are defined as antibody positive (Fig. 2).

Standard curve: Optionally a standard curve can be included. Use serial dilutions of a positive control sera for generating a standard curve on every plate (e.g., if several plates are run on 1 day or on several days), e.g., use 1:50 as starting dilution and dilute serial 1:2 or 1:3 to obtain a sigmoidal-shaped standard curve.

6 PURIFICATION OF HIS-TAGGED RECOMBINANT TENASCIN-C

Overview: Purification of His-tagged recombinant murine and human tenascin-C

Duration: 2–3 weeks for collecting the CM and 1 week for the protein purification

6.1 PREPARATION OF CM

Materials

- HEK293 cells expressing His-tagged huTNC (Huang et al., 2001) or muTNC
- Selection medium: DMEM high glucose + 10% FCS + puromycin at 2.5 µg/mL
- Collection medium: DMEM high glucose without puromycin and FCS
- 15 cm diameter cell culture dishes
- Sterile filter with 0.22 µm pore size

Method

1. Seed and expand HEK293:TNC cells in 15 cm cell culture dishes in selection medium (30 mL). Grow cells to confluency.
2. Carefully wash poorly adhesive cell layer with PBS and add 30 mL of collection medium.
3. After 48 h incubation, collect the supernatant and add selection medium to the cells for 24 h.

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- o0785 4. Centrifuge the collected supernatant at $1000 \times g$ for 10 min at 4°C and then remove cells and debris by filtration ($0.22 \mu\text{m}$ pore size).
- o0790 5. For storage, freeze the filtered supernatant in plastic tubes (e.g., 50 mL Falcon tubes) at -80°C .
- o0795 6. Repeat steps 2–5 to collect more CM. Up to 8 repetitions can be done on cells seeded in the same dish.
- o0800 7. If cells start to detach, discard plate and start again with freshly expanded cells in 15 cm dishes.
- o0805 8. A final volume of 3 L of CM is appropriate to purify TNC in the mg range.

s0085 6.2 PROTEIN ENRICHMENT FROM CM BY AMMONIUM SULFATE PRECIPITATION

Equipment

- u0600 • Ultracentrifuge ($12,000 \times g$)

Materials

- u0605 • Ammonium sulfate (final concentration 2.20 M)
- u0610 • Dialysis tubing (nominal MWCO 12,000 Da)
- u0615 • Dialysis buffer: PBS, 0.01% Tween-20

Method

- o0810 1. Thaw CM overnight at 4°C .
- o0815 2. To precipitate the proteins, add ammonium sulfate (291 g/L of CM) and stir for 2 h at 4°C .
- o0820 3. Sediment the protein precipitated by spinning at $12,000 \times g$ for 20 min at 4°C .
- o0825 4. Resuspend precipitate in dialysis buffer (50 mL for 1 L of CM) and transfer into dialysis tubing.
- o0830 5. Exchange solution by dialysis with the dialysis buffer in a 2-L beaker at 4°C with 3 changes (2 times 2 h and overnight).

s0090 6.3 REMOVAL OF CONTAMINATING FIBRONECTIN

Equipment

- u0620 • Ultracentrifuge ($12,000 \times g$)
- u0625 • AktaPrime plus FPLC apparatus (GE Healthcare Life Sciences)

Materials

- u0630 • Akta Tricorn column
- u0635 • Gelatin agarose beads (Sigma Aldrich)
- u0640 • Washing buffer: distilled water
- u0645 • Equilibration buffer: PBS, 0.01% Tween-20 (150 mL)
- u0650 • Elution buffer: 4 M solution of urea in PBS (100 mL)
- u0655 • Storage buffer: 50 mM boric acid at pH 8, 150 mM NaCl (75 mL)

Method

- o0835 1. Collect previously dialyzed solution and separate precipitates by centrifugation at $12,000 \times g$ for 20 min at 4°C . Collect the supernatant and keep at 4°C .
- o0840 2. Fill the Tricorn column with the gelatin agarose beads to 80% of its capacity and insert in the Aktaprime plus FPLC apparatus. Program the protocol in order to do the following.
- o0845 3. Wash the beads with 30mL of washing buffer.
- o0850 4. Equilibrate the column with 35 mL of Equilibration buffer.
- o0855 5. Load the supernatant from step 1 onto the gelatin beads-filled column and collect the flow-through (which contains the TNC) on ice. This step allows separation of contaminating fibronectin (present in the supernatant) from TNC.
- o0860 6. Wash the column with 40 mL of Equilibration buffer.
- o0865 7. To regenerate the column, remove fibronectin from the gelatin agarose beads by washing with 80mL of elution buffer.
- o0870 8. Wash the column with 85 mL of washing buffer.
- o0875 9. For storage, fill the gelatin beads column with 50 mL of storage buffer and store column at 4°C for future use.
- o0880 10. The flow-through solution, collected in step 5, is now adjusted to a final concentration of 450mM NaCl, 250mM sodium phosphate, 20mM imidazole, and 500mM urea. This solution is used for the next steps of the TNC purification.

