


STATE-OF-THE-ART REVIEW

Collagen VI as a driver and disease biomarker in human fibrosis

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Fibrosis of visceral organs such as the lungs, heart, kidneys and liver remains a major cause of morbidity and mortality and is also associated with many other disorders, including cancer and metabolic disease. In this review, we focus upon the microfibrillar collagen VI, which is present in the extracellular matrix (ECM) of most tissues. However, expression is elevated in numerous fibrotic conditions, such as idiopathic pulmonary disease (IPF), and chronic liver and kidney diseases. Collagen VI is composed of three subunits $\alpha 1$, $\alpha 2$ and $\alpha 3$, which can be replaced with alternate chains of $\alpha 4$, $\alpha 5$ or $\alpha 6$. The C-terminal globular domain (C5) of collagen VI $\alpha 3$ can be proteolytically cleaved to form a biologically active fragment termed endotrophin, which has been shown to actively drive fibrosis, inflammation and insulin resistance. Tissue biopsies have long been considered the gold standard for diagnosis and monitoring of progression of fibrotic disease. The identification of neoantigens from enzymatically processed collagen chains have revolutionised the biomarker field, allowing rapid diagnosis and evaluation of prognosis of numerous fibrotic conditions, as well as providing valuable clinical trial endpoint determinants. Collagen VI chain fragments such as endotrophin (PRO-C6), C6M and C6M $\alpha 3$ are emerging as important biomarkers for fibrotic conditions.

Introduction

Collagen VI is a ubiquitously expressed interstitial collagen involved in diverse homeostatic functions from cell adhesion to migration, in addition to forming an important structural scaffold in many organs. The macromolecular structure of collagen VI is highly complex and was initially described as a triple helical monomer composed of three genetically distinct shorter chains, $\alpha 1$, $\alpha 2$ and the significantly longer chain of $\alpha 3$ [1–3]. Subsequently, three new collagen VI chains closely

resembling the $\alpha 3$ chain were identified and termed $\alpha 4$, $\alpha 5$ and $\alpha 6$. Each of these novel chains is encoded by a distinct gene (*COL6A4*, *COL6A5* and *COL6A6*) [4,5] and exhibits a more restricted expression pattern than the $\alpha 3$ chain. They can substitute for the $\alpha 3$ chains to form $\alpha 1\alpha 3\alpha 4$, $\alpha 1\alpha 2\alpha 5$ or $\alpha 1\alpha 2\alpha 6$ heterotrimers. However, collagen VI $\alpha 4$ chain is not functional in humans, as the *COL6A4* gene is disrupted by a chromosome break creating two pseudogenes [4].

Abbreviations

BMP-1, bone morphogenic protein 1; BTHLM1, Bethlem myopathy; CKD, chronic kidney disease; CNS, central nervous system; COPD, chronic obstructive pulmonary disease; ECM, extracellular matrix; ETP, endotrophin; HCC, hepatocellular carcinoma; HFD, high-fat diet; ILD, interstitial lung tissue; IPF, idiopathic pulmonary fibrosis; MMP, matrix metalloproteinase; NAFLD, nonalcoholic fatty acid liver disease; NASH, nonalcoholic steatohepatitis; PAH, pulmonary arterial hypertension; PCSK, proprotein convertase subtilisin/kexin; SSc, systemic sclerosis; TF, transcription factor; THD, triple helix domain, LRP, leucine-rich proteoglycans LRP; TSS, transcription start site; UCMD, Ullrich congenital muscular dystrophy; vWF-A, von Willebrand factor A domain homologue; WAT, white adipose tissue.

The intricate quaternary structure of collagen VI is mirrored by a highly complex assembly process. Each chain contains a central triple helix domain (THD) of 335–336 amino acids with repeating Gly-X-Y motifs flanked by large N- and C-terminal globular domains. Formation of the triple helix monomers occurs in stages, first by formation of anti-parallel dimers stabilised by inter-chain disulphide bonds. A single conserved cysteine (30th Gly-X-Y) (Fig. 1) in the THD of $\alpha 1$ (exon 14) or $\alpha 2$ (exon 11) chains interacts with a cysteine residue in the C-globular domain is thought to be responsible for the assembly or stability of dimers, which are then aligned through more disulphide bonds to form tetramers [3,6,7]. There is also a key cysteine (17th Gly-X-Y) in the $\alpha 3$ chain that is critical to tetramer formation [8]. The tetramers are secreted into the extracellular space and form the characteristic beaded linear microfibrils connected end to end, with a bead repeat of 105 nm [9]. Collagen VI is unique amongst the collagens in having a short triple helix with terminal ends enriched in globular regions [10] that contain several von Willebrand factor A domain homologues (vWF-A). The C-terminal domains of each chain are thought to be involved in chain association and selection [11], and the N-terminal vWF-A domains are required for microfibril assembly [12] (Fig. 1).

Collagen VI microfibrils form large networks in a tissue-specific manner and interact with a number of other ECM proteins and cell surface molecules, producing a highly filamentous meshwork that encircles the fibres of collagens I, II, III and IV [13]. The complex structure and variety of domains enable collagen VI to bind multiple components of the ECM and thus play an important role in organising and maintaining three-dimensional tissue architecture [14]. In the human body, collagen VI is a core structural component of the ECM of numerous tissues including muscle, skin, nerves, tendons, cartilage, blood vessels, the lobes and portal tracks of the liver, lung and adipose tissue [15].

In addition to playing an important structural role, collagen VI influences a panoply of cellular functions from adhesion, migration, autophagy, apoptosis and proliferation [14]. These pleiotropic effects likely reflect its emerging role across a range of disease systems, including cancer and fibrosis. For example, collagen VI has been found to be a key driver of fibroblast activation in human fibrosis and acts to regulate cell cycle progression [16], favouring mammary tumour cell survival and cancer progression [17–19]. These studies showed that secretion of collagen VI by adipocytes enhanced tumour growth and was critical to tumour-

associated macrophage recruitment in breast cancer. Subsequently, these findings have been confirmed in a range of malignancies where collagen VI depletion attenuated metastasis and the invasion in cell lines isolated from patients with triple negative breast cancer (TNBC) [20], colorectal cancer [21] and gastric cancer [22]. Taken together, these studies provide increasing evidence for a central role of collagen VI in human health and disease. This review focuses on the mechanisms by which collagen VI signalling promotes fibrosis, the pathways that modulate this process and emerging therapeutic opportunities for the inhibition of collagen VI-driven human fibrosis.

Genetic abnormalities associated with collagen VI

Dominant and recessive autosomal mutations in the three major collagen VI genes, *COL6A1*, *COL6A2* and *COL6A3*, are associated with congenital myopathies [23]. Bethlem myopathy (BTHLM1) is a dominantly inherited disorder which usually follows a relatively benign course and is characterised by proximal muscle weakness and joint contractures mainly involving the upper limb and ankles [24,25]. Collagen VI-related myopathies represent a unique class of skeletal muscle disease as the mutated proteins downstream of the primary genetic defect are not produced by the myofibres themselves, but by fibroblasts [26]. Typical mutation types seen in BTHLM1 are missense mutations of glycine residues of the Gly-X-Y motif at the N-terminal end of the triple helical domain (THD) [27,28]. Ullrich congenital muscular dystrophy (UCMD) is predominantly an autosomal recessive condition, causing an early-onset severe muscle weakness with proximal joint contractures, pronounced hyper-elasticity of distal joints and early respiratory failure [29,30]. The first mutations identified were recessive null mutations, leading to an absence of collagen in muscle biopsies [29,31]. Subsequently, more mutations were characterised, most leading to premature termination codons [23,30] and splice-specific mutations which can lead to exon skipping [32,33]. In Western countries, the proportion of BTHLM1 and UCMD cases attributed to *COL6A1* and *COL62* mutations is approximately 38% and 44%, respectively. However, mutations in *COL6A3* are less common at only 18% of the total [34]. A further study of a large Chinese cohort of 60 patients showed a similar distribution of mutations at 34%, 46% and 19% in *COL6A1*, *COL6A2* and *COL6A3*, respectively [35]. In agreement with previous reports, most dominant cases were clustered around the cystine residue critical to dimer ($\alpha 1/2$ chains) and

