IN VITRO TENDON TISSUE ENGINEERING

A THESIS SUBMITTED TO THE
UNIVERSITY OF OXFORD

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TRINITY TERM 2010

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisors Dr. Afsie Sabokbar, Prof. Andrew J, Carr and Dr. Zhidao Xia for their invaluable encouragement, guidance and funding for this research project.

I would also like to thank everyone who helped me at the Botnar Research Centre. In particular:

Mr. Xiao Wang for his advice and help with the silk degumming procedure; Miss. Hannah Cornell for all her advice and help with the real time RT-PCR procedure; Mr. Hao Zhang for all his support with scanning electron microscopy; Dr. Raj Rout for providing the tendon samples.

I would like to thank Novartis Pharmaceuticals (Basel, Switzerland) and SOHO Company Ltd., (Jiangsu, China) for the kind gift of TGFβ-3 and *Bombix silk*, respectively.

Finally, my deepest gratitude must go to my wife and my parents who have been a constant source of support and encouragement.

ABSTRACT

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Trinity Term, 2010

Tendon, ligament, and joint capsular injuries represent 45% of the 32 million musculoskeletal injuries each year in the United States. Tendon injuries are especially common, requiring surgical repair for the shoulder's rotator cuff tendons (51,000 per year), the Achilles tendon (44,000 per year), and the patellar tendon (42,000 per year). Tissue engineering provides an alternative in the treatment of tendon lesions through replacement of an injured tendon segment. The purpose of this study was to develop a tendon construct *in vitro* for clinical reconstructive surgery. Human tenocytes were isolated from hamstring tendons of patients who had undergone anterior cruciate ligament (ACL) surgeries. These tenocytes were cultured with culture media (α-MEM) supplemented with various concentrations of foetal bovine serum (FBS) (0%, 1%, 5% and 10%) and in the presence of different growth factors such as $PDGF_{BB}$ (0, 5, 10 and 50ng/ml), basic FGF (0, 5, 10 and 50ng/ml), IGF-1 (0, 10 and 50ng/ml) and TGFβ-3 (0, 1 and 10ng/ml). Fractional factorial design was utilized to select the combinations of growth factors that supported the following criteria: **(1)** the maximal cell proliferation with a minimum differentiation of the tenocytes in the presence of the least concentration of FBS possible and **(2)** maintaining cell survival and promoting tenocyte differentiation in FBS free culture media. The results have shown that:

(i) The tenocytes cell number cultured for 14 days in media supplemented with 1% FBS, 50ng/ml $PDGF_{BB}$ and 50ng/ml bFGF matched that of the positive control (10% FBS-treated cells). Not only the collagen synthesis was significantly reduced in these growth factor-treated cultures compared to positive control

tenocytes, but also a significant inhibition of the mRNA expression of various tenocyte differentiation markers (Scleraxis, Tenomodulin, Collagen type I and Decorin) was evident. IGF-1 did not promote significant cell proliferation under low serum conditions but did induce tenocyte differentiation *in vitro*. Examination of the cell morphology confirmed that tenocytes were capable of less differentiation when cultured with 1% FBS, 50ng/ml $PDGF_{BB}$ and 50ng/ml bFGF, this culture condition was termed **"the expansion phase"**;

(ii) The cell survival was maintained for up to 14 days in serum free culture media supplemented with 50ng/ml IGF-1 and 10ng/ml TGFβ-3 whilst cell differentiation was enhanced and evident by the increase in collagen synthesis and cell morphology. Furthermore, mRNA expression of the aforementioned cell differentiation markers were also significantly increased, this culture condition was termed **"the differentiation phase"**;

(iii) By combining the culture condition optimized for the **expansion** and **differentiation** phase **sequentially**, it was possible to maintain a long term 2-D tenocyte culture *in vitro* for up to 28 days. In these cultures, the presence of dense collagen formation was clearly evident whereas in positive control group (10% FBS group) such observation was not noted even after prolonged culturing period of up to 45 days. These results suggested that the sequential treatment of tenocytes with growth factors identified for the **expansion** and **differentiation** phases was significantly more superior than the standard 10% FBS treatment;

(iv) By combining the **expansion** and **differentiation** phases optimized for the 2-D cultures, it was possible to maintain human tenocytes in a 3-D scaffold (*Bombix* silk) for up to 28 days. The tendon like constructs that were formed, macroscopically and microscopically resembled the human hamstring tendon.

This observation was confirmed by using H&E staining, scanning electron microscopy and by detecting collagen type I immunohistochemically;

(v) It was possible to further validate these findings using *in vivo* animal models. This was undertaken by implanting the tenocytes cultured **sequentially** in the defined culture media described above, into the quadriceps of *Balb/c* nude male mice for up to 30 days. The nature and specificity of the tendon like structure that was formed after this implantation was investigated by H&E staining and immunohistochemistry. It was revealed that the culture condition that was optimized during the **expansion** and **differentiation** phases were suitable for generating a human tendon reconstruct; a finding which is of significance due to its potential in the tendon reconstructive surgery.

STRUCTURE OF THE THESIS

The thesis is presented in the following structure:

Chapter 1 introduces basic tendon biology; tendon injuries and the need for *in vitro* tenocyte-based tendon tissue engineering; reviews current challenges of *in vitro* tenocytes culture and proposed a possible solution; outlines recent important findings of tenocyte phenotypic and differentiation marker and the important growth factors which have potential effect in tenocyte expansion and differentiation.

Chapter 2 outlines the materials and methods employed in this research project and control experiments performed to validate those methodologies.

Chapter 3 reports *in vitro* experiments conducted to develop a low serum culture medium that promotes tenocyte **expansion** and controls **differentiation**.

Chapter 4 reports *in vitro* experiments conducted to develop a serum free culture medium that maintains tenocyte survival and promotes **differentiation**.

Chapter 5 reports *in vitro* experiments in which tendon like construct is generated in 2-D and 3-D culture by combining the aforementioned culture media **sequentially**.

Chapter 6 reports *in vivo* experiments that ascertain tenocytes cultured from above conditions can aid in neo-tendon formation in *Balb/c* nude mice.

Chapter 7 summarises the important results and conclusions of the research outlined in the thesis and provide plans for future investigations.

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ABBREVIATION

α-MEM	α minimal essential medium
ANOVA	analysis of variance
ACL	anterior cruciate ligament
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
BMSC	bone marrow stromal cell
bp	base pairs
°C	degree Centigrade
cDNA	complementary DNA
cm	centimetre
CO_2	carbon dioxide
COL-I	collagen type I
COL1A1	collagen, type I, alpha 1
COL-II	collagen type II
COL2A1	collagen, type II, alpha 1
Ct	cycle threshold
Cys	cystine
DAB	3'-3'diaminobenzidine
DCN	Decorin
DNA	deoxyribonucleic acid
Ethd-1	ethidium homodimer-1
ECM	extracellular matrix
FBS	foetal bovine serum
FGFR	fibroblast growth factor receptor
GAP	GTPase-activating proteins
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
H&E	haematoxylin and eosin
HCl	hydrochloride
H_2O	water
IGF-1	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
Ig	immunoglobulin
kDa	kilodalton
kV	kilovolt
L	litre
M	molar
MAPK	mitogen-activated protein kinase
mins	minutes
ml	millilitre
mm	millmetre
mM	millimolar
mN	micronewton
mRNA	messenger RNA
MSC	mesenchymal stem cell

MSTN	myostatin
NaOH	sodium hydroxide
ng	nanogram
nm	nanometre
nM	nanomolar
O.D.	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
Pt	platinum
RFU	relative fluorescent unit
rhIGF-1	recombinant human insulin-like growth factor-1
RNA	ribonucleic acid
RNase	Ribonuclease
rpm	revolutions per minute
RT-PCR	reverse transcriptase PCR
RUNX2	Runt-related transcription factor 2
SCX	Scleraxis
SEM	standard error of the mean
Smad	cytoplasmic proteins mothers against decanentalegic
SOX9	SRY (sex determining region Y)-box 9
TGF-β	transforming growth factor-β
TM	trade mark
TNMD	Tenomodulin
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
u	unit
UK	United Kingdom
USA	United States of America
v/v	volume/volume
w/v	weight/volume

CHAPTER ONE:

INTRODUCTION

1.1 BASIC TENDON BIOLOGY

Tendons are dense fibrous connective tissues that connect muscle to bone and their function is to transmit forces and stabilize joint structures [1-4]. Tendons are stronger than muscles, are subjected to both tensile and high compressive forces, and can sustain 17 times body weight. They act as energy storage sites, shock absorbers and help to maintain posture through their proprioceptive properties [5].

Achilles tendon **(Figure 1-1)**, was the first human tendon identified and recorded in 1693 by Flemish Phillippe Verheyen, a Professor of anatomy and Surgery at the University of Louvain, Belgium [6].