s0095 6.4 PURIFICATION OF RECOMBINANT MURINE AND HUMAN TNC

Equipment

- u0660 • Aktaprime plus FPLC apparatus (GE Healthcare Life Sciences)

Materials

- u0665 • Akta Tricorn column
- u0670 • Nickel beads
- u0675 • Equilibration buffer: 250mM sodium phosphate buffer pH 7.4, 450mM NaCl, 0.01% Tween-20, 20mM imidazole, 500mM urea (500mL)
- u0680 • Washing buffer: 250mM sodium phosphate buffer pH 7.4, 450mM NaCl, 0.01% Tween-20, 20mM imidazole (60mL)
- u0685 • Elution buffer A: 250mM sodium phosphate buffer pH 7.4, 450mM NaCl, 0.01% Tween-20, 300mM imidazole (100mL)
- u0690 • Elution buffer B: 250mM sodium phosphate buffer pH 7.4, 450mM NaCl, 0.01% Tween-20, 300mM imidazole (100mL)
- u0695 • Storage buffer: Ethanol 20% v/v
- u0700 • Dialysis buffer: PBS, 0.01% Tween-20

Method

- o0885 1. Nickel beads are used for purification of the His-tagged TNC molecules. Put the beads in 50 mL Falcon tubes and fill the tubes with equilibration buffer. Shake for 5 min and centrifuge at $1000 \times g$ for 5 min. Carefully, discard the supernatant and repeat this equilibration step 2 more times.

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- o0890 **2.** Incubate the equilibrated nickel beads with the flow-through solution previously obtained, at 4°C overnight on an overhead rotator. This step ensures an optimal binding of TNC to the beads.
- o0895 **3.** The next day, fill a clean Tricorn column with the nickel beads obtained from step 2. The flow through is kept for future use.
- o0900 **4.** Insert the column to the AktaPrime plus FPLC apparatus and program the purification.
- o0905 **5.** Load the whole volume of the flow-through solution from step 3 onto the column.
- o0910 **6.** Wash the column with 70 mL of Equilibration buffer followed by 60 mL of washing buffer.
- o0915 **7.** Elute column with 60 mL of elution buffer A. The flow through is collected in fractions of 0.5 mL and stored on ice. The fractions that have an OD > 0.05 are those that contain TNC. These fractions are pooled together and kept on ice. The resulting solution is called “TNC solution.”
- o0920 **8.** Regenerate the column with 60 mL of Elution buffer B.
- o0925 **9.** Wash and reequilibrate the column with 85 mL of Equilibration buffer.
- o0930 **10.** Load column with 50 mL of storage buffer and store column separately at 4°C for future use.
- o0935 **11.** Change solution of the “TNC solution” from step 7 by dialysis using the dialysis buffer. Change volume 3 times in a 2-L beaker at 4°C (2 times 2 h plus overnight dialysis).
- o0940 **12.** The next day, the dialyzed solution is collected. The concentration of the “TNC solution” is determined by OD at 280 nm. The actual TNC concentration in mg/mL is equal to an OD at 280 nm multiplied by a factor of 0.78. To avoid spontaneous precipitation/aggregate formation of TNC, dilute the solution to a final concentration lower or equal to 100 µg/mL.
- o0945 **13.** Aliquot the TNC solution and store at –80°C until use. The defrosted solution can be kept at 4°C for about a week.

s0100 **6.5 USE WESTERN BLOT TO CONFIRM PURITY OF TNC**

p1895 Use 100 ng of purified TNC and probe western blot with a TNC-specific antibody as described in [Section 3](#) (Fig. 3). Absence of contaminating fibronectin is assayed by western blotting for fibronectin (Sigma Aldrich, F3648).