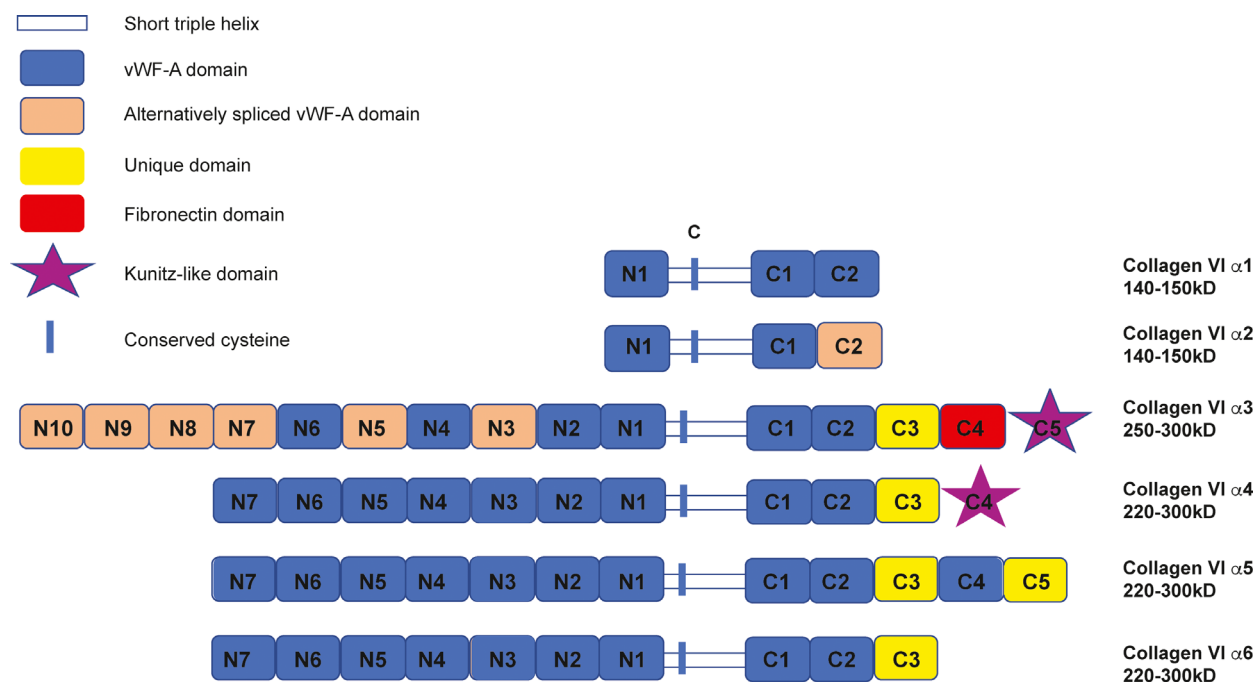


Fig. 1. Schematic representation of domain structure of collagen VI. All collagen VI chains contain a relatively short central collagenous triple helix domain (THD), flanked by large N and C-terminal globular regions which are homologous to Willebrand factor type A (vWF-A) domains. The $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 6$ chains are much larger than the $\alpha 1$ and $\alpha 2$ chains, containing up to 10 repeating vWF-A domains within the N-terminal region (N1–10), which are important in microfibril assembly, and a further 2 vWF-A domains are found within the C-terminal region. Three additional domains C2–5 are found within $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 6$ chains, with the C5 Kunitz domain of $\alpha 3$ chain being cleaved to release endotrophin. A single conserved cysteine (C) (30th Gly-X-Y) in the THD of the $\alpha 1$ or $\alpha 2$ chains interacts with a cysteine residue in the C-globular domain is thought to be responsible for the assembly or stability of dimers, and a key cysteine (17th Gly-X-Y) in the $\alpha 3$ chain is critical to tetramer formation. This figure was adapted with permission from Cescon *et al.* [14] Copyright The Company of Biologists Ltd.

trimer formation ($\alpha 3$ chain) within the N-terminal TND domain. Recessive mutations were located near in the C-terminal end of the α chain. The authors were also able to correlate the phenotype with the genotype. Patients with glycine substitution mutations in critical region of G-X-Y had a relatively severe phenotype whilst patients with mutations outside this region were mostly of the less severe BTHLM1 phenotype.

The clinical manifestations of these myopathies reflect a primary defect in the structural integrity of muscle and tendon, within which interstitial fibroblasts [26] and residential tendon fibroblasts are the principal sources of collagen VI. Using fibroblasts isolated from patient biopsies showed that a reduction or absence of collagen VI in the matrix leads to the loss of mechanical anchoring between the matrix and basement membranes [36,37]. Further studies using patient samples have shown mitochondrial dysfunction and spontaneous apoptosis of muscle fibres [38]. This was subsequently attributed to a deficiency in autophagy [39], an important homeostatic mechanism for the recycling or removal of cellular components. Identification of

autophagy dysfunction within these patients has led to a clinical trial evaluating low protein diet to reactivate autophagy in BTHLM1/UCMD patients (<https://clinicaltrials.gov/>, NCT01438788). Overall, monogenic disorders have illuminated several important functions of collagen VI in musculoskeletal physiology. In addition, they provide a means to correlate genotype with phenotype and ascribe distinct cellular functions to specific protein domains.

Mouse models of collagen VI deficiencies

The most extensively studied collagen VI disease model are the *Col6a1* knockout mice in which deletion of the $\alpha 1(VI)$ chain results in absence of the triple helical collagen VI molecules [40]. These mice develop spontaneous apoptosis of muscle fibres as the result of an accumulation of abnormal mitochondria and sarcoplasmic reticulum due to a defect in autophagy pathways [39]. An excellent review of the critical role collagen VI plays in autophagy has been recently been

published [41]. In a hypomorph model of *Col6a1* deficiency in which G283R mutation was introduced, the mice showed mild muscle weakness, impaired muscle contractile forces and a reduction in the numbers of myofibres [42]. Interestingly, in this model the authors were unable to observe pronounced apoptosis and only limited muscle fibre necrosis. Therefore, the reduction in muscle fibres size was not due to fibre atrophy, but more likely due to defective increase of myofibre number during the neonatal period.

Further studies have shown a diverse range of phenotypes in *Col6a1* knockout mice. As they aged, *Col6a1*^{-/-} mice exhibited accelerated development of osteoarthritis, delayed secondary ossification and lower bone mineral density [43], suggesting changes in the mechanical properties of chondrocytes. Mechanical tests on skin from these mice showed impaired tensile strength and abnormalities in collagen I fibril formation in wounds [15]. Collagen VI has also been shown to have a role in the central and peripheral nervous systems. *Col6a1* mice^{-/-} mice have hyper-myelinated axons in the sciatic nerves, in the absence of any axon damage or inflammation. The mice exhibit defective nerve conduction and impaired motor control [44], suggesting that collagen VI plays an essential role in the structural integrity and function of peripheral nerves. In keeping with this, nerve injury induces robust upregulation of collagen VI, whereas *Col6a1*^{-/-} mice showed delayed peripheral nerve regeneration through impaired CD68/F4/80-positive macrophage migration and reduced anti-inflammatory (M2) phenotype polarisation [45]. Within the CNS, *Col6a1*^{-/-} neural cells show increased apoptosis and dysregulated autophagy, with subsequent increased oxidative damage, suggesting a protective role of collagen VI [46]. Together, these data suggest a central role for collagen VI in neural homeostasis and regeneration, and a thorough review on the wide ranging role collagen VI plays within the CNS has recently been published [47].

Collagen VI is the most abundant collagen expressed within the lung [48] and an early study using the *Col6a1*^{-/-} mice demonstrated an increased lung tissue elasticity [49]. More recently, a study showed that the *Col6a1*^{-/-} mice exhibited a distorted airway morphology due to an increased lung volume with larger and fewer alveoli and increased epithelium thickening [50]. These alterations suggest that collagen VI may be important in branching morphology, resulting in a state that resembles an immature lung.

Collagen VI is also highly expressed in the osteogenic lineage [51]. *Col6a1*^{-/-} mice display reduced trabecular bone density and an increase in trabecular structure compared with *WT* mice. Specifically, *Col6a1*

deficiency altered the shape and arrangement of osteoblasts on the bone surface, suggesting that collagen VI plays a significant role in regulating normal bone homeostasis [52,53]. Recent work has shed a light on the role of *Col6a2* in bone homeostasis [54]. *Col6a2*^{-/-} mice showed lower whole-body bone mineral density (BMD), lower fat body mass and lower fat content compared with *WT* mice. Specifically, whilst cortical bone formation remained unaffected, trabecular bone mass in the spine and femur was significantly reduced in the *Col6a2*^{-/-} mice. Bioinformatic pathway analysis of RNA Seq data suggested a role of TNF signalling pathways in this phenotype. The authors subsequently confirmed a role for collagen VI in sequestering TNF within the ECM whereby inhibiting TNF-induced osteoclastogenesis. Whilst it is known that collagen VI expression diminishes with age, currently very little is known about how collagen VI affects bone formation in humans. It is of interest that patients with mutations in *COL6A1*, *COL6A2* or *COL6A3* all have BMD Z scores significantly below the norm [55], suggesting a role in human bone homeostasis.

Important insights have also been uncovered by two knock-in *Col6a3* murine models which both display a mild myopathic phenotype. The first description was of a mutant *Col6a3*^{hm/hm} mouse that produced a non-functional collagen VI protein. The key phenotypic features were muscle and tendon defects similar to those seen in human collagen VI UCMD [56]. More recently, tendon defects in patients with UCMD and BTHLM1 were compared and both were found to have similar abnormalities in the tendon matrix and defective cell polarisation *in vitro* [57]. These mice were deficient in extracellular collagen VI microfibrils and exhibited myopathic features, including decreased muscle mass and contractile force. The second *Col6a3*^{+d16} mouse, generated by an exon 16 deletion, resulted in an in-frame deletion, mimicking the most common defect found in UCMD. Like the *Col6a1*^{-/-} mice, alterations of mitochondria and sarcoplasmic reticulum were reported [58]. Interestingly, the absence of normal $\alpha 3$ chain did not result in the compensatory upregulation any of the three $\alpha 3$ like ($\alpha 4$, $\alpha 5$ or $\alpha 6$) chains in skeletal muscle or any other organs during development. This might be due to the tight spatial transcriptional control of collagen VI.

In summary, the generation of multiple collagen VI deficiency models (Table 1) has revealed both severe and mild myopathic features similar to the spectrum observed in the human myopathies and highlighted the diverse role collagen VI plays in multiple tissues. Despite the insights these mechanistic studies have allowed us to gain into the pathophysiological

Table 1. Summary of key phenotypes observed in murine models of collagen VI disorders.