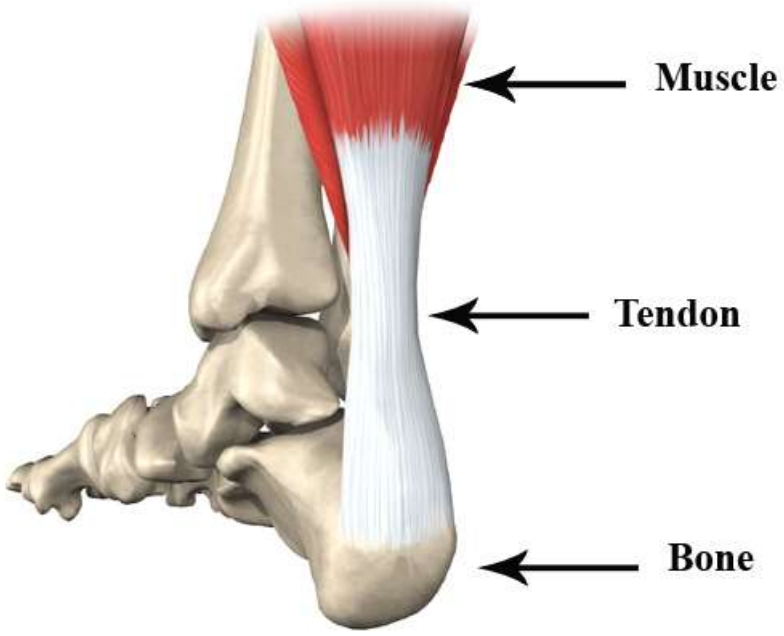

Figure 1-1: Schematic diagram of human Achilles tendon. (Modified with permission from: http://patientsites.com/media/img/565/foot_achilles_tendon_anatomy01a.jpg accessed 10/06/2010)

Tendons are composed of closely packed parallel collagen fibre bundles and are high in tensile strength. The strength of a tendon depends on the number, size and orientation of the collagen fibres and the thickness and internal fibrillar organization [7]. Tendons are comprised mainly of collagen type I molecules that are hierarchically organized into structural units: collagen molecules aggregate to form fibrils; bundles of fibrils form fibres; fibres group into primary fibre bundles or fascicles; and fascicles group together to form tertiary fibre bundles [8-12] **(Figure 1-2)**. Endotendinous connective tissue (endotenon) surrounds the fibre bundles and contains blood vessels, lymphatics, and nerves. The whole tendon, composed of multiple bundles and endotenon, is surrounded by the epitenon, a thin layer of connective tissue that is contiguous with the endotenon [8, 12, 13] **(Figure 1-2)**. The functional properties of the tendons are derived from the structure and components of the extracellular matrix (ECM) [14, 15].

When diseased or injured, tendons do not heal to the same regenerative capacity of embryonic tissue, but exhibit a highly disorganized matrix that consequently affects normal tissue function [16-19]. The reason that tendon regeneration does not recapitulate embryonic tissue development is not fully understood. This is probably due largely to our limited understanding of the mechanisms of tendon regeneration [12, 20-22].

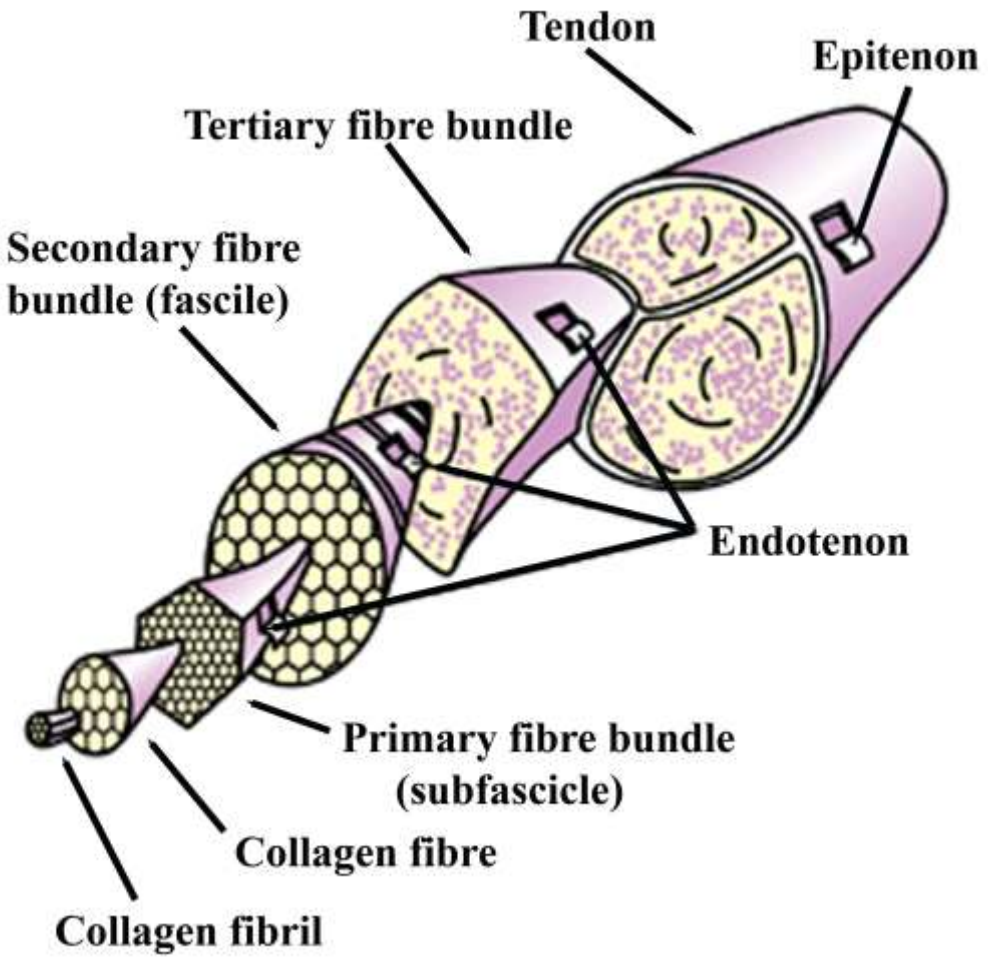

Figure 1-2: Structural hierarchy of tendon. Collagen molecules aggregate to form collagen *fibrils*; collagen fibrils group together to form collagen *fibres*; collagen fibres bundle together to form *fascicles*; fascicles group together to form tertiary collagen fibre bundles which act as the primary tendon unit (*endotenon*) (Figure modified from Kastelic, J., Galeski, A. and Baer, E. (1978). The multicomposite structure of tendon. Connect Tissue Res 6, 11-23, reproduced by permission from Taylor & Francis, Inc.)

1.2 TENDON INJURIES

Tendon, ligament, and joint capsular injuries represent 45% of the 32 million musculoskeletal injuries each year in the United States [23]. Tendon injuries are especially common, requiring surgical repair for the shoulder's rotator cuff tendons (51,000 per year), the Achilles tendon (44,000 per year), and the patellar tendon (42,000 per year) [24-34].

Large, irreparable rotator cuff tears present a demanding problem for orthopaedic surgeons. These large tears are associated with weakness, persistent defects, and poor outcomes. Tendons have very poor natural regenerative capabilities, and in spite of intensive remodelling, complete regeneration is barely achieved and the strength of tendon and ligament remains as much as 30% lower than normal, even months or years following an acute injury [35-39].

The treatment options for patients with large rotator cuff tears include non-operative treatment, surgical debridement via open or arthroscopic methods, and partial or complete surgical repair or reconstruction [35-39]. Non-operative treatment has yielded inconsistent results and has been especially ineffective for patients with symptoms lasting longer than 6 months [35-39]. Non-operative treatment of large rotator cuff tears is mainly reserved for elderly patients whose primary symptoms do not involve significant pain [35-39]. Improved function may be obtained with activity modification, physical therapy with an emphasis on training of the anterior deltoid muscle, and cautious use of steroid injections. However, the reports on the effectiveness of non-operative treatment of large rotator cuff tendon injuries have been varied [35-39].

Operative treatment options for large rotator cuff tears are limited if a direct repair cannot be accomplished. Open or arthroscopic debridement is another option for elderly patients with the predominant symptom of pain. Although this procedure may relieve pain and associated symptoms; it does not restore strength or function to the incapacitated shoulder [40, 41].

In order to achieve maximal functional restoration, a surgical repair of the rotator cuff tear could be used as an alternative approach. However, if a tear cannot be anchored primarily to the tuberosities despite all mobilization techniques, then the patient may require a reconstruction in order to restore some level of function and reduce pain [40]. The use of tendon transplantation to reconstruct the biomechanics of the rotator cuff may be a viable treatment option for patients with irreparable rotator cuff tears. A tendon transfer procedure may increase the likelihood of a functional recovery. Many tendon transfer techniques with various donor tendons have been described to treat patients with large irreparable rotator cuff tears, including the *subscapularis*, *latissimus dorsi*, *teres major*, *pectoralis major*, *deltoid*, *triceps*, and *trapezius* [35]. However, this technique may cause further damage and injuries to the patients. In order to obtain the optimal results of tendon transfer surgery without causing unnecessary damage to the patients, *in vitro* artificial tendon generation by tissue engineering methods could be a more practical and successful option.

1.3 TENOCYTE-BASED TENDON TISSUE ENGINEERING

Tendon tissue engineering provides an alternative in the treatment of rotator cuff tendon lesions through replacement of an injured tendon segment [42]. Tendons seem to be the

least complex of the connective tissues with respect to their composition and architecture and this leads to the expectation that they would be more amenable to tissue engineered approaches than other tissues. There are number of approaches of tendon tissue engineering that have been reported: (a) local injection of stem cells or growth factors [43, 44], (b) gene transfer [45] and (c) *in vitro* tissue engineering and production of bioengineered tendons to be further transplanted in the lesion site [42, 46-48]. So far, only a few experimental or clinical studies have been done on tendon tissue engineering compared to the extensive work on other tissues of orthopaedic interest, such as bone and cartilage, probably due to the unsatisfactory biomechanical properties of the engineered tendons.