s0105 **6.6 FUNCTIONAL ASSAY TO TEST ANTIADHESIVE PROPERTIES OF TNC**

Materials

- u0705 • Human glioblastoma T98G cells (ATCC: CRL-1690)
- u0710 • Culture medium: DMEM high glucose, 10% FCS, 1% Pen/Strep, 40 µg/mL gentamicin
- u0715 • Purified TNC, recombinant fibronectin (FN)
- u0720 • 1% BSA (Cohn’s fraction V) solution in PBS, heated for 10 min at 70°C

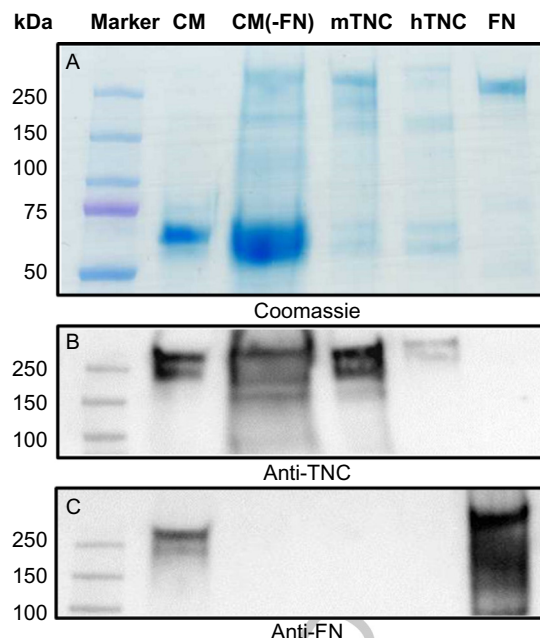


FIG. 3
Purification of His-tagged recombinant TNC. (A) Coomassie-stained SDS gel with samples from the different steps of the purification: conditioned medium (CM), CM after the gelatin column (CM(-FN)), purified murine TNC (mTNC) (100ng), human TNC (hTNC) (100ng), and horse FN (100ng). (B, C) Immunoblots upon probing with antibodies against TNC (TNC1.2) (B) and fibronectin (C).

Method

1. TNC is described as an antiadhesive ECM molecule. Human glioblastoma T98G cells are known to be sensitive to this property of TNC which delays their spreading in culture (Lange et al., 2007, 2008).
2. Coat 6-well cell culture dishes with purified TNC, or fibronectin or with TNC and fibronectin consecutively at a coating concentration of $1\text{ }\mu\text{g}/\text{cm}^2$ in duplicates. Saturate the uncoated surface with 1% BSA solution for 1 h. Remove solutions, wash with PBS, and air dry under UV lamp. Leave 2 wells uncoated (will be referred as “Plastic” control).
3. Seed 1 million T98G cells per well in cell culture medium and observe the kinetics of the spreading behavior of the cells over 24 h. The antiadhesive effect of TNC is well seen in the TNC-coated wells, whereas cells spread on the fibronectin or uncoated surfaces. On the mixed TNC/fibronectin substratum cells also remain round but eventually spread (Fig. 4).

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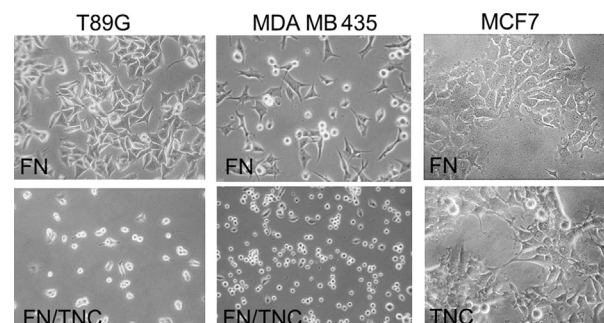


FIG. 4

Cell adhesion assay. Phase contrast picture of human glioblastoma T89G, human melanoma MDA MB 435, and human breast cancer MCF7 cells upon adhesion to the indicated substrata (1 mg/cm²) for 4 h in 1% FCS-containing medium. Note that cell adhesion on FN, TNC, and FN/TNC is cell specific.