Model	Key phenotypes	Refs
<i>Col6a1</i> ^{-/-} Insertion of the <i>neo</i> cassette into the second exon interrupts the <i>Col6a1</i> coding sequence. No triple helix collagen VI produced	First description of <i>Col6a1</i> ^{-/-} mice, which induced an early-onset myopathy (hypercontracted/necrotic muscle fibres, loss of contractile strength) that strongly resembles Bethlem myopathy Mitochondrial dysfunction and apoptosis of muscle fibres, due to major defect in autophagy Inability of muscle to regenerate after injury due to impairment of satellite cells self-renewal Decreased tensile strength of the skin and an altered collagen fibril and basement membrane architecture Improvement of the metabolic phenotype in the context of both a high-fat diet and a challenge with the <i>ob/ob</i> mutation Attenuation of hyperplasia and primary tumour size in mammary tumour virus/polyomavirus middle T oncogene (MMTV-PyMT) murine model Collagen VI plays a protective role in myocardial infarction Impaired peripheral nerve regeneration due to defects in macrophage polarisation and migration. Increased apoptosis and abnormal regulation of autophagy in the CNS. Altered neuromuscular transmission, with electrophysiological defects, due to impaired AChR clustering and synaptic gene expression Delayed hair cycling and growth Abnormal tendon fibril structure, resulting in a reduction in maximum load and stiffness Accelerated development of osteoarthritis, delayed secondary ossification and lower bone mineral density Bone loss, changes in osteoblastic morphology Protection from bleomycin-induced lung fibrosis Increased lung tissue elasticity, altered airway morphology; larger but fewer alveoli and increased epithelium thickening Decreased intracranial melanoma progression, basal laminin deposition and pericyte maturation and vascular defects	[40] [39,194] [195] [15] [162] [17] [196] [44–46,197] [198] [199] [43] [52,53] [109] [48–50] [200]
<i>Col1a1</i> ^{GT/GT} G283R mutation in exon 9. Collagen VI not detected in all tissues	Impaired muscle growth, twitch and tetanic contractions and limb weakness. Reduced number of myofibres	
<i>Col6a2</i> ^{-/-} The insertion of Velocigene cassette ZEN-Ub1 created a deletion	Trabecular bone mass in the spine and femur significantly reduced with increased osteoclast differentiation	[54]
<i>Col6a3</i> ^{hm/hm} Insertion of the <i>Pgk-Neo</i> cassette within exon 15 caused a premature translational termination codon. Collagen VI deficient in ECM, retained within the cellular compartment	Mild myopathic features, including decreased muscle mass and contractile force and tendon abnormalities	[56]
<i>Col6a3</i> ^{+/-d16} Generated by exon 16 deletion, the most common defect found in UCMD. Mutant Collagen VI secreted as tetramer but no microfibrils assembled	Mild myopathic features, abnormalities in muscle and tendon and compromised muscle functions	[58]

functions of collagen VI, a therapeutic strategy for the treatment of muscular dystrophies remains elusive. Two approaches are currently being evaluated. Anti-sense oligonucleotides (ASO), also known as molecular patches, have recently been shown to correct mutations associated with exon skipping in *COL6A1*. These ASO were shown to restore functional matrix deposition in cells isolated from patient samples [59].

Secondly, adult-derived stem cells (ADSC) express significant quantities of collagen VI, and in murine proof-of-concept studies, intramuscular transplantation of these cells restored muscle function in collagen VI-related myopathies [60]. However, an effective therapy would require transplantation of ADSC to the entire musculature. The team recently performed a proteomic screen using muscle biopsies from patients with

collagen VI-related myopathies (CMD) and *Col6a1*^{-/-} mice to identify chemokines/chemokine receptors which could be specifically activated to promote disease-specific homing of the circulating ADSC to skeletal muscle [61]. CCR2 and CXCR2 emerged as putative targets, as they provided directional migration of the ADSC into injured muscle in CDM murine models. Hopefully, these initial studies will pave the way for further mechanistic studies, providing the basis for the translation of novel strategies in the treatment of collagen VI-related myopathies.

Receptors/interaction partners for collagen VI

Protein–protein interactions coordinate diverse molecular mechanisms underlying cellular function and are often perturbed in disease states. Understanding these associations is of great significance, and recent work has explored their role in collagen VI signalling. The multidomain structure of collagen VI permits interaction with several matrix components, including fibronectin [62], biglycan [63], decorin [64], von Willebrand factor (vWF) [65], vWF-A domain-related protein (WARP) [66], heparin sulphate [67], fibulin [68] and type I and type IV collagen [69,70] and within the cellular compartment, Annexin A2 [49]. Annexin 2 knockout mice failed to secrete collagen VI which remained within the late Golgi–microsomal compartment. Annexin 2, along with the SNARE proteins SNAP-23 and VAMP2, formed a complex within secretory vesicles and facilitated collagen VI secretion in bronchial epithelia cells and tissue fibroblasts. Annexin A2 mediated secretion of collagen VI and its subsequent adhesion to the basement membrane and was shown to be critical to the prevention of apoptotic cell dropout and normal pulmonary function.

The leucine-rich proteoglycans (LRP) biglycan and decorin have been shown to interact with collagen VI via the N-terminal region of the triple helix. Both of these LRP proteoglycans form complexes with the matrix protein matrilin-1 to anchor collagen VI to collagen II, with aggrecans serving as adapter proteins connecting hexagonal macromolecular networks in the extracellular matrix [64,71,72]. Transforming growth factor β I (TGF β I) has also been shown to form a complex with biglycan and decorin, enhancing their interaction with collagen VI. In addition, TGF- β I has been shown to induce the rapid aggregation of the pepsin–collagen VI tetramers to large molecular weight complexes [73]. Biglycan is also able to bind to sarcoglycan and α -dystroglycan complex, the latter being upregulated in the dystrophic *mdx* mouse and

suggested as a putative receptor for this complex [74,75]. However, the physiological roles of collagen VI–biglycan–sarcoglycan, and α -dystroglycan complexes have yet to be demonstrated.

vWF-A domain-related protein (WARP) is another protein now established as exhibiting a close high-affinity interaction *in vivo* with heparan sulphate, perlecan and collagen VI [76]. The overlapping expression profile of perlecan, WARP and collagen VI in many tissues such as peripheral nerves, cartilage and skeletal muscle suggests a relationship between WARP and collagen VI signalling. Accordingly, in a WARP-deficient murine model, collagen VI expression was found to be unchanged in skeletal muscle fibres and cartilage, but significantly diminished in peripheral nerves and displaying an altered morphology [77]. Thus, it appears WARP may play a role in collagen VI processing *in vivo*. This work also illuminates an important paradigm in collagen VI signalling in that it displays profound tissue specificity. Therefore, exploring the function of collagen VI in different tissues could help elucidate core pathogenic pathways associated with specific diseases [66].

Collagen VI is abundantly expressed in the arterial subendothelium and in the extracellular matrix component has been shown to be responsible for vWF-dependent platelet adhesion and aggregation under high shear forces. This suggests that collagen VI plays an important role in the haemostatic process triggered upon damage of the blood vessel wall [78]. This interaction is mediated via the globular amino-terminal portion of the α 3(VI) chain of intact collagen VI tetramers.

Early studies showed that pepsin solubilised collagen VI binds to fibronectin via the triple helix [62], and this interaction has also been confirmed using native collagen VI tetramers [70]. Subsequent studies have shown abnormal fibronectin deposition in the ECM from fibroblasts both Bethlem myopathy and UCDM patients, as well as fibroblasts from *Col6a1* null mice [79,80]. Furthermore, matrices from these *Col6A1*-deficient fibroblasts produced more aligned fibronectin fibres [81,82], collectively demonstrating that collagen VI regulates the 3D organisation of fibronectin fibrillogenesis within the ECM.

Despite increasingly knowledge of collagen VI-binding partners, there are limited descriptions of functional receptors. Potential receptors include NG2 chondroitin sulphate proteoglycan (NG2) [83], integrins [84,85] and the CMG2/ANTXR2 receptor [86]. Collagen VI contains 13 Arg-Gly-Asp- (RGD) sequences within the triple helical domains [87], and their involvement in collagen VI binding has been

shown to be mediated via unfolded $\alpha 2$ and $\alpha 3$ chains [88]. This group further demonstrated that the triple helix is the major-cell binding domain of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins [84]. Interestingly, in corneal fibroblasts, attachment to type VI collagen has also been shown to be RGD-independent unless the molecule is denatured [89].

NG2 was initially shown to tether collagen VI to the cell surface, and its absence led to a loss of collagen VI from the peri-cellular matrix [90]. More recent work has shown that the NG2–collagen VI axis plays a critical role in tendon fibroblast polarisation and migration, regulating cell behaviour during *in vitro* wound healing [91]. Collagen VI–NG2 colocalises at the trailing edge of migrating cells, providing an anchorage to the substrate. The physiological importance of the collagen VI–NG2 axis was confirmed using fibroblasts isolated from UCMD patient tendons. NG2 expression was significantly reduced in these cultures and cells failed to polarise during migration [57]. The NG2–collagen VI axis has also been shown to play a key role in soft tissue sarcoma invasion and adhesion, through co-operation with $\alpha 2\beta 1$ integrin [92]. More recently, collagen VI has been shown to be crucial in driving TNBC cell invasion. Small interfering RNA (siRNA)-mediated NG2 depletion partially attenuated both adhesion and migration of epithelial breast cancer cell lines [20] and, interestingly, no role for $\beta 1$ integrin was found in this system. However, collagen VI-induced migration of these cells was also shown to be mediated via epithelial growth factor receptor (EGFR) signalling, suggesting tyrosine kinase receptor (TKR) crosstalk with NG2 to augment collagen VI signalling, as is observed with other ECM components.

Hyaline fibromatosis syndrome (HFS) is a genetic disorder caused by mutations in the *ANTXR2* gene, also termed *CMG2*, which encodes the transmembrane receptor CMG2/ANTXR2. CMG2 has been shown to be a receptor for collagen VI [86] and binding to the THD of collagen VI facilitates its lysosomal degradation. In *Antxr2*^{−/−}-deficient mice, excessive collagen VI deposition was observed, leading to progressive uterine fibrosis that was reversed by crossing with *Col6a1*^{−/−} mice. Subsequently, it was reported that CMG2 binds to actin indirectly via talin and vinculin. The downstream effects of this are the recruitment of RhoA and its effectors Src, mDia1 and MYL12A, resulting in CMG2 receptor-mediated endocytosis of collagen VI and its subsequent trafficking to the lysosomes (Fig. 2) [93]. The ANTXR1/ tumour endothelial marker (TEM) 8 was identified via a yeast two-hybrid screen, of interacting with the cleaved C5 domain of collagen

VI $\alpha 3$, and confirmed using immunoprecipitation approaches [94]. To explore the physiological relationship, immunohistochemical studies showed a high degree of colocalisation of ANTXR1 and collagen VI, in malignant (but not corresponding matched normal) samples from colonic tumour, lung and oesophageal cancer, suggesting the expression levels were co-ordinated regulated. However, no subsequent studies have further investigated this relation or pathophysiological consequence of TEM8 receptor engagement by the cleaved C5 fragment. Collectively, these results highlight the myriad of protein interactions mediated by collagen VI enabling it to function within a sequential signalling hub to guide a broad set of cellular processes in many different tissues (Fig. 2).