There are three key factors which are critical in tenocyte-based tendon tissue engineering: cells, host responses and the choice of scaffold. An understanding of tenocyte proliferation and differentiation *in vitro* will therefore enable the development of rational approaches to improve current therapies and tissue engineering strategies for regeneration of "*tendon constructs*" with properties that more closely mimic those seen in normal development and function.

1.3.1 Cells

Although mesenchymal stem cells (MSCs) have been studied extensively for tendon tissue engineering [49-51], there are still certain limitations to fully exploring the measures which drive the MSCs to differentiate to a tendon like structure. Therefore, it is reasonable to expect that the tenocyte should be studied as the main cellular component for tendon tissue engineering. Tenocytes, the cells of tendon, are capable of differentiating *in vitro* to secrete fibrous proteins such as collagen fibrils and extracellular matrices required to regenerate

tendon tissue architecture [52-57]. Tenocytes are aligned longitudinally between the collagen fibres along the tendon axis [58, 59] and have the potential to communicate with one another via specific cellular processes and gap junctions that could form the basis of a load sensing system, thereby allowing the tenocytes to modulate their extracellular matrices [60, 61]. The isolated tenocytes could also regenerate tendon-like structures after extended expansion *in vitro* and implantation in nude mice [62].

In spite of the promising potential of tenocyte-based tendon tissue engineering, there are two difficulties in *in vitro* tenocytes culture:

Firstly, the limited cell number isolated from human tendon samples and prolonged cultures are likely to cause phenotypic drift [63, 64]. This was evident by studies showing that rabbit tenocytes exhibited phenotypic drift at early passage numbers *in vitro* [64], and tenocytes isolated from human Achilles tendon had significant phenotypic changes after 8 passages *in vitro* [63]. In these studies, changes are reflected in growth characteristics and alterations to the composition of the extracellular matrix. Thus, the maintenance of the tenocyte phenotype is essential for tendon tissue regeneration. Moreover, for tissue engineering purposes, it is desirable to maintain the tenocytes in a less differentiated state while **expanding** *in vitro*, as early cell differentiation may relate to asymmetric cell division and apoptosis [65-67]. In order to obtain enough cell number without phenotypic drift, rapid tenocytes **expansion** is necessary.

1.3.2 Host Responses

The second challenge is to eradicate the continuous use of non human sera *in vitro* culture which would make any future tendon transplantation to patients, impractical, due to the

host vs. graft responses [68, 69] as well as the potential cross species disease transmission [70, 71].

Recent researches have demonstrated successful *in vitro* tendon tissue engineering with avian [46], canine [72] and rat [73] tenocytes or rat mesenchymal stem cells (MSCs) [42] on different scaffolds (further details on the latter are discussed in **Chapter 5 and 6**). Those studies were performed with the supplementation of 7- 10% serum in the culture medium.

Serum, a complex medium supplement, contains potent stimulators for *in vitro* cell growth including amino acids, growth factors, vitamins, proteins, hormones, lipids, and minerals. It is common practice to use 10% serum in routine cell culture for cell expansion and survival. However, absence or reduced levels of serum may prove beneficial as serum contains unknown growth factors which make the culture media less defined for specific purposes. Moreover, the risk of disease transmission to the engineered products and patients would be increased [70, 71]. Human serum might be a feasible alternative choice for the substitution of the use of FBS; nevertheless, unless autologous serum is used in the culture, there is still a potential antigenicity problem with the engineered products. Hence the optimization procedure developed in this research project is based on the growth factors supplemented in tenocyte culture media that requires minimum amount of non-human serum. The study of tenocyte survival, expansion and differentiation under defined culture conditions will provide evidence and basis for tendon tissue engineering objectives. There have been several attempts to control tenocytes expansion and differentiation by supplementing different growth factors in the culture media with some success [74-78], but

still, little is known about the growth factors synergized effect in serum free conditions over a **long term** culture period.

1.3.3 Choice of Scaffold

A suitable scaffold for tendon tissue engineering should be carefully selected. There has been several tendon tissue engineering attempts which employed different scaffolds, such as acellularized tendon allograft [79], polyglycolic acids [46], umbilical veins [42], small intestine sub mucosa [72] and hyaluronan hybrid polymer fibre [80] and chitosan micro channel scaffolds [81]. Those findings showed structural resemblances to natural tendon. However, the mechanical properties of their engineered constructs were not satisfactory for any future tendon reconstruction. In addition, these experiments employed the use of synthetic materials such as hyaluronan hybrid polymer fibre, polyglycolic acids and chitosan micro channel scaffolds as they are easy to produce. Nevertheless, some of these synthetic materials may biodegrade which could initiate host foreign body responses making them unsuitable for long term reconstructive surgery [46].

Bombix silk has been used as surgery suture material for centuries and has been explored in various tissue engineering purposes [48, 82-85] due to its biocompatibility, toughness and elasticity. The main constituents of silk are (i) silk fibroin, a filament core protein, and (ii) silk sericin, a family of glue-like coating proteins [86]. As silk has been reported to have a high tensile strength after implantation *in vivo* in various animal models [87-90] and as it has been reported to have a slow rate of biodegradation [85], it is reasonable to consider silk as a possible choice of scaffold for the tendon tissue engineering. One main concern with the use of silk for biomedical scaffold is the potential host immune responses which

may be associated with sericin and therefore affecting its biocompatibility [91-93]. Recent studies have been focused on resolving this problem by chemically removing sericin and as such reducing any potential antigenic effects, a process which is known as "*degumming*" [94]. Hence, the silk scaffolds used in the 3-D culture reported in this thesis are degummed *Bombix* silk

With the aim of overcoming these practical challenges, it is essential to have a better understanding of the phenotypic/differentiation markers of tenocytes and those growth factors that may have an effect on tenocyte **expansion** and subsequent **differentiation**. Fibrillar and non-fibrillar components of the extracellular matrix and mediators of matrix–cell interaction can be used as markers for tenocyte phenotypic drift and differentiation [17, 64, 95]. Those markers and growth factors will be discussed in detail in the following sections.

1.4 TENOCYTE PHENOTYPIC AND DIFFERENTIATION MARKERS

1.4.1 Collagen type I (COL-I)

Although small amounts of other collagens (for example: collagen -II, -III, -IV and -V) are found within tendon, collagen type I is the predominant collagen type found within tendon and is responsible for >70% of the dry weight of the tendon structure [8]. Collagen is arranged hierarchically within the tendon in longitudinal arrays, grouped as microfibrils, subfibrils and fibrils bound together into fascicles that contributes to most of the tendon's mechanical properties [8]. Collagen type III is a member of the fibrillar collagen family

[16, 96-98] and is similar in structure to collagen type I, with the exceptions that it is a homotrimeric molecule (collagen type I is a heterotrimeric molecule consisting of two type I and one type II collagen chains). Also the monomer chains in collagen type III are joined together with permanent disulphide bonds, as opposed to the transitory C-propeptide disulphide bonds found in collagen type I [99]. Collagen type III has been proposed to play a significant role in collagen type I fibrillogenesis because of its frequent colocalization with collagen type I and has even been detected within the same fibril as collagen type I [9, 98-103]. Collagen type III is found in the tendon in small amounts, however, its content has been reported to increase significantly in injured or ruptured tendons and this is believed to deleteriously affect the mechanical properties of the tendon [2, 17, 104, 105].

Extended monolayer culture of rabbit tenocytes has been reported to lead to decreased levels of collagen type I production with increasing number of passages *in vitro* [64]. Other studies have shown that significant increases in the type III:I collagen ratio after prolonged culture of human Achilles tenocyte [63]. It has been suggested that, in order to maintain the tenocyte phenotype after prolonged *in vitro* culture, it is essential to keep the collagen type I production to a maximum level within specific period [63].

1.4.2 Scleraxis (SCX)

Scleraxis (SCX) is a basic helix-loop-helix (bHLH) transcription factor and it is a highly specific marker for all the connective tissues that mediate attachment of muscle to the bone. Embryos homozygous for a targeted disruption of the SCX gene do not form mesoderm, fail to undergo gastrulation and die at E8.5 [106]. SCX expression is also

observed at later stages in various mesodermally derived tissues [107]. SCX was first used as a specific tenocyte marker by Schweitzer in 2001 and he showed that early SCX expression marks the progenitor cell populations in these tissues [108]. By using embryonic mice model, it was discovered that the presence of SCX expression during embryonic development of tendon precursor cells in close association with that of SOX9 expression in chondrogenic cells in skeletal tissues [109]. Recently, SCX has been found to express selectively in tenocytes and not only does it regulates type I collagen gene expression in murine tendon fibroblasts [110] but it can also positively regulate the expression of a murine tenocyte differentiation marker, Tenomodulin [111] (**see 1.4.4**). SCX gene knockout studies have demonstrated that SCX plays a vital role in tendon development [14]. SCX null mice have shown that tendon defects severely limit leg, back, and tail mobility [14]. Furthermore these knockout mice displayed a reduced and disorganized tendon matrix, as well as cellular disorganization within tendon [14]. Consequently, SCX has been suggested to be associated/required for normal tendon differentiation and formation [14].