7 KNOCKDOWN OF TENASCIN-C EXPRESSION

Overview: Knockdown of the expression of TNC in cancer associated fibroblasts (CAFs)

Duration: 15–20 days

Equipment

- 6-well cell culture plates

Material

- Human colorectal CAF CT5.1 (De Boeck et al., 2013)
- MISSION lentiviral particles (TRC2, Sigma Aldrich). The ShRNA sequences selected are as follows:
 - hTNC sh1 (Clone ID TRCN0000230785): 5'-CCGGGGAGTACTTTATCCG TGTATTCTCGAGAATACACGGATAAAGTACTCCTTTTG-3'
 - hTNC sh2 (Clone ID TRCN0000230787): 5'-CCGGCAGGCGCAAACGGGCATAAATCTCGAGATTTATGCCCGTTT GCGCCTGTTTTTG-3'
- Negative control ShRNA particles: MISSION pLKO.1-puro empty vector control (SHC001V) and MISSION pLKO.1-puro Non-Mammalian shRNA Control Transduction Particles (SHC002V)
- Polybrene: 2 mg/mL stock solution (Sigma Aldrich)
- Culture medium: Culture medium: DMEM 4.5 g/L glucose culture medium + 10% FCS
- Puromycin: 10 mg/mL stock solution (Sigma Aldrich)

p2000 **Method**

p2005 Day 1

o0965 **1.** Seed CAFs at a density of $2-4 \times 10^6$ cells per well in culture medium.
Note that the growth rate of cells may vary. The number of cells to be seeded should be adjusted so that just before transduction the cells are at 70%–80% confluency.

o0970 **2.** Incubate cells at 37°C.

p2020 Day 2

o0975 **3.** Remove culture medium from wells and add 1 mL of fresh culture medium containing polybrene at a concentration of 5 µg/mL. Gently swirl the plate to ensure proper mixing.

o0980 **4.** Add the corresponding lentiviral particles to the appropriate wells with a MOI of 2–5. Gently swirl the plate to ensure proper mixing.

o0985 **5.** Incubate cells at 37°C.

p2040 Day 4

o0990 **6.** Remove media from wells.

o0995 **7.** Add 1 mL of fresh culture medium supplemented with 1 µg/mL puromycin to each well.

o1000 **8.** Incubate cells at 37°C.

p2060 Day 5 and onward

o1005 **9.** Change medium with fresh culture medium supplemented with 1 µg/mL puromycin every 2 days

o1010 **10.** Passage cells when needed.

o1015 **11.** After 5 passages postinfection, TNC protein expression is assessed by western blot (Fig. 5).

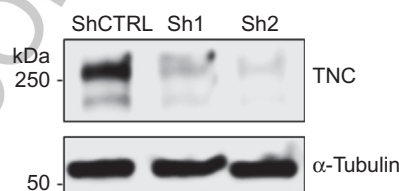


FIG. 5

TNC shRNA efficiency. Expression of TNC in CAFs transfected with shCTRL, sh1 TNC, and sh2 TNC by immunoblotting, with α-tubulin as control 48h after plating. Western blot obtained from William Erne.

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s0115

8 PREPARATION OF CELL-DERIVED MATRIX BY FIBROBLASTS EXPRESSING OR LACKING TNC

p2080

Overview: Preparation of cell-derived matrix by mouse embryonic fibroblasts (MEFs) expressing or lacking TNC (adapted from [Rupp et al., 2016](#)).

p2085

Duration: 11 days

Equipment

u0770

- 10 cm cell culture dishes

Materials

u0775

- Wild-type murine lung embryonic fibroblasts MEF WT and MEF Knockout for TNC (KO) ([Brellier et al., 2011](#))

u0780

- PBS (~1 L). Autoclave for cell culture use

u0785

- Glycine 1 M (Sigma Aldrich) in PBS (~500 mL)

u0790

- Glutaraldehyde 1% (Sigma Aldrich) (~50 mL)

u0795

- L-Ascorbic acid 50 mg/mL (Sigma Aldrich) in PBS (~50 mL)

u0800

- Gelatin 1% m/v (Sigma Aldrich) solution in distilled water. Autoclave for cell culture use (~500 mL)

u0805

- DMEM 4.5 g/L glucose culture medium (~500 mL)

u0810

- Culture medium: DMEM 4.5 g/L glucose culture medium + 10% FCS + 1% Pen/strep

u0815

- Extraction buffer: 20 mM ammonium hydroxide + 0.5% Triton X-100 (Euromedex) in PBS (~50 mL)

u0820

- 100 U/mL DNase I recombinant, RNase-free (Invitrogen)