Transcriptional control of collagen VI expression

Collagen VI is expressed in several extracellular matrices, including tendon, muscle, cartilage, lung [95], adipose tissue [96], as well as in the central and peripheral nervous systems [47]. It is often expressed in a discrete anatomical niche, for example in and adjacent to basement membranes of myofibres and intramuscular nerves. Fibroblasts are the best characterised source of collagen VI and are the principal source in skeletal muscle [26,97] and the dermis [98,99]. Less well-studied sources of collagen VI include astrocytes [100] and macrophages. Cytokines associated with alternatively activated macrophages, such as IL-4, IL-10 and TGF- $\beta 1$, induce expression of collagen VI, with TGF- $\beta 1$ exhibiting the most significant regulatory role [101]. Interestingly, despite the abundant expression of collagen VI by differentiated macrophages, these cells could not assemble collagen VI into beaded filaments as observed in fibroblasts. This has been proposed to be due to low expression of proteins required for filament assembly; these cells all express comparatively low levels of biglycan, decorin, fibronectin, fibromodulin and lumican. Again, this highlights how the spatial distribution of collagen VI and the subsequent range in local tissue levels could guide distinct functional roles *in vivo*.

Given the intricate spatial patterning of collagen VI within organs in the human body, it is likely that sophisticated regulatory mechanisms orchestrate its expression. Collagen VI regulation appears independent to that of other ECM components such as collagen I/III and fibronectin [102], although few studies to date have looked at the transcriptional control of collagen VI. Like most collagens, collagen VI mRNA turnover is relatively slow compared with protein

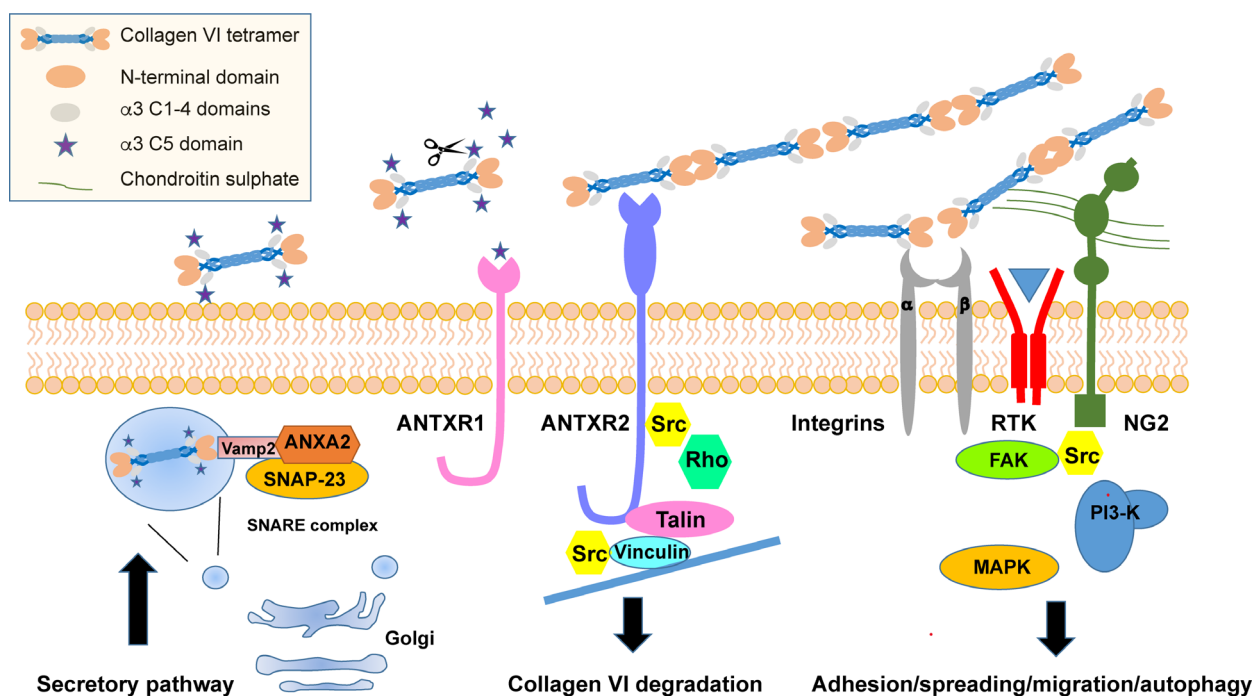


Fig. 2. Schematic representation of the binding partners of collagen VI. Collagen VI tetramers are secreted through their interaction with the SNARE complex of annexin 2, SNAP-23 and VAMP2 into the extracellular matrix and form characteristic beaded microfibrils in linear pattern with end-to-end connections. The C5 domain is proteolytically cleaved and can bind to the ANTXR1 receptor. ANTXR2 binds to the triple helix domain of collagen VI, resulting in the release of Src and subsequently talin, vinculin and actin release from ANTXR2. RhoA then binds to ANTXR2 and active Src, leading to phosphorylation of ANTXR2, endocytosis and subsequent collagen VI lysosomal degradation. Co-operative integrin-mediated binding to the N-terminal domains of collagen VI, NG2 chondroitin sulphate and receptor tyrosine kinase (RTK) leads to PI-3 kinase-dependent cytoskeletal changes, resulting in enhanced cell spreading and adhesion.

turnover, with the half-life of *Col6a1* mRNA being ~ 35 h compared with < 3 h for collagen VI $\alpha 3$ protein [103]. Early studies identified a transcription start site (TSS) proximal element (-82 and $+8$) termed growth arrest responsive region (GARR), which contained Sp1 and SREBP transcription factor (TF)-binding sites. Both transcription factors were subsequently shown to drive *Col6a1* gene expression [104,105]. An in-depth analysis of cis-acting regulatory elements in *Col6a1* found multiple cis-acting elements in genomic regions up to 7.5 kB from the TSS that were found to be critical for high levels of gene expression in several tissues [106]. More recently, we have identified epigenetic control of *COL6A1/2/3* by the histone acetylase EP300 and BRD4 [107] (Fig. 3). Both of these epigenomic readers colocalise with lineage-defining transcription factors on active enhancers [108]. Depletion of collagen VI from these myofibroblasts significantly impaired the contractile forces exerted by these cells, via EP300 transcriptional co-ordination. Also, we observed high levels of both H3K4 methylation and H3K27 acetylation in the distal genomic regions of the

COL6A1 and *COL6A2* with a strong peak of EP300 binding, highly suggestive of an active enhancer within this region in Dupuytren's disease myofibroblasts (Fig. 3). BRD4 also marks the TSS within both promoters, again with elevated levels of H3K27 acetylation reflecting high levels of active transcription of these genes. We also performed EP300 Chip-Seq de novo motif analysis of the EP300-enriched loci and identified known consensus binding sequences for the transcription factor Fra-1/FOSL1 and subsequently confirmed a role of this TF in *COL6A3* gene expression [107]. Further evidence supporting a direct role of this transcription family in the regulation of collagen VI gene expression was recently described by Ucero *et al.* [109]. Here, Fra-2 was shown to directly regulate all 3 isoforms of *Col6a* in murine macrophages. Using ChIP-qPCR, the authors were able to show direct binding at putative AP1/Fra-2 TPA response elements (TRE) with promoter proximal regions of *Col6a1*, *Col6a2* and *Col6a3*. Interestingly, they did not find a role for Fra-2 in TGF- $\beta 1$ -induced *Col6a* gene expression in fibroblasts, suggesting either a distinct isoform-

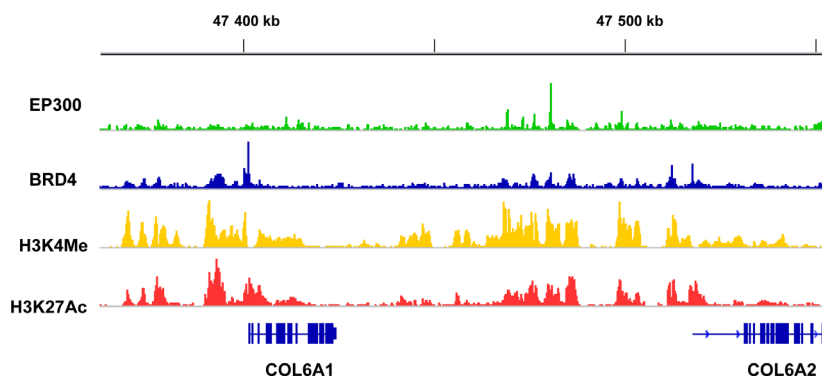


Fig. 3. ChIP-Seq profiles of genomic locations of *COL6A1* and *COL6A2* reveal presence of distal enhancer elements.

or spatial-specific Fra-dependent mechanism control *Col6a* gene expression.

Through correlation analysis of human cohorts, a recent study identified the paired related homeobox 1 (*PRRX1*) as a novel positive regulator of *COL6A3* gene expression in differentiating human adipocytes [110]. However, whilst TGF- β 1/SMAD induced strong activation of *COL6A3* in these cells, this transcriptional response was not mediated by *PRRX1* and the detailed mechanism remains to be elucidated.

Taken together, these data indicate the growing appreciation of how collagen VI expression is regulated through genetic and epigenetic mechanisms. Indeed, alongside the complex post-translational modification of collagen VI, its expression now appears intricately orchestrated in the human body. These data highlight potential therapeutic strategies to co-opt processes that control collagen VI expression. As in many other diverse disease systems, disordered protein expression underpins the pathological sequence that ultimately compromises organ function in fibrosis.