1.4.3 Decorin (DCN)

Decorin is a low molecular weight proteoglycan that is present in the ECM of various connective tissues, including tendon [112, 113]. DCN is expressed abundantly in the tendon [112, 114, 115] and has been found to be located within a specific region near the C-terminus of collagen type I, in close proximity to one of the major intermolecular cross linking sites [112, 116]. DCN isolated from tendon has been shown to reduce the diameter

of collagen fibrils and inhibit collagen types I and II fibrillogenesis, a process which involves the secretion of collagen fibrils by tenocytes [112, 114, 117, 118]. However, DCN is not solely an inhibitor of fibrillogenesis [119]. It has been suggested that DCN can regulate the fibrillogenesis process in various species [118-122]. The mechanism for such regulation has yet to be fully elucidated, it has been hypothesized that DCN acts to inhibit lateral fibril fusion, resulting in uniformly thinner fibrils [10, 112, 114, 118]. DCN is thought to stabilize as well as align collagen fibrils during fibrillogenesis [123, 124]. Furthermore, it is believed that DCN contributes to the strength and elasticity of tendon via interactions with the surface of collagen fibrils [116, 125-127].

1.4.4 Tenomodulin (TNMD)

Tenomodulin is a member of a new family of type II transmembrane glycoprotein. It is predominantly expressed in tendons, ligaments, and eyes. The research conducted by Shukunami *et al.* also revealed that the expression of TNMD mRNA was not associated with muscle fibres, but was tightly associated with epimysium and tendon, both of which are classified as dense connective tissue having little vascularity [128]. By using an adenovirus expression system, Oshima *et al.* has also demonstrated that the C-terminal domain of TNMD exhibits both anti-angiogenic and anti-tumour activities when expressed in a secreted form [129]. Recent studies have suggested that TNMD-deficient mice exhibit a severe decrease in proliferating cells in newborn tendons, as such indicating that TNMD is necessary for tenocyte proliferation and maturation [130]. Furthermore, Shukunami *et al.* reported that TNMD expression is closely associated with the appearance of tenocytes

during chick development and is positively regulated by SCX [111]. Their results also suggested that TNMD is a late marker of tendon formation and that SCX positively regulates TNMD expression in a tendon cell lineage-dependent manner [111]. Research showed that the expression of SCX and TNMD was decreased in myostatin null mice compared with wild type mice, but the expression of SCX and TNMD was increased after the tenocytes were treated with myostatin, which activated the p38 MAPK and Smad2/3 signalling cascades [131].

1.5 GROWTH FACTORS WHICH MAY PROMOTE *IN VITRO* TENOCYTE EXPANSION AND DIFFERENTIATION

Growth factors are cell secreted peptide hormones that control cellular functions in an autocrine or paracrine manner. They initiate intracellular signal transduction cascades upon binding to cell surface tyrosine kinase receptors [132]. Several growth factors, including basic fibroblast growth factor (bFGF), insulin-like growth factor-I (IGF-I), platelet-derived growth factor ($PDGF_{BB}$) and transforming growth factor β-3 (TGFβ-3) have been shown to play a significant role in regulating **expansion** and **differentiation** of tenocytes and extracellular matrix formation *in vitro*/*in vivo* [133-138]. It is plausible that by combining a number of growth factors, the biological effect on tenocyte **expansion** and **differentiation** can be achieved at low or serum free culture conditions [135]. Although there are numerous growth factors which have been identified for musculoskeletal regeneration, the following sections will focus on those which have a pivotal role in tendon tissue engineering (*in vitro* and /or *in vivo*).

1.5.1 Insulin-Like Growth Factor-1 (IGF-1)

Insulin-like growth factor was discovered initially as “sulfation factor”, a serum factor that stimulated cartilage sulfation, a downstream effect of growth hormone [139]. It later was renamed “somatomedin” after it was shown to stimulate other cellular processes [140]. Simultaneously other studies identified a serum factor that exhibited insulin-like activity but that could not be suppressed by anti-insulin antibodies and therefore was termed “non suppressible insulin-like activity” [141]. Eventually these factors were cloned and found to be identical [142, 143]. The amino acid sequence was found to have structural homology with human proinsulin, and the protein was renamed IGF-1. Upon secretion, IGF-1 either can act on local tissues in an autocrine/paracrine fashion or can enter the circulation and exert distant endocrine effects. Locally acting IGF-1 can bind directly to its receptor, whereas circulating IGF-1 largely is bound in the serum to 1 of 6 different IGF binding proteins (IGFBP-1-6), predominantly IGFBP-3 [140]. When IGFBP-3 is cleaved, the circulating IGF-1 is aided by other binding proteins to leave the intravascular space and enter target tissues, where it then binds to cell membrane-bound IGF-1 receptor, a tyrosine kinase receptor, that activates intracellular signal transduction pathways [140].

Circulating IGF-1 is mainly produced by the liver, where its release is induced by growth hormone [140]. Locally acting IGF-1 is produced in a variety of tissues such as keratinocytes, osteoblasts, and fibroblasts, where it is regulated by growth hormone or parathyroid hormone, as well as in the uterus, where it is regulated by sex steroids such as estrogens [144]. It is present in most tissues in the human foetus [145, 146], where it plays

a major role in development. This is consistent with experimental findings that IGF-1 is mitogenic for fibroblasts, chondrocytes, keratinocytes, osteoblasts, skeletal muscle cells, smooth muscle cells, mammary epithelial cells, thymic epithelial cells, thyroid follicular cells, neuronal cells, mesangial erythroid progenitor cells, oocytes, granulose cells, spermatogonia, and Sertoli cells [147-149]. Given the large range of cell types that bind IGF-1, it is not surprising that IGF-1 has widespread physiological and pathological effects. Locally acting IGF-1 also is involved in wound healing, its absence in rat has been shown to impair wound healing [150]. Furthermore, exogenously applied IGF-1 can reverse the deleterious effect of steroids in rat [151].

Several studies have examined the role of IGF-1 in tenocyte proliferation and differentiation. Separate extracts from the epitendon and internal compartment of avian tendons have been found to contain IGF-1, indicating that IGF-1 is expressed in normal uninjured tendons [152]. IGF-1 have been shown to stimulate rabbit tenocyte DNA synthesis [153] and collagen synthesis *in vitro* [154]. The DNA synthesis was inhibited by anti IGF-1 antibody, indicating that IGF-1 was the primary factor responsible for the extract's mitogenic activity [153] . These experiments, however, were performed in non-injured tendons and therefore did not provide direct evidence for the role of IGF-1 in tendon healing. Although IGF-1 levels have been found to be increased transiently after vibration-induced injury in rat Achilles tendon models [155], no published studies have specifically documented an increase in IGF-1 in **human** tendons after injury. *In vitro* studies have shown that matrix synthesis and cell proliferation were stimulated dose dependently by human recombinant IGF-I at doses between 10 and 250ng/ml. Thus, IGF-I

has the ability to stimulate matrix synthesis and cell proliferation in rabbit tenocytes [156]. In a collagenase-induced equine model of tendonitis, IGF-1 administration was found to improve tendon wound healing compared to saline-injected controls, as evidenced by reduced swelling, increased mechanical stiffness of the tendon, and decreased lesion sizes after 4 weeks of treatment, and increased cell proliferation and collagen I and III content [157].

1.5.2 Basic fibroblast growth factor (bFGF)

Basic fibroblast growth factor (also known as FGF-2) initially was identified as a substance purified from bovine pituitary with mitogenic activity for fibroblasts [158]. It since has been found also to modulate cellular processes such as angiogenesis, wound healing, and embryonic development. Basic FGF is produced by endothelial cells, fibroblasts, smooth muscle cells, chondrocytes, and mast cells [159] and it acts on a wide variety of cell types, reviewed by Bikfalvi *et al*. [160]. Basic FGF is involved in the development, remodelling, and disease states of almost every organ system [160]. Basic FGF is active in angiogenesis, atherogenesis, endothelial healing after vascular injury, lung development, neural development, eye development, prostate growth, muscle growth and development, as well as wound healing [160].

Basic FGF is one member of the heparin-binding growth factor family encompassing over 20 members, each of which plays a different and specific role in processes as varied as embryonic development, organogenesis, and tumorigenesis [161]. bFGF most commonly is an 18-kD, 146-amino acid, single-chain polypeptide, although larger forms of bFGF (22,

22.5, and 24 kD) have been identified [162]. Basic FGF binds to heparin and heparan-sulfate proteoglycans on cell surfaces and the extracellular matrix. When heparan sulfate proteoglycans are degraded, bFGF bound to extracellular matrix is released and then can bind to 1 of 4 specific cell surface receptors (FGFR1-4), each of which has variants based on alternate splicing. Basic FGF receptor tyrosine kinase then initiates further signalling.

Duffy *et al.* first showed in 1995, that bFGF was present constitutively expressed in normal intrasynovial flexor tendons [163]. Chang *et al.* showed that bFGF levels were increased in the rabbit tendon and sheath during healing from post repair day 1 to 56 [164]. The highest levels of bFGF were seen from rabbit epitendon tenocytes as well as inflammatory cells and fibroblasts from the tendon sheath. This temporal and spatial organization of bFGF levels after injury suggested that bFGF was active in normal tendon healing. Exogenously applied bFGF increased canine tenocyte expression of integrins [165], molecules that mediate communication between cells and extracellular matrix, also suggesting a possible mechanism by which physiologic increases in bFGF after injury coordinate the tendon healing process. In tendons, exogenously applied bFGF has been shown to accelerate wound closure in an *in vitro* rat patellar tendon wound healing model, and the likely mechanism was suggested to be via the stimulation of tenocyte proliferation [166]. This was consistent with *in vivo* studies showing that bFGF injected into rat healing patellar tendons also increased collagen type III production and cell proliferation [76]. *In vitro* study of bFGF's effects on rat tenocytes has shown that high-dose of bFGF (30 ng/ml) can lead to significantly lower proliferation, cell density, and gene expression levels than low dose bFGF (3 ng/ml) [166]. High-dose bFGF even showed a tendency to express less

Collagen I, Collagen III and fibronectin after 14 and 28 days compared with the low dose bFGF group. Therefore, a dose-dependent influence of cellular activity was hypothesized by Chan *et al*. [166].