p2145

Method

p2150

Day 1: All steps are carried out in sterile conditions

o1020

1. Coat culture dishes with 6 mL of 1% gelatin solution, cover them with their lids, and incubate for 1 h at 37°C.

o1025

2. Wash culture dishes 2 × with 10 mL of PBS and discard PBS.

o1030

3. Add 5 mL of 1% glutaraldehyde to each culture dish and incubate the culture dishes for 20 min at room temperature.

o1035

4. Wash as described in step 2.

o1040

5. Add 5 mL of glycine 1 M to each culture dish and incubate the culture dishes for 30 min at room temperature.

o1045

6. Wash as described in step 2.

o1050

7. Wash culture dishes once with 10 mL of DMEM each and discard the medium.

o1055

8. Plate the cells (MEFs) at a density of $\sim 3 \times 10^6$ cells in 9 mL of culture medium. Note that MEF WT grow faster than MEF TNC KO. To reach the same cell density at the end of the experiment MEF WT are plated at a lower density: $\sim 2 \times 10^6$ cells in 9 mL of culture medium.

o1060

9. Incubate culture dishes overnight at 37°C.

- p2200 Day 2–8
- o1065 **10.** On day 2, change cell culture medium to culture medium supplemented with 50 µg/mL of ascorbic acid.
- o1070 **11.** Allow cells to grow for 7–8 days while renewing the supplemented cell culture medium every day.
- p2215 Day 9
- o1075 **12.** The following steps are carried out to remove the cells from the coated plates while keeping the cell-derived matrix of the fibroblasts.
- o1080 **13.** Wash the cell culture dishes 2 × with PBS.
- o1085 **14.** Add very gently 5 mL of extraction buffer to each dish and incubate at 37°C for 15 min.
- o1090 **15.** Incubate the cell culture dishes at 4°C overnight.
- p2240 Day 10
- o1095 **16.** Wash very gently the cell culture dishes 3 × with PBS.
- o1100 **17.** Add 10 mL of PBS in each culture dish and incubate the culture dishes at 4°C overnight.
- p2255 Day 11
- o1105 **18.** Treat culture dishes with 100 U/mL DNase I recombinant, RNase-free and incubate the culture dishes at 37°C for 1 h.
- o1110 **19.** Wash very gently 1 × with PBS.
- o1115 **20.** The cell-derived matrices are now ready to use.
- o1120 **21.** If the cell-derived matrices are not used the same day, they can be kept at 4°C up to 3 days. Fig. 6 shows immunofluorescence staining of cell-derived matrices isolated as described earlier.

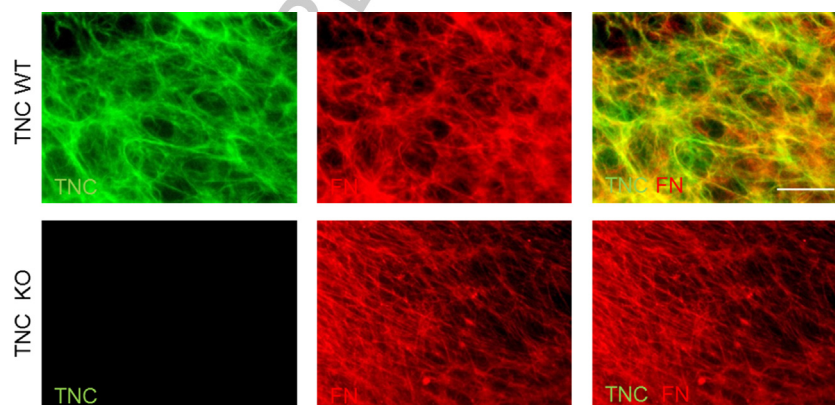


FIG. 6

Immunofluorescence analysis of TNC and FN expression in cell-derived matrices produced by MEFs obtained from lungs of a WT or TNC KO mouse. Scale bar: 50 µm. Pictures were obtained from Tristan Rupp.

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s0120

9 SUMMARY

p2280

Studying tenascin-C expression poses a number of technical challenges created by the biological complexities associated with this multimodular, multifunctional molecule that simultaneously make it such an interesting matrix component. Challenges remain to be solved, including the development of reagents and protocols for easy and reproducible isolation of tenascin-C from tissues that will enable further examination of endogenously synthesized matrix.

ac0010

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p2285

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Non-Print Items

Keywords: Tenascin-C tissue expression, qPCR, Splicing, Western blot, Immunofluorescence, Immunohistochemistry, ELISA, Purification, shRNA-mediated knockdown, Cell-derived matrix

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