Proteolytic processing of collagen VI chains

The N- and C-terminal portions of collagen VI are to a large extent cleaved off during post-translational processing of type VI collagen fibrils. The C5 Kuntz-like domain is not detected in mature type VI collagen fibrils within fibrous tissues [111] and is cleaved off from collagen VI immediately after secretion in articular cartilage [112]. However, the identity of enzyme(s) responsible for the generation of the cleavage products has not been extensively studied. Until recently, the only class of enzymes shown to be capable of enzymatic cleavage was members of matrix metalloproteinase (MMP) family, specifically MMP2, 7 and 9, cleaving collagen VI α chain 1 and 2 within the short triplex helix domain [113–116]. MMP11 has been

shown to cleave native α 3 chain of collagen VI and MMP11-deficient mice showed incorrectly folded collagen VI surrounding adipocytes [117]. Immortalised human adipocytes also show sensitivity to MMP11-mediated collagen VI α 3 degradation [118]. More recently, it has been shown that overexpression of MMP14 can digest collagen VI α 3 within its C5 domain to produce endotrophin (ETP) in adipose tissue from pre-existing obese mice [119].

A recent comprehensive analysis of tissue distribution of collagen fragments found that C-terminal cleavage products of the collagen VI α 3 chain vary in size and that their composition is tissue-specific [120]. Furthermore, bone morphogenetic protein 1 (BMP-1) metalloproteinase was identified as being responsible for cleavage of the α 3 chain between C4 and C5 domains, generating the ETP fragment. In addition, a furin-like proprotein convertase (PCSK3) cleaved the protein between the C1 and C2 domains. Proprotein subtilisin/kexin, of which there are nine family members in total (PCSK1–9) with tissue-specific expression, can be found within the endoplasmic reticulum, Trans-Golgi network (TGN), at the plasma membrane and in the extracellular space [121,122]. With the exception of PCSK8 and 9, these enzymes recognise and cleave their diverse proprotein substrates at the C-terminal end of the basic sequence RXR/KR, leading to the activation of protein precursors. *In vitro* assays have shown significant biochemical redundancy between the family members, but the phenotypes of PCSK-deficient mice and patients carrying an inactive PCSK allele argue for a specific biological function [123–129]. Further work will be needed to identify potential tissue-specific PCSK-mediated collagen VI α 3 processing. Overall, the post-translational modification of collagen VI and downstream cellular effects of discrete proteolytic pathways is only recently being studied in detail. Looking forward, defining the specific enzymes involved in the cleavage processes of collagen VI

within the tissue niche will be crucial to better understand collagen VI protein dynamics and may guide potential therapeutic strategies.

Collagen VI and fibrosis

Fibrosis is the result of a disordered ECM deposition and remodelling. Fibroblasts play a crucial role in ECM homeostasis by controlling ECM turnover, ensuring degradation and secretion of collagens and other matrix components are finely balanced. During fibrosis, this equilibrium is disturbed and the continuous activation of fibroblasts and their differentiation into myofibroblasts produce a relative excess of ECM proteins. Collagen VI substrates are potent inducers of myofibroblast differentiation [130], and collagen VI expression is tightly associated with pro-fibrotic myofibroblasts [131]. Indeed, a study of cellular phenotype and ECM in palmar fibromatosis demonstrated that the distribution of collagen types IV, VI, laminin and fibronectin was confined to the myofibroblast compartment. Moreover, whilst the $\alpha 1$, $\alpha 2$ and $\alpha 3$ changes are ubiquitously expressed, $\alpha 6$ seems to be specifically upregulated in fibrotic areas in Duchenne muscular dystrophy [132], suggesting that interactions between myofibroblasts and the surrounding ECM may be critical in the pathogenesis of fibrosis in an α chain-specific manner.

Liver fibrosis

In the adult human liver, collagen VI expression is localised within the portal spaces and forms a continuous lamina in the sinusoids. Interestingly, adult hepatocytes exhibit low expression of collagen VI in contrast to foetal hepatocytes that stain strongly for the protein [133]. Together, these results highlight the dynamic expression of collagen VI during human development and how its function may adapt to support discrete functions at different stages. As collagen VI is now recognised as a putative marker of mesenchymal activation, its expression pattern in the healthy and fibrotic adult liver may provide clues to its role in the disease process.

Collagen VI has been proposed as an indicator of early architectural remodelling in liver fibrosis [134,135]. In accordance with this, in alcoholic fibrosis and cirrhosis, high levels of collagen VI have been detected in the fibrous septa that compartmentalise areas of nodular generation. Specifically, most ECM fibres within fibrotic foci contained collagen VI, indicating that this protein forms an integral component of these structures [133]. Furthermore, studies in

cadaveric human livers confirmed collagen VI expression in fibrous septa, areas of bridging fibrosis and within the diffuse architectural remodelling of cirrhosis colocalised with collagens I, III and V [136]. Collectively, these results indicate that collagen VI is a key scaffold within various fibrotic structures in the liver. As the complex cellular and mechanisms orchestrating cirrhosis are better appreciated, it will become important to understand the role collagen VI in the profound structural reorganisation of the liver in fibrosis. Serum collagen VI levels have also been shown to be elevated in patients with hepatic fibrosis, although there was no a clear correlation with disease severity [135,137]. Children with cystic fibrosis also display high levels of collagen VI in serum but only in patients with significant liver disease [138].

Several publications have shown in multiple animal models of liver fibrosis that collagen VI is elevated following injury. In an earlier report, collagen VI expression was detected 3 days following administration of carbon tetrachloride (CCl_4), an acute injury model of liver fibrosis, with initially localising around the central veins [139]. In rat models following administration of CCl_4 , collagen VI protein was also detected within 3 days of acute injury [139]. Subsequent studies in rats showed that collagen VI expression was elevated in two distinct peaks in different models of liver fibrosis: at 8 weeks in the CCl_4 model and 2 weeks in the bile duct ligation model [140]. We also found that in the 5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet murine model of cholangitis and biliary liver fibrosis [141], similar to bile duct ligation, strong expression of collagen VI around the biliary canaliculi was observed at 10 days following commencement of the diet (Fig. 4).

Skin fibrosis

In healthy adults, collagens I and III comprise the major ECM components within the skin. Collagen VI is a minor component although $\alpha 1$ – $\alpha 3$ chains are present throughout the dermis, with $\alpha 5$ and $\alpha 6$ chains showing a more restricted pattern of expression. The $\alpha 5$ chain is strongly associated with the papillary dermis and both $\alpha 5$ and $\alpha 6$ chains localise to the blood vessels [15,142]. A wide spectrum of skin changes has been observed in patients with collagen VI-related disorders, including abnormal scarring, such as keloids and ‘cigarette paper’ scars, rough or dry skin, and striae rubrae [143,144]. Indeed, collagen VI has been shown to be highly expressed at the edges of keloid scars and in healing wounds [81,145]. Using a biopsy punch murine model on shaven backs, $\alpha 1$ – $\alpha 3$ chains are

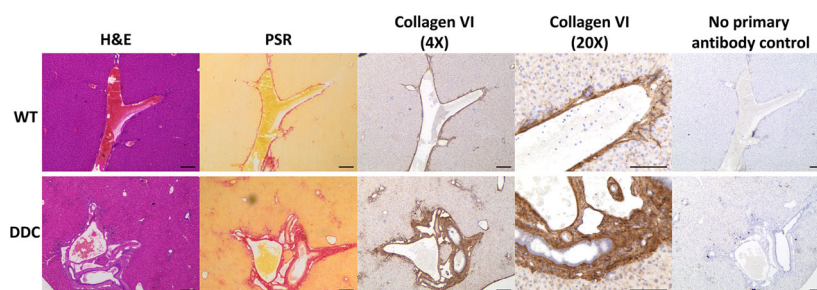


Fig. 4. Increased expression of collagen VI in the DDC diet-induced liver fibrosis model. C57BL/6 mice were fed with control diet (WT), or the 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet for 10 days. Representative images of liver sections stained with H&E, Picro Sirius red (PSR) or immunohistochemical staining for collagen VI $\alpha 1$ chain visualised using horseradish peroxidase and haematoxylin counterstain. Images were captured using Leica Microscope Systems (Milton Keynes, UK). Scale bar = 200 μ m, inset = 100 μ m.

strongly expressed throughout the dermis and epidermis by day 4 [15], and following completion of epithelialisation, their expression is mainly restricted to the newly formed epidermis. These authors also investigated collagen VI expression following local administration of bleomycin. Increased $\alpha 3$ chain expression was observed throughout the dermis and $\alpha 5$ expression was also increased in the perivascular regions [15]. Tight skin (Tsk) 1 and Tsk2/+ mice recapitulate some features of systemic sclerosis (SSc) and have been used to study the mechanisms of skin fibrosis, such as increased thickening of the dermis and excessive deposition of thick collagen fibres. Early studies using Tsk1^{+/-} mice showed increased collagen VI expression in the spontaneously fibrotic skin [146]. However, using the Tsk2/+ model, a gain-of-function mutation in the *Col3A1* gene [147], collagen VI does not appear to be dysregulated. Limited data exist regarding expression of collagen VI in clinical samples from fibrotic skin conditions. Using *in situ* hybridisation, enhanced expression of collagen $\alpha 2$ was observed in five patients with diffuse, rapidly progressive SSc compared with normal skin samples [148]. An early study using immunohistochemistry showed high levels of collagen VI expression within the subcutaneous fat in SSc patients [149], although no subsequent reports have been published.

Kidney fibrosis

Collagens are differentially expressed throughout the kidney under physiological conditions. Collagen type IV is predominantly located within the glomerular basement membrane (GBM). Mesangial ECM differs substantially from GBM and mainly comprises fibronectin, collagen type IV and collagen V. Collagen VI is minimally expressed in the interstitial matrix [150],

and the GBM was weakly positive for type VI collagen in the adult human kidney [151]. Deposition of both collagen V and VI was found to be significantly increased in patients with kidney disease of diverse aetiology when compared with control patients [152,153]. Collagen type VI was markedly increased in diabetic glomeruli, associated with α -smooth actin-positive myofibroblasts [154].