1.5.3 Platelet-Derived Growth Factor (PDGF)

Platelet-derived growth factor (PDGF) was initially discovered in 1974 as a platelet-derived mitogen that was capable of stimulating fibroblast and smooth muscle cell proliferation [167]. Since then it has been studied extensively, mostly for its role in wound healing and cell proliferation. PDGF is one of the first growth factors expressed after tissue injury in porcine wound model [168]. PDGF consists of a group of dimers consisting of 2 polypeptide chains, A and B, whose various combinations form 3 different isoforms (AA, AB, and BB). These bind to 2 different tyrosine kinase receptors, α and β [169, 170], where all 3 PDGF isoforms bind to the α receptor whereas only the BB isoform ($PDGF_{BB}$) binds to the β receptor.

PDGF is produced and secreted primarily by platelets but also by macrophages, endothelial cells, fibroblasts, and keratinocytes [171, 172]. It acts as a chemoattractant and mitogen for fibroblasts, smooth muscle cells, and endothelial cells, as well as a stimulant for production of fibronectin and hyaluronic acid. The β receptor is the predominant PDGF receptor in fibroblasts, vascular smooth muscle cells, and endothelial cells [173]. Receptor activation ultimately leads to rabbit fibroblast proliferation and differentiation, collagen deposition, and angiogenesis [174]. Much research has been directed toward elucidating the role of PDGF in wound healing in particular because it is one of the first detectable growth factors

after acute tissue injury. In early wound healing PDGF induces synthesis of other factors (IGF-1) in porcine animal models [175] and is one initial signal in the cascade of events including rabbit fibroblast proliferation and differentiation, collagen deposition, and angiogenesis [176, 177]. *In vivo* studies have revealed that, PDGF is increased in healing tendon compared with uninjured tendons in a canine tendon transection model [163]. Similarly, in *in vitro* investigations, $PDGF_{BB}$ has been shown to stimulate collagen, proteoglycan, and DNA synthesis in rabbit tenocytes [178]. Because $PDGF_{BB}$ up-regulates IGF-1 and IGF-1 receptors, it is possible that $PDGF_{BB}$ may exert at least some of its effects indirectly through the actions of IGF-1 [175]. Researchers have shown that 10ng/ml of $PDGF_{BB}$ significantly reversed the effects caused by 10^{-6}M dexamethasone, a concentration that can significantly reduce human tenocytes proliferation and collagen synthesis [75]. Therefore PDGF has a variety of activities including stimulation of cell proliferation and collagen synthesis.

1.5.4 Transforming Growth Factor-β (TGF-β)

Transforming growth factor-β was discovered in 1983 as a placenta-derived substance that was capable of stimulating rat fibroblasts to proliferate in soft agar [179]. Since its initial discovery, it has been the focus of intense studies because of its wide-ranging biologic activity and distribution throughout the entire body. There are 3 mammalian isoforms of TGF-β (-β1, -β2, and -β3) that are expressed in different tissues. The isoforms are 60% to 80% homologous and are dimers of 12-kD polypeptides that are cleaved proteolytically from larger precursors [180]. The TGF-β family of proteins binds to 3 distinct membrane

receptors termed RI, RII, and RIII [180]. Ligand binding causes these receptors to interact with each other, ultimately activating intracellular serine-threonine protein kinase activity. TGF-β is produced by nearly every cell type in the body, and nearly every cell type in the body exhibits a cellular response to it, reviewed by Blobe *et al.* [181]. TGF-β plays different roles in each cell type. Some of its general roles include cell-cycle regulation, embryogenesis, and organ development. TGF-β (1, 2 and 3) is released from degranulating platelets and secreted by all of the major cell types participating in the healing process including lymphocytes, macrophages, endothelial cells, smooth muscle cells, epithelial cells and fibroblasts. All isoforms of TGF-β stimulates chemotaxis, promotes angiogenesis and regulates the transcription of a wide spectrum of matrix proteins including collagen, fibronectin, glycosaminoglycans, matrix-degrading proteases and their inhibitors and integrin receptors [182, 183]. In several experimental models exogenous TGF-β has been shown to reverse age- and steroid-associated impairment of rat [184] and rabbit [185] tendon wound healing. Chang *et al*. found increased levels of TGF-β1 mRNA in both the tendon sheath as well as in the rabbit tendon injury model, primarily localized to the more cellular epitendon [186]. *In vitro* studies have shown that at concentration of 1 and 5ng/ml of each three TGF-β isomers, the collagen production by rabbit tenocytes can be increased. But this effect was not mirrored in the tenocyte cell number as the latter was decreased in TGF-β-treated cultures [187].

1.6 SUMMARY

In this Chapter, the following topics were outlined:

- ❖ The basic tendon biology and the problems which pose the biggest challenges to orthopaedic surgeons after tendon injuries.
- ❖ Tenocyte-based tendon tissue engineering could be a viable solution toward tendon reconstructive surgeries.
- ❖ One of the difficulties in tenocyte-based tendon tissue engineering is the use of non-human serum in tenocyte culture.
- ❖ The study of tenocyte **expansion** and **differentiation** in low/no serum culture conditions could provide practical rationale for tendon tissue engineering.
- ❖ Several fibrillar and non-fibrillar components of the extracellular matrix of tenocytes that will be used as "tenocyte markers" for the study of tenocyte **expansion** and **differentiation**. Those tenocyte markers are useful in determining the functional characteristics and the differentiation potential of tenocytes.
- ❖ Some growth factors and their combinations that could potentially control tenocyte **expansion** and **differentiation** *in vitro*/*in vivo*.

1.7 OVERALL OBJECTIVE OF THE RESEARCH PROJECT

1.7.1 The main objectives

The scope of this research project is therefore to develop a transplantable human tendon construct for future clinical application by using tenocytes cultured with an ideal culture medium and a suitable scaffold.

The *in vitro* generated construct should fit following criteria:

- The minimum usage of FBS.
- To regulate cell differentiation during the cell **expansion** stage.
- Cell **differentiation** should be achieved after cell expansion is completed.
- The histological morphology and microstructure of the construct should resemble human natural tendon.
- The construct should have the mechanical properties that match or be superior to human natural tendon.
- The tenocytes cultured under such defined conditions should have the ability to **differentiate** *in vivo*.

1.7.2 Specific Objectives

Based on the above mentioned aims, the following specific objectives are outlined:

- To utilize a fractional factorial design to optimize the culture media with growth factors that promote tenocytes **expansion** and control **differentiation** with the least possible serum. It is hypothesized that the culture media supplemented with low serum concentration and combination of growth factors will sustain less differentiated tenocytes **expansion** *in vitro*.
- To optimize the culture media with growth factors which promote tenocytes differentiation with the least possible serum. It is hypothesized that the culture media supplemented with no serum and combination of culture media will maintain cell survival and promote the cell **differentiation**.
- To generate tendon like constructs by combining the two steps sequentially in 2-D and 3-D culture using degummed *Bombix* silk as a scaffold. The hypothesis is that the sequential application of those growth factors will promote tenocyte **differentiation** *in vitro* and a tendon like construct will be generated.
- To verify that the tenocytes cultured under defined conditions could maintain **differentiation** potentially towards neotendon formation *in vivo*. It is proposed that the human tenocytes cultured with defined culture media could be able to generate tendon like structure under *in vivo* condition.

CHAPTER TWO:

MATERIALS AND METHODS

GENERAL

For all tissue culture studies reported in this thesis, the following materials were employed. Culture media, Foetal Bovine Serum (FBS), antibiotics, growth factors and supplements: 20.34g α-MEM powder (Gibco, Invitrogen, UK), 4.4g of Sodium Bicarbonate were dissolved in 2L milli-Q water and sterilized by 0.2μm filter (PALL life Science, PALL Corporation, UK). Heat deactivated FBS (Biosera, East Sussex, UK) was supplemented to the culture media with the concentration of 0%, 1%, 5% and 10% (v/v). Human recombinant growth factors (IGF-1, bFGF, $PDGF_{BB}$ [Invitrogen, Paisley, UK] and TGFβ-3 [Novartis pharmaceuticals, Surrey, UK]) were diluted with serum free α-MEM medium to 1μg/ml working solutions according to manufacturer's instructions. All culture media was supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (Lonza, Belgium). Unless otherwise noted, all tissue culture materials were purchased from Invitrogen (Paisley, UK), all other reagents were of analytical grade from Sigma Chemical Company (Poole, UK), and cell culture plates, flasks and cell scrapers were purchased from BD falcon, BD bioscience, California, USA.

The following subheadings will describe in detail the experimental methodologies and protocols employed in this thesis.

2.1 Human tenocyte *in vitro* culture

Human tendon biopsies were obtained from consenting, non-smoking, male patients <30 years of age who were undergoing right anterior cruciate ligament (ACL) reconstruction with hamstring graft at the Nuffield Orthopaedic Centre, Oxford, and followed the

approved guidelines set by the Oxford Research Ethics Committee C (09/H0606/11)[#]. All tendon samples were collected by the same surgeon within one hour and were kept in cold sterile α-MEM medium on ice before processing. In general, 2 cm tendon segments from the centre of the tendon biopsies from the distal end of the muscle-tendon conjunction were harvested.

The tenocytes isolation method was adapted and modified from Bi *et al.* [62]. The tendon samples were diced into $1mm^3$ pieces and treated by incubating in 4mg/ml dispase (w/v) (Roche, Hertfordshire, UK) and 300u/ml collagenase type II (w/v) (Gibco, Invitrogen, Paisley, UK) in serum free α-MEM medium at 37 °C for 16 hours; this was to prevent deactivating of collagenase/dispase by components in serum. After enzymatic digestion, equal volumes of α-MEM medium, supplemented with 10% FBS were added to quench the collagenase/dispase and filtered through cell strainers (70 μm nylon, BD falcon, BD bioscience, California, USA). The filtered cell suspension was centrifuged at 1500 rpm for 5 min, and the supernatant was discarded. The cell pellet was re-suspended with α-MEM medium with 20% FBS and cultured in T75 ($75cm^2$) TC flasks, at 5% CO_2, 37 °C. The cells were sub-passaged after reaching 80% confluence.

Following expansion to passage 3, the tenocytes were trypsinized using 0.017% trypsin/EDTA solution (Lonza Wokingham Ltd, Berkshire, UK) and counted by using a hematocytometer under the inverted microscope (Nikon, Japan). The cells were then seeded in 96/48 well culture plates with the initial cell density of $5\times10^3/cm^2$; and fed with 200μl/well of the designated media (see **Table 3-1** and **Table 4-1**), with media changes twice a week. Concentrations of the growth factors were selected based on previously

Thanks to Dr. Raj Rout for kindly providing the tendon samples.

published articles [78, 135, 138, 178, 188-192]. Six replicates were used in each treatment group and the results are expressed as means ± SEM for each treatment. To avoid batch to batch serum variation, all studies were conducted using the same serum batch.

2.2 Tenocyte proliferation assay

AlamarBlue™ is a cell viability indicator that uses the natural reducing power of live cells to convert resazurin to the fluorescent molecule, resorufin. The active ingredient of AlamarBlue™ (resazurin) is a nontoxic, cell permeable compound that is blue in colour and virtually non-fluorescent. Upon entering cells, resazurin is reduced to resorufin, which produces very bright red fluorescence. Viable cells continuously convert resazurin to resorufin, thereby generating a quantitative measure of cell viability. AlamarBlue™ is a widely used test to examine the metabolic activity of the cells hence to calculate the cell number. However, the limitation of using AlamarBlue™ to analyze the cell number being that, AlamarBlue™ is based on the assumption that, all the cells maintainsimilar metabolic activities throughout the experiments. There are several other methods that could be employed to accurately assess cell proliferation, such as H^3 Thymidine incorporation assays and BrdU incorporation assays to analyze the DNA synthesis. However, these methodologies are endpoint assays which were not considered suitable for the current projects.

AlamarBlue™ assays were performed according to manufacturer's protocol at day 1, 7 and 14 to examine the cell number expansion. In brief, 200µl of 5% AlamarBlue™ (Biosource Europe, Nivelles, Belgium) was added in each well after removal of the culture media and

washed with 0.1M PBS (Lonza Wokingham Ltd, Berkshire, UK). The cells were incubated at 37 ºC, 5% CO_2 for 2 hours. The AlamarBlue™ solution was transferred to another plate with 5 wells of 5% AlamarBlue™ as blank and the relative fluorescent unit (RFU) was measured with SoftMax Pro software using a SPECTRAmax GEMINI microplate spectrofluorimeter (Molecular Devices, Berks, UK) at the excitation wavelength of 530 nm and the emission wavelength of 590 nm, with a cut off at 570 nm.

2.2.1 Assays validating the correlation between RFU and the cell number

The estimated cell number was calculated by using a standard curve created by incubating 5% AlamarBlue™ under the same conditions and time with doubling dilution of the cell number ranging from 7900 to 30 per well (7900, 3950, 1970, 980, 490, 240, 120, 60 and 30). The cell number was then plotted against the relative fluorescent unit (RFU) **(Figure 2-1)**. These standard curves provided excellent reproducibility and there was a strong linear correlation between the cell number and their RFU measured at the above wavelengths. The linear regression analysis showed there was a very high degree of correlation: the Pearsons's correlation coefficient was 0.9967. This process was performed repeatedly with each microplate in order to calculate the cell number.

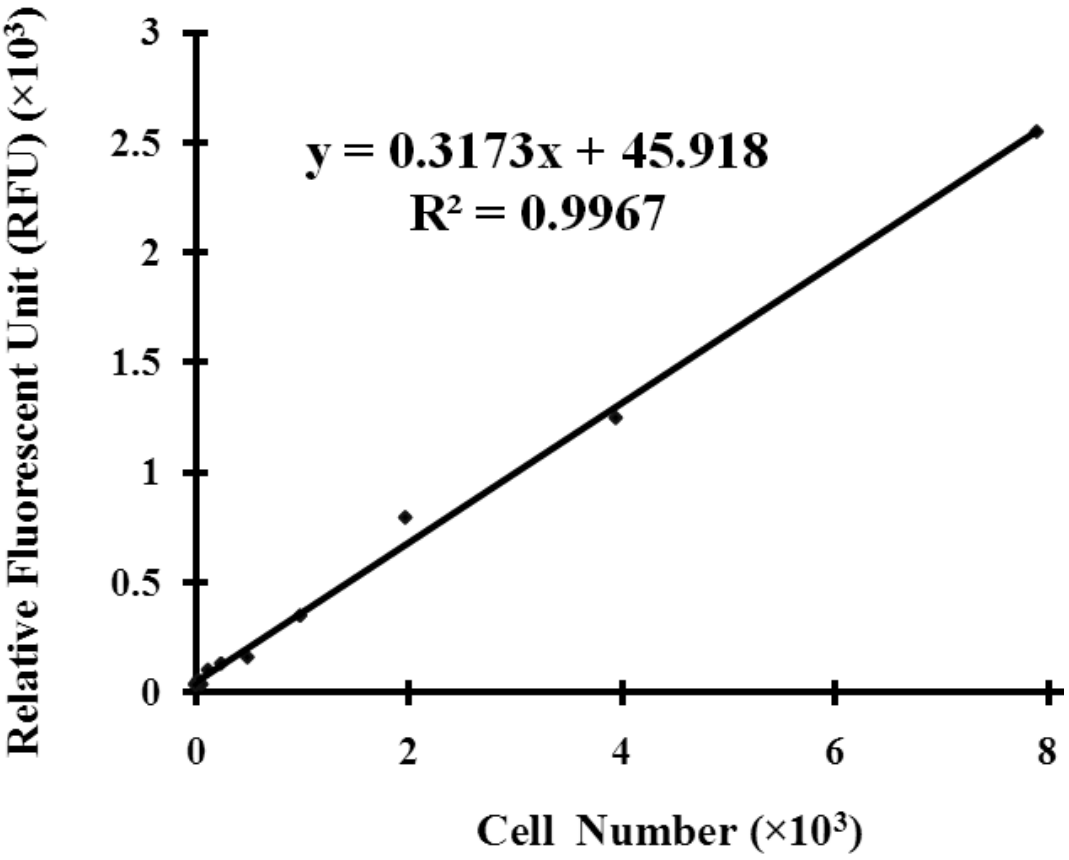

Figure 2-1: The relative fluorescent unit (RFU) plotted against the increasing number of tenocytes. This is a representative standard curve which was used for any subsequent cell number measurements. Similar standard curves were set up for every cell number measurement. The equation indicates the linear relationship between RFU and cell number. R^2 is the Pearsons's correlation coefficient.

2.2.2 Validation of the RFU reflects the living cell activities

In order to ascertain whether RFU measurement from AlamarBlue™ assay truly reflects the number of viable tenocytes, the following experiments were performed using the following groups:

(***a***) The control **live tenocytes** group (cells cultured in α-MEM);

(***b***) **Dead tenocytes** group (the tenocytes were treated with acetone/methanol, a known cytotoxic agent);

(***c*) No cell** group (5% AlamarBlue^{TM} solution without cells).

The RFU values for these three groups are illustrated in **Figure 2-2**.

The result showed that there was no significant difference in RFU values between the dead cell group and the no cell group (5% AlamarBlue^{TM} solution only) ($p=0.999$); whereas there was a statistically significant difference between the live cell group and the blank as well as versus the dead cell group ($*p<0.05$, One-way ANOVA). Thus, the results of the control experiments showed that any RFU value above the blank RFU value reflected metabolic activity of **live cells**.