Lung fibrosis

In normal lungs, collagen VI is a key component of the basement membrane and is present in the bronchial and vascular walls, as well as in the interstitial space [69]. It is distributed on the basal surface of the respiratory epithelium and is believed to be an important adhesive substrate for the pathogens *S. pyogenes* and *S. pneumoniae* [155]. The expression of collagen VI is increased in lung fibrosis, and levels appear to be independent of the aetiology of fibrosis [69]. Analysis of gene expression data from the Lung Genomics Research Consortium (LGRC) database of patients with interstitial lung tissue (ILD), chronic obstructive pulmonary disease (COPD) and control lung tissue from patients undergoing thoracic surgery showed increased *COL6A1* expression in patients with both ILD and COPD compared with controls. *COL6A3* expression was only increased in ILD samples, but not in COPD patient samples [109]. However, in a study of 11 patients with COPD, immunohistochemistry revealed increased collagen VI $\alpha 3$ chain staining in ECM of the airway mucosa of COPD patients compared with healthy smoker controls [156]. A similar analysis of idiopathic pulmonary fibrosis (IPF) samples from a smaller independent sample set (GSE2052) also found significantly increased mRNA expression of *COL6A1*, *COL6A2* and *COL6A3* in IPF lung samples

compared with healthy lungs. Expression of collagen VI $\alpha 1$ [157] and collagen VI $\alpha 3$ chains [107] was found to be upregulated on immunohistochemical staining of lung tissue from patients with IPF and was associated with the fibrotic foci containing a myofibroblast core and procollagen I [157].

Collagen VI protein is the most abundant collagen expressed in the mouse lung [48], and expression of *Col6a1*, *Col6a2* and *Col6a3* was upregulated early in the lungs of mice injected with LPS intraperitoneally [158], supporting the view that collagen VI expression may be an early rather than a late phenomenon in pulmonary fibrosis [69]. A recent elegant study has shown collagen VI plays a critical role in two distinct models of lung fibrosis: intratracheal administration of bleomycin and Fra2-transgenic (Tg) mice. The AP-1 transcription factor Fra-2 has been shown to be elevated in several chronic lung conditions, such as systemic sclerosis (SSc) [159], interstitial lung disease (ILD) and idiopathic pulmonary fibrosis [109,160], and in pulmonary macrophages from chronic obstructive pulmonary disease (COPD) patients [161]. Previous studies, using a transgenic construct for ectopic expression of Fra-2 driven by the ubiquitous H2Kb promoter, showed that these Fra2 mice developed spontaneous fibrosis in multiple organs, with the lung and skin showing the highest degree of fibrosis, resembling systemic sclerosis [160]. The lungs of these mice showed massive collagen deposition and an accumulation of ECM producing myofibroblasts. Fra-2 was expressed by mesenchymal cells, alveolar epithelial type II (AEC2) cells and cells of monocytes/macrophage lineage within the lungs of these mice. The latter are thought to be major contributors to the production of secreted factors critical to the development of lung fibrosis. Exosomes are enriched in RNAs, and proteins form precursor cells and macrophages are known to be major secretors of pro-inflammatory cytokines in the bronchoalveolar lavage fluid (BALF) exosomes. Analysis of microsomes isolated from BALF from both Fra-2 Tg mice and bleomycin treated wild-type mice, showed an enrichment of all three collagen VI chain peptides. Similarly, their respective mRNAs were also upregulated in lung tissue of these two murine models. However, whilst PDGFR α ⁺ and EpCAM⁺ cells constitutively expressed high levels of all three collagen chains, only the F4/80⁺ cells (macrophage) upregulated all three chains in response to bleomycin administration. Immunohistology revealed that collagen VI was co-expressed with YM-1 positive alternatively activated population of macrophages within the lung. Further experiments demonstrated the important contribution of Fra-2 expression within the

myeloid/macrophage compartment; using mice in which Fra-2 had been specifically inactivated in the macrophage compartment (Fra-2 ^{Δ mac}), bleomycin treatment resulted in an attenuated fibrotic response, as shown by a reduction in Sirius red staining, hydroxyproline content and improved pulmonary function.

To address the relative contribution collagen VI played in the development of lung fibrosis, *Col6a1*^{-/-} deficient mice were exposed to intratracheal bleomycin administration. These mice developed a much less severe phenotype, with better lung function and corresponding lower levels of collagen I and fibronectin deposition [109]. Specifically, transplantation of bone marrow cells isolated from *Col6a1*^{-/-} deficient mice provided protection from bleomycin-induced fibrosis. Collectively, these data show the important role collagen VI plays in driving fibrotic lung disease, and the critical role the AP-1 transcription factor Fra-2 plays in driving this phenotype. Importantly, this raises the possibility of utilising small molecular inhibitors of AP-1 for the treatment of fibrotic lung disease.

In conclusion, collagen VI appears to be an important driver in a range of fibrotic disorders, in both murine models and human systems. Corresponding to the pleiotropic functions of collagen VI in human physiology, its roles in fibrosis seem equally broad. As with other members of the collagen superfamily, there is growing recognition that excessive collagen VI deposition in organs undergoing fibrosis is not merely an end product of the disease process but may be a master regulator orchestrating disordered protein turnover in addition to multiple cellular mechanisms that sustain the pathological ecosystem, including chemotaxis, cell migration, proliferation and apoptosis.

Endotrophin

ETP and adipose tissue

Collagen VI is abundantly expressed in white adipose tissue (WAT) in mice. It is the most highly expressed collagen by differentiated adipocytes and undergoes significant structural remodelling during adipogenesis. The absence of collagen VI (*Col6a1*^{-/-}) leads to expansion of adipocytes on the *ob/ob* background, and these mice show reduced mRNA levels of key fibrotic genes such as TGF- β 1, lumican, decorin, elastin and multiple matrix metalloproteinases (MMP-1, MMP-3, MMP-7, MMP-13 and MMP-25), ultimately resulting in a looser and more disordered adipose tissue ECM [162]. Several studies have demonstrated that collagen VI levels are positively correlated with hyperglycaemia and insulin resistance [163], suggesting an important

metabolic role in adipose tissue. ETP was initially identified as a cleavage product involving the C5 domain of collagen VI $\alpha 3$ secreted by fully differentiated 3T3-L1 adipocytes, but not by the preadipocytes, and has been shown to be upregulated in fat pads from *ob/ob* mice compared with lean littermate [18]. ETP plays a pivotal role in shaping a metabolically unfavourable microenvironment in adipose tissue during consumption of a high-fat diet (HFD). It serves as a powerful costimulator of pathologically relevant pathways within the 'unhealthy' adipose tissue milieu, and neutralising ETP ameliorated metabolic adverse effects and effectively reversed metabolic dysfunction induced by a high-fat diet (HFD) [164]. Recent work using *Col6a1*, *Col6a2* and *Col6a3*-deficient 3T3-L1 cell lines showed that whilst all three lines showed attenuated adipocyte differentiation, only the *Col6a3*-deficient lines showed a decrease in adipocyte lipolysis and lipolytic capacity, and a significant reduction in IL-6 production. Conversely, adenoviral-driven expression of ETP facilitated ER stress-driven IL-6 expression, JNK activation, cellular apoptosis and insulin resistance. Adipose tissue-specific overexpression of ETP using the APN-ETP Tg mice fed long term (23 weeks) on HFD showed elevated expression of genes associated with inflammation and lipolysis, namely leptin, IL-6, ATGL and HSL [165]. Collectively, these findings indicate that ETP exerts a major influence in adipose tissue, eventually resulting in systemic elevation of pro-inflammatory cytokines and insulin resistance.

In human adipose tissue, *COL6A3* expression increases in obesity, independent of diabetes. Obese subjects with high *COL6A3* have increased adipose tissue inflammation and increased visceral adipose tissue mass [166]. A further large study showed spatially heterogeneous collagen VI expression pattern in human adipose tissue, with tracks of collagen VI associated with abundant macrophages. At the mRNA level, there were strong correlations between the gene expression of collagen VI and CD68, and both *COL6A1* and *CD68* mRNA levels were associated with body mass index (BMI) and inversely correlated with insulin sensitivity. A higher percentage of fibrosis was found in the adipose tissue from obese compared with lean subjects (86 patient biopsies) and most of these fibrotic areas expressed collagen VI [167]. In contrast, a smaller study using adipose tissue biopsies showed that *COL6A3* showed a trend (although not significant) to be lower in obese people in a study of 15 paired obese *vs* lean patients. Further arms of this study looked at *COL6A3* expression after very low-calorie diet or bariatric surgery and reported an

increase in *COL6A3* expression after either intervention. In the seminal study by Sun *et al.*, ETP levels were measured in mesenteric adipose tissue from BMI age-matched healthy obese, with normal insulin sensitivity HOMA-IR < 1.7 or those with HOMA2-IR > 2.6. ETP was found to be significantly elevated in the latter group, this metabolically challenged group was also exhibiting increased overall fibrosis within the adipose tissue, as measured by percentage positive area of Trichrome C staining [164]. In summary, ETP is now an emerging biomarker in obesity within which its expression correlates with structural aberrations in the adipose tissue matrix that occurs during excessive fat storage.

ETP and cancer

Endotrophin is highly expressed by both cancer cells and tumour stromal cells within the tumour microenvironment in human breast and colon cancer biopsies [17,168]. ETP is a potent pro-fibrotic factor influencing the tumour niche and a trigger of tumour stromal expansion within the tumour microenvironment. ETP overexpression can trigger fibrosis, with high levels of myofibroblast accumulation within tumour tissues and acts as a chemokine to enhance stromal expansion, with endothelial cells and macrophages being particularly responsive in migration assays [18]. Furthermore, endothelial cells exposed to ETP formed more organised vascular structures in angiogenesis assays. ETP is also involved in mammary tumour progression, fibrosis and chemokine upregulation and through enhanced TGF- β 1-dependent endothelial-mesenchymal transition (EMT) [169]. Hepatocellular carcinoma (HCC) patients with mutations in *COL6A3* exhibited a mortality rate 3.5-fold higher compared with those without mutations in *COL6A3*, indicating *COL6A3* plays a crucial role in HCC pathogenesis. The same authors showed that ETP overexpression augmented *N*-nitrosodiethylamine (NDEA)-induced HCC progression, with more tumour lesions evident in Alb-ETP mice than in controls [170]. The role of endotrophin in cancer has been comprehensively evaluated elsewhere [171].