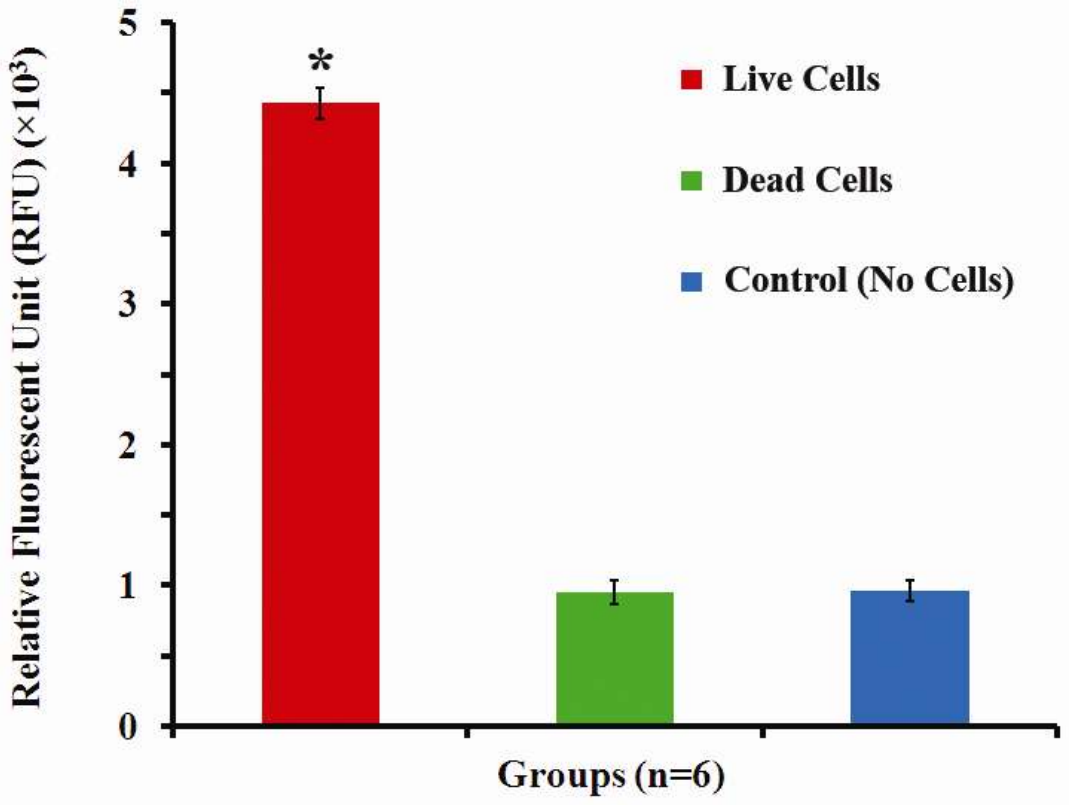

Figure 2-2: Relative fluorescent unit of the AlamarBlue™ assay for live, dead tenocytes and the negative control group (i.e. those with no added cells). Data were presented as means ± SEM.
*** Indicates significant differences ($p<0.05$) between the treated group compared to negative control (i.e. those with no added cells) and the dead cell group.**

2.3 Total collagen quantification

The procedure of total collagen quantification was adapted and modified from Tullberg-Reinert, H. and G. Jundt [193] where Sirius Red was used as target dye for this task. Briefly, after 14 days of incubation (either in 96 or 48 well plates), the culture media from the tenocytes were removed and the cell layers were washed extensively with PBS before

they were fixed with 100µl of Bouin's solution (which consists of 71% of saturated picric acid; 24% of 36% formaldehyde; and 5% of 0.5M acetic acid) for 1 hour. After fixation, the cell layers were washed under tap water for 15 mins and was air dried before stained with 100µl 0.1% Sirius red (RAYMOND A. LAMB, Eastbourne, UK) dissolved in saturated picric acid (w/v) for 1 hour. Sirius red binds specifically to collagen and can be redissolved in NaOH solution. The optical density (O.D.) of the NaOH that redissolves bound Sirius red can be measured with a microplate reader. After staining, the unbound dye was removed by washing with 200µl 0.01M HCl for 5 times. The bound dye was redissolved with 200µl 0.1M NaOH at room temperature for 30 mins. The dye solution was transferred to another 96 well plate and the optical density (O.D.) was measured with a Bio-Rad microplate reader (DYNEX technologies, Channel Islands, UK) at 570 nm. Wells containing 0.1 M NaOH were used as blanks. The actual collagen synthesis of the tenocytes was calculated by the standard curve described in the following section.

2.3.1 Validation of the correlation between O.D. and total collagen synthesis

In order to ascertain whether the O.D. measurement from Sirius Red truly correlates with the collagen synthesis, the following validation procedures were undertaken.

Soluble rat tail collagen type I (Sigma-Aldrich, UK) was added to the microplates in sequential doubling dilution ranging from 100µg to 3.3µg (100µg, 50µg, 25µg, 12.5µg, 6.7µg and 3.3µg) and air dried before the collagen quantification method was performed. The actual amount of collagen was plotted against the O.D. values measured at the same wavelength mentioned previously. The linear regression analysis showed that there was a

high degree of correlation between the O.D. and the amount of collagen added; the Pearsons's correlation coefficient was 0.9951 (**Figure 2-2**). This process was performed repeatedly with each microplate in order to calculate the actual collagen production with time in the cultures.

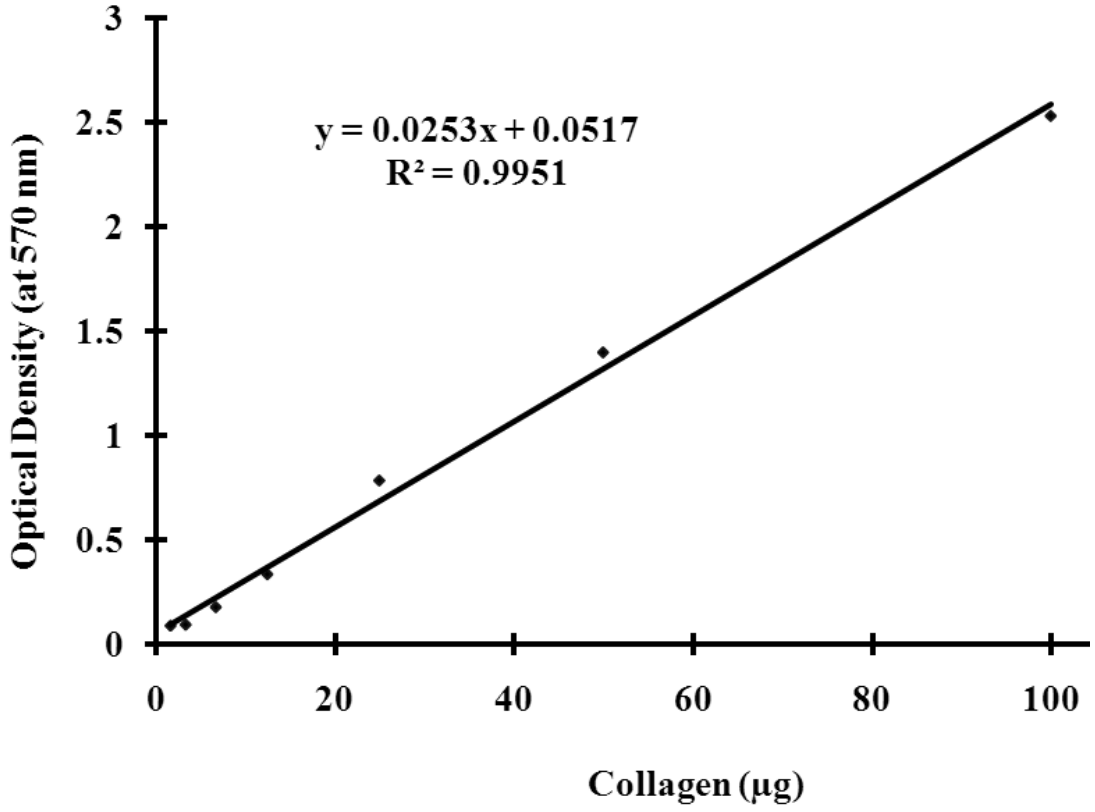

Figure 2-3: The Sirius red staining of rat tail collagen I as determined by O.D. measurements at 570 nm. This is a representative standard curve which was used for any subsequent collagen measurements. Similar standard curve was set up for every collagen assay measurement. The equation indicates the linear relationship between O.D. and collagen amount. R^2 is the Pearsons's correlation coefficient.

2.3.1 Validation of the collagen quantification method

This control experiment was performed to validate that the FBS constituents had no significant effect on the experimental results. The non-collagen producing cell RAW 264.7 murine macrophages cultured in α-MEM were used in this experiment. The optical density values were measured in 6 groups:

(***a***) α-MEM supplemented with **no FBS**;

(***b***) α-MEM supplemented with **1% FBS**;

(***c***) α-MEM supplemented with **5% FBS**;

(***d***) α-MEM supplemented with **10% FBS**;

(***e***) **No cell** group (α-MEM only without cells);

(***f***) **Blank** group (0.01M NaOH).

The optical densities of the six groups are illustrated in **Figure 2-4**. The data were examined by One-way ANOVA. The result showed that there was no significant difference in O.D. values between each of the above groups ($p>0.05$). These results indicate that there are no constituents present in FBS that bind to the Sirius red and interfere with the assay, and these control experiments validate the results of the collagen synthesis by using the Sirius red staining method.