ETP and fibrosis

There is increasing evidence that ETP accounts for many of the pro-fibrotic properties of collagen VI observed in various fibrotic diseases [18,164,170,172]. Ectopic ETP expression induces upregulation of TGF- β 1, adipose tissue fibrosis, angiogenesis and inflammation. ETP has recently been shown to play a critical

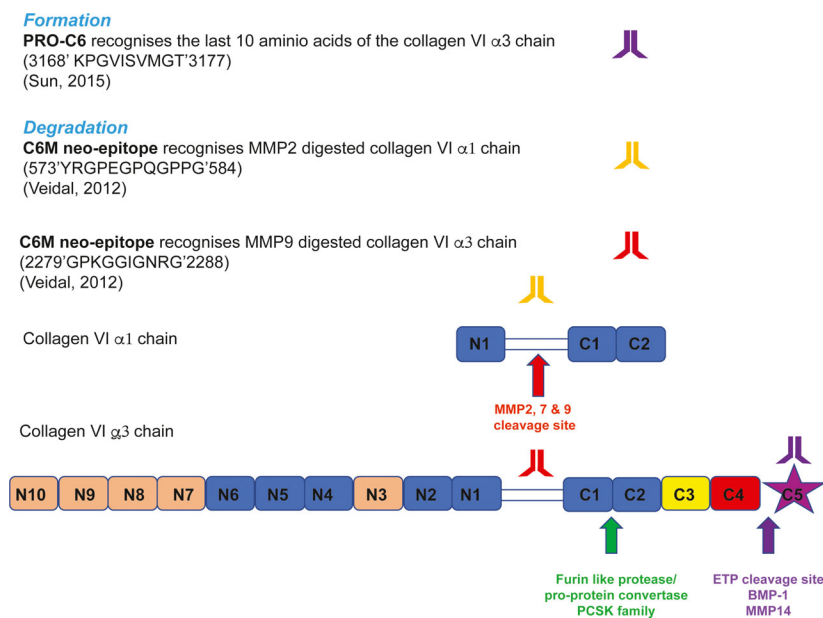


Fig. 5. Schematic representation of collagen VI chains with enzymatic cleavage sites and the epitopes detected by PRO-C6, C6M and C6M $\alpha 3$ antibodies.

role in the pathogenesis of chronic liver disease as a signalling molecule rather than a structural component of the ECM [170]. Here, when ETP expression was driven in a hepatocyte-specific manner (Alb-ETP), the mice showed limited phenotypic changes in the liver pathology, indicating that ETP alone is insufficient to trigger pathological changes. However, ETP significantly enhanced the progression of hepatic fibrosis by agents such as CCl_4 , suggesting that it acts to promote the deleterious effects of other stimuli that drive liver injury and fibrosis. Further work crossing Alb-ETP-hepatocyte-specific transgenic mice with the sterol regulatory-binding protein (SREBP) 1a transgenic mice (which display a lipodystrophic phenotype, characterised by enlarged fatty livers and insulin resistance [173]), fed on a dox chow diet showed enhanced inflammation, fibrosis and liver damage but only after chronic exposure for 10 months. Furthermore, 4/5 of these mice developed cancerous nodules [174].

Further studies using human cells have now provided confirmation of many of the pro-fibrotic properties initially observed in murine systems. ETP was shown to act as a chemokine to both monocytes and macrophages, as well as human umbilical vein endothelial cells (HUVECs) [168]. Additionally, ETP treatment also stimulated tube formation of HUVEC cultures, underlining its pro-angiogenic role previously observed in mice [18]. Collectively, the early studies identifying ETP as an important mediator of fibrosis within the tumour stroma in murine models have now been translated to human cells within the context of numerous pathophysiological conditions, which will

support the development of effective therapeutic strategies targeting ETP.

Collagen VI fragments as serum biomarkers of fibrosis

Veidal and colleagues were the first to demonstrate cleavage of collagen VI α chain by MMP-2 and MMP-9. These two metalloproteinases generated several fragments, initially termed CO6-MMP [114,116]. They showed the CO6-MMP, now shortened to C6M, neo-epitope was highly associated with liver fibrosis in two animal models, suggesting type VI turnover may be a central player in fibrogenesis. As discussed previously, the C-terminal portion of the collagen VI $\alpha 3$ chain is immediately cleaved following secretion of the collagen microfibrils to produce ETP. The terminal 10 amino acids of the collagen VI $\alpha 3$ chain termed PRO-C6 have been used to generate antibodies and assays based on these developed [175]. This assay was initially used to investigate the effect of immobilisation on ECM remodelling in muscle in response to loading forces and to show that both PRO-C6 and PRO-C3 increase with mobilisation over time. More recently, MMP-9 digested collagen VI $\alpha 3$ chain fragments have been used to generate another ELISA to detect a neo-antigen, termed C6M $\alpha 3$ [176]. Given collagen VI turnover and remodelling are linked to tumour progression, the initial validation of this biomarker was performed in a large cohort of cancer patients to determine its potential as a biomarker, alongside C6M and PRO-C6. Only C6M and the novel neo-antigen

Table 2. Summary of studies using collagen VI fragments as serum biomarkers.

Biomarker	Disease	Association	Ref
PRO-C6	Type 1 diabetes	Increased arterial stiffness	[201]
PRO-C6	Type 1 diabetes	Mortality	[202]
PRO-C6	Type 2 diabetes	Cardiovascular events/mortality	[203]
PRO-C6	Type 2 diabetes	Responsiveness to PPAR γ agonists	[204]
PRO-C6	Chronic kidney disease	Mortality	[186]
PRO-C6	Chronic kidney disease	Disease progression	[184]
PRO-C6	Chronic kidney disease	Disease progression	[185]
PRO-C6	NASH	Fibrosis stage	[192]
C6M	Hepatitis C liver fibrosis	Fibrosis progression	[193]
PRO-C6	Systemic sclerosis	Organ involvement	[182]
C6M/PRO-C6	Systemic sclerosis	Biomarker	[181]
PRO-C6	Pulmonary arterial hypertension	Biomarker	[182]
C6M/PRO-C6	COPD	Biomarker/quality of life	[205]
C6M	COPD	Exacerbations	[206]
C6M	Interstitial lung disease	Biomarker	[182]
PRO-C6	IPF	Disease progression	[180]
C6M	IPF	Disease progression	[179]
C6Ma3	Ulcerative colitis, Crohn's disease and colorectal cancer	Biomarker	[207]

C6M α 3 were found to be elevated in this cohort of various cancers ($N = 65$), compared with healthy controls ($N = 13$). Collectively (Fig. 5), these three assays have now been extensively used to noninvasively monitor collagen VI formation (PRO-C6), turnover and remodelling (C6M and C6M α 3) within large patient cohorts with a variety of fibrotic conditions (Table 2). Their value as diagnostic and prognostic tools is discussed below.

Accurate disease biomarkers for monitoring disease progression and treatment are still desperately needed in IPF. For example, in clinical trials of IPF, the regulatory-approved end point of forced vital capacity (FVC) is insensitive to short-term physiological changes and, therefore, is recorded at 52 weeks. In addition, changes in FVC do not necessarily reflect response to antifibrotic therapy [177,178]. Hence, identification of new markers is urgently required as more effective endpoints. The PROFILE study assessed a panel of 11 MMP-degraded ECM proteins neoantigens as potential biomarkers in IPF. In a discovery cohort

of 55 patients, 6 neoepitopes (BGM, C1M, C3A, C3M, C6M and CRPM) were significantly elevated in patients with progressive IPF ($n = 32$) compared with those with stable disease ($n = 23$). Moreover, when assessed longitudinally, concentrations of the same neoepitopes were significantly higher in patients with progressive IPF ($n = 71$) compared with patients with stable disease ($n = 60$). By 6 months, rising concentrations of collagen degradation markers C1M, C3M, C6M and CRPM were associated with an increased risk of overall mortality [179]. In an extension to this study, longitudinal change in markers of ECM synthesis (PRO-C3 and PRO-C6) was assessed in 145 newly diagnosed individuals with IPF. Both of these markers were elevated in IPF patients compared with controls at baseline, and progressive versus stable disease during follow-up (PRO-C3 $P < 0.001$; PRO-C6 $P = 0.029$). However, their levels were not correlated with mortality [180]. Together, these results support the potential for soluble collagen VI fragments as disease markers in IPF.

Type VI collagen gene and protein levels have been shown to be upregulated in systemic sclerosis (SSc) patients [148], and a cross-sectional study demonstrated turnover of type VI collagen products (PRO-C6 and C6M) as promising biomarkers to differentiate between asymptomatic and early diffuse SSc [181]. Building on these reports, a recent study found that PRO-C6 and C6M were higher in the serum of SSc patients compared with control subjects [182]. A key objective of the study was to determine the predictive value of these biomarkers in determining organ involvement. Serum levels of C6M were found to be higher in patients with interstitial lung disease. Furthermore, serum levels of PRO-C3, PRO-C6 and C6M were higher in patients with pulmonary arterial hypertension (PAH). Indeed, all patients with PAH had significantly elevated levels of these biomarkers [182].

Progressive loss of kidney allograft function following transplantation is associated with interstitial fibrosis and adversely affects graft survival. This fibrosis is a pathological response to injury, representing an imbalance between ECM formation and turnover [183]. In a study of 78 patients undergoing renal transplant surgery, increased collagen type VI expression was observed in fibrotic lesions. In addition, there was a progressive increase of PRO-C6 in the plasma of renal transplant recipients with circulating PRO-C6 levels reflecting the stage of chronic kidney disease (CKD), levels significantly increasing with disease severity. PRO-C6 levels are also correlated with estimated glomerular filtration rate (eGFR), a key metric used to define renal function and determine the stage

of kidney disease. The authors suggested that collagen VI could be a useful marker to study renal ECM turnover to noninvasively identify patients with more active disease [184]. Progressive fibrosis is the major pathophysiological process in CKD. In the RIISC study, including a cohort of 499 patients, PRO-C6 detected in the urine was used as a marker for disease progression [185]. In an extension to this study, serum PRO-C6 levels were found not to be significantly associated with progression to end-stage renal disease over a three-year period and were independently associated with mortality after adjustment of confounding factors [186].

As with other fibrotic diseases, there is an urgent need for identification of noninvasive biomarkers for the diagnosis and management of patients with nonalcoholic fatty liver disease (NAFLD). The two principal phenotypes are nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). The latter is characterised by histological lobular inflammation and hepatocyte ballooning and is associated with faster fibrosis progression than NAFL [187]. Currently, there are no approved treatments for NASH patients. New drugs for NASH can be conditionally registered if they are shown to improve NASH without worsening of liver fibrosis or if they improve fibrosis without worsening NASH as assessed by paired liver biopsy samples, whereas full registration depends on the drug effect on clinical outcomes. Therefore, there is a reliance on biopsies to assess the severity of NAFLD and monitor disease progression [188]. Imaging approaches such as ultrasonography, transient elastography (TE or FibroScan) and MRI are also used to detect stenosis, scarring/stiffness and fat mapping of the entire liver, respectively, and have provided an alternative to tissue biopsies to assess liver fibrosis in patients with hepatic failure. Despite this, each technique has their own limitations and one example for TE is potential inaccuracies in the setting of severe obesity and ascites. Therefore, serum biomarkers including collagen VI may help to provide diagnostic or prognostic information alongside imaging techniques and could help us better understand the underlying biology during liver fibrosis progression. Owing to the modest accuracy of individual serum markers of NASH, combinations of markers in panels have been examined to increase diagnostic utility.

A major component of the assessment of NASH is the determination of the extent of liver fibrosis which has been linked to the risk of mortality and liver-related clinical outcomes [189]. Cirrhotic livers contain up to ten times more collagen than healthy livers. Moreover, liver scarring occurs over a long period and

is largely asymptomatic [190]. However, damage of ECM and the resultant change in homeostasis of its constituents can be detected very early in liver disease [191]. Therefore, the development of the panels of antibodies to collagen neoepitopes can provide an early assessment of perturbations in the balance between collagen formation and degradation, as disease progresses. A panel of collagen neo-epitope biomarkers (PRO-C3, P4NP7S, PRO-C5, PRO-C6, C3M and C4M) of collagen formation and degradation were evaluated in a discovery cohort of 141 patients with biopsy-proven NASH and 23 patients with NAFL. Only PRO-C3 and PRO-C6 levels were found to be significantly elevated in the serum of patients with advanced fibrosis stage 3–4 compared with those with fibrosis stage 0–2. However, further analysis of PRO-C3 and PRO-C6 in a smaller independent validation cohort ($N = 41$) subsequently found only PRO-C3 levels were significantly associated with fibrosis stages [192].

A cohort of 52 patients with moderate-stage hepatitis C were followed over 52 weeks using the serological biomarkers of collagen VI formation (PRO-C3, PRO-C4, PRO-C5) and collagen degradation (C3M, C4M and C6M) to identify liver disease patients likely to regress or progress in absence of any therapeutic intervention. Only high baseline PRO-C3 and C6M were found to be independent predictors of progression of fibrosis, with odds ratios of 19.4 ($P = 0.003$) and 11.6 ($P = 0.011$), respectively; interestingly, C6M levels were not correlated with regression of fibrosis [193].

Conclusion

The importance of collagen VI to human health and disease is becoming increasingly recognised. Endotrophin has emerged as a crucial driver of fibrotic diseases influencing multiple aspects of fibrosis such as inflammation, chemotaxis, apoptosis, angiogenesis and myofibroblast accumulation leading to a vicious cycle exacerbating tissue damage as summarised in Fig. 6. Consequently, it is a promising therapeutic target and humanised neutralising antibodies targeting ETP are being developed for the treatment of fibrotic diseases, as well as numerous tumour settings where collagen VI expression has been associated with disease progression.

The use of PRO-C6, C6M and C6M α 3 in panels of serological biomarkers of collagen formation and degradation is increasingly being considered invaluable to noninvasively assess the progression of numerous fibrotic diseases. These types of quantitative assays

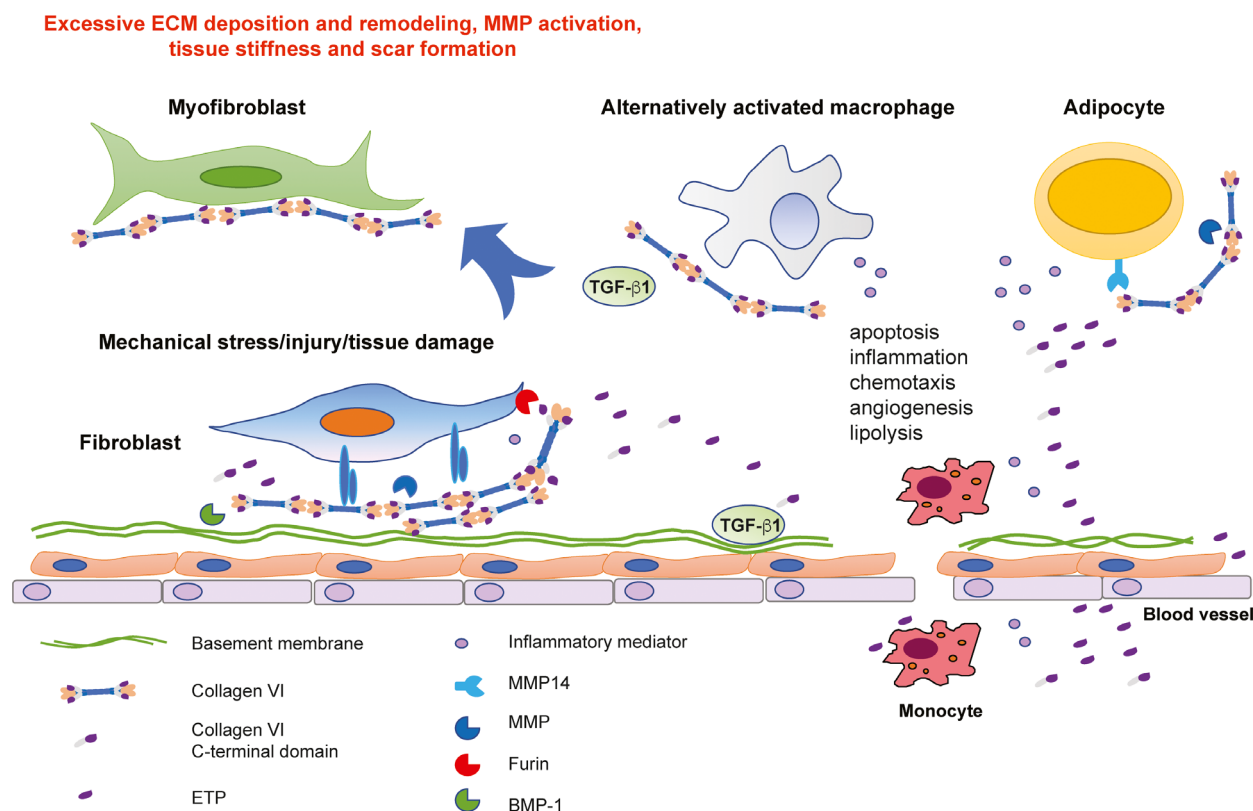


Fig. 6. Schematic of the key cell types driving myfibroblast differentiation within fibrotic lesions and the role of collagen VI and its proteolytic fragments. Localised tissue injury leads to excessive deposition of ECM, enhanced by release of cytokines such as TGF- β 1 by damaged epithelial/endothelial cells and by alternatively activated macrophages. Collagen VI acts as a substrate to facilitate adhesion of fibroblasts and their differentiation into myofibroblasts. Resident macrophages, adipocytes and recruited inflammatory cells drive the chronicity through the release of inflammatory mediators and chemokines to attract more inflammatory cells, leading to further damage of the interstitial and basement membrane and dysregulated ECM turnover. Enzymatic cleavage of collagen VI leads to the release of bioactive peptides that exacerbates the fibrotic process. These peptides also enter the systemic circulation where their levels can be used to monitor the stage and progression of fibrotic diseases.

should hopefully translate to more effective clinical monitoring of disease progression and form a prognostic tool in the clinic and facilitate comparisons in therapeutic response and adverse effects within clinical trials to allow the rapid assessment of efficacy of new antifibrotic therapies.

Summary and key questions to be resolved

What transcriptional cues drive enhanced Collagen VI gene expression in fibrosis?

Collagen VI expression has been shown to be associated with fibrosis of a wide range of organs, including the lung, liver and kidney in both humans and mice. Fibrogenic factors such as TGF- β 1 and other cytokines associated with alternatively activated

macrophages can drive expression, but what other soluble factors are involved? Do mechano-sensors or epigenetic mechanisms also control expression of collagen VI specifically within the fibrotic foci.

What proteinases control the generation of these fragments and are they tissue-specific?

Free ETP is scarce under physiological conditions, suggesting that the proteolysis of collagen VI α 3 is tightly regulated and may be driven through pathological processes. Several candidate proteinases, including MMP11, MMP14 and BMP-1, have been shown to cleave collagen VI α 3 chain to produce ETP. MMP11 and MMP14 were specifically studied within adipose tissue. Is this a universal mechanism or do other tissue-specific proteinases also control this key regulatory mechanism?

Do collagen VI fragments have distinct biological roles or are they simply a consequence of enhanced matrix turnover. What, if any receptors do, they engage?

Endotrophin has been shown to drive pathological processes, including fibrosis and carcinogenesis. However, as the PRO-C6 ELISAs detects the terminal 10 amino acids of the collagen VI C5 domain, this assay will detect all proteolytic fragments, not just ETP. Given the spectrum of fragments containing the C5 domain across different tissues [120], it is important to understand how and where these fragments are generated to gain a greater understanding of their role in driving disease pathology. As yet, ANT XR1 is the only candidate receptor for ETP; is this functionally relevant; or are there more receptors to be identified? Given its key role in driving fibrosis, identification of any receptors capable of binding ETP and other biologically active C5 domain-containing peptides raises the interesting possibility of novel classes of targeted therapeutics.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

LW, TL, MF and JN wrote paper; NY performed experiments.

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