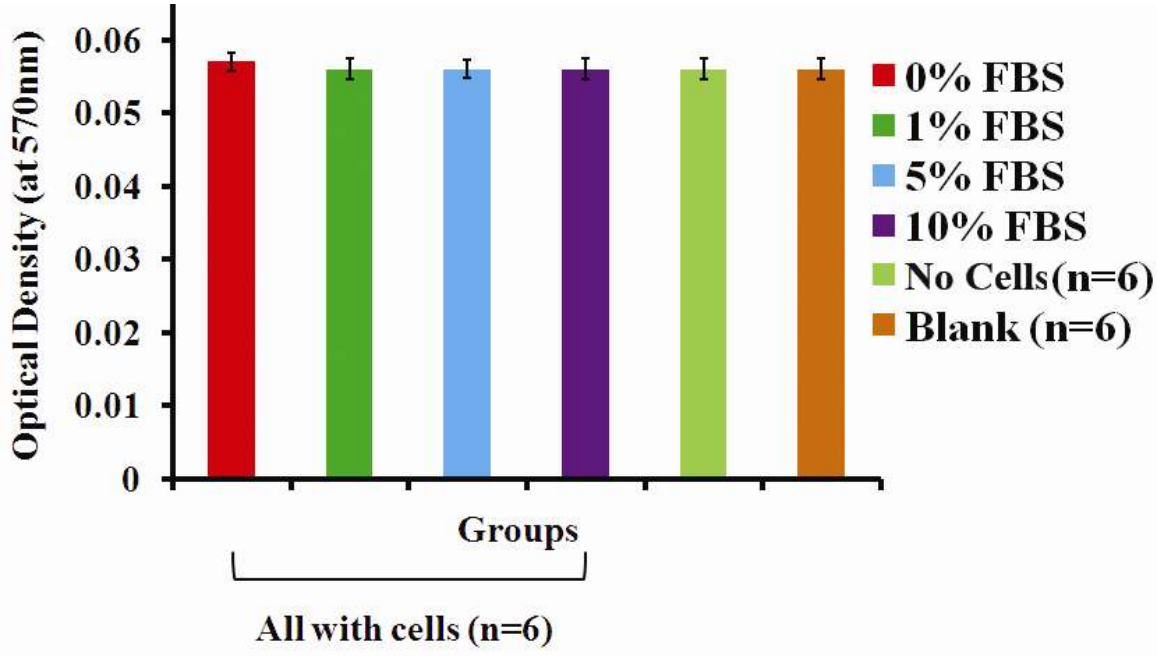

Figure 2-4: Validation of Sirius red staining using RAW 264.7 murine macrophages supplemented with various concentrations of FBS in order to ascertain that FBS does not interfere with the Sirius red staining. The data is expressed as means ± SEM of the O.D. measurement at 570nm for each group.

2.3.2 Validation of the calculation of collagen synthesis per live cell

In order to ascertain the collagen synthesis measurement was truly produced by "live" tenocytes and **not** any "dead" cells at the time of the culture, Live/Dead Viability/Cytotoxicity Kit (Invitrogen, USA) was employed for validation purposes.

The basis of this assay is the use of two fluorescent dyes; (1) Calcein which stains the cytoplasm of living cell as fluorescent green, (2) whereas dead or damaged cell's nuclei are stained fluorescent red by Ethidium homodimer-1 (Ethd-1). The cells were observed under inverted fluorescent microscope (Nikon, Japan). The effect of various treatments (± FBS and ± growth factors) at different concentrations on the collagen synthesis was investigated using this approach. The results indicated that majority of the cells attached to the culture

plates were live cells and that only a few dead cells could be observed and hence were neglected.

A representative photograph of the cells cultured in the presence of 10% FBS, 1% FBS + 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF is illustrated to demonstrate the viability of the cells after 14 days of culture **(Figure 2-5)**. Therefore, the results of this validation experiment showed that the collagen layers were synthesized by the living cells attached to the culture plates and not by any dead cells. Based on these findings, any subsequent collagen synthesis per cell calculation was conducted using collagen synthesis was normalized by Sirius red staining method.

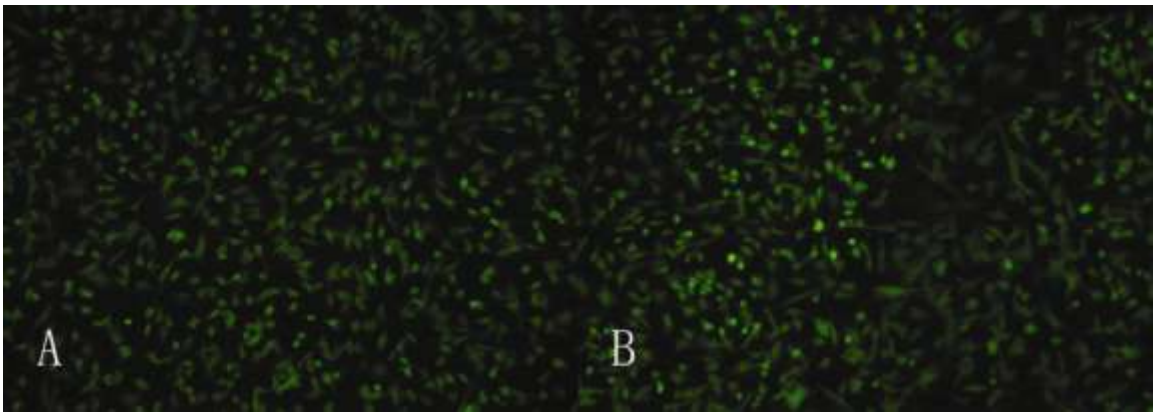

Figure 2-5: A representative photomicrograph of the live/dead staining of the tenocytes cultured in 48 well plates for 14 days in the presence of 10% FBS group (A); 1% FBS + 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF (B) (Magnification ×100)
Green colour stained cells indicate live tenocytes;
Red colour stained cells indicate dead tenocytes.

2.4 RNA extraction and real-time RT-PCR

Tenocytes were cultured in the same seeding density ($5\times10^3/cm^2$) in 10 cm culture dishes in triplicate for each treatment group. Total RNA was extracted from tenocytes on day 14

using RNeasy Kits (Qiagen, West Sussex, UK) in accordance with the manufacturer's protocol **(Appendix A)**. Briefly, the RNA was solubilised in sterile RNase free water provided in the kit. The RNA concentration and purity was determined by Nanodrop spectophotometry (Thermo scientific, East Sussex, UK). Total RNA (500 ng) was reverse transcribed to complementary DNA (cDNA) by using qScript® cDNA Supermix (Quanta bioscience, Gaithersburg, USA) following the manufacturer's protocol **(Appendix B)**. Template cDNA was then used in gene specific real time PCR for human specific Collagen type I (COL-I), Scleraxis (SCX), Tenomodulin (TNMD) and Decorin (DCN). The gene coding for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous reference gene. The sequences of primer sets were designed by and purchased from QuantiTech primer assay (Qiagen, West Sussex, UK). Entrez Gene ID, Cat. No. and expected sizes of resulting PCR products are shown in **Table 2-3**. The primers were validated for the same amplification efficiency prior to the experiments as described previously [194]. For Quantitative real-time reverse transcription PCR (real-time RT-PCR), PerfeCta^{TM} SYBR green Supermix (Quanta bioscience, Gaithersburg, USA) was used to prepare the reaction mixture following manufacturer's protocol **(Appendix C)**. Amplifications were performed in the Rotor gene RG-3000 (Corbett Research, Cambridgeshire, UK). All the reactions were performed in triplicate and included a negative control (without primers). The relative quantification of the mRNA levels of the target genes (quantity of transcripts of the target gene in the experimental groups relative to control group) was determined by using the $2^{-\Delta\Delta Ct}$ method [194]. Briefly, the Ct of the target gene was normalized to the Ct of the endogenous reference gene (GAPDH). ΔCt =

Ct (target gene) - Ct (GAPDH) for the experimental group and control group. The ratio of the target gene expression level was calculated as $R = 2^{-[\Delta Ct\ (experimental\ group) - \Delta Ct\ (control\ group)]}$ [194]. The final results were expressed as the ratio in gene expression relative to that of the control group. For different culture media supplemented with different growth factors, the values of gene expression were expressed as means ± SEM of triplicate samples from three separate experiments relative to the expression level found in cells cultured in control group (10% FBS supplemented in α-MEM).

2.5 Scanning Electron Microscopy (SEM)

The microstructures of engineered constructs were examined under scanning electron microscopy (JEOL JSM-840F Scanning Electron Microscope, Peabody, MA, USA) on day 7, 14, 21 and 28. Constructs were immediately rinsed in 0.1 M PBS (pH 7.3) and fixed in 4% glutaraldehyde overnight at 4ºC. Samples were then dehydrated through a gradient of alcohol followed by hexamethyldisilazane (Sigma-Aldrich, USA) and allowed to air dry in a fume hood. After dehydration, the constructs were examined using DMC-LX2 digital camera (Panasonic, Japan) after 7, 14, 21 and 28 days in culture and then sputter-coated with Platinum using a Polaron SC502 Sputter Coater (Fison Instruments, UK) before examination at 3kV.

The results of SEMs are presented in **CHAPTER 5, Section 5.2.4**.

Table 2-1: Real-Time RT-PCR Primer Sequences of Target Genes

Gene (Homosapient)	Gene Symbol	Qiagen Cat. No	Entrez Gene ID	Length of detected transcript (bp)	Amplicon length (bp)
Scleraxis	SCXB	QT01529507	642658	606	91
Collagen type I, α 1	COL-IA1	QT00037793	1277	5927	118
Collagen type II, α 1	COL2A1	QT00049518	1280	5087	94
Tenomodulin	TNMD	QT01024590	64102	1360	85
Decorin	DCN	QT00032459	1634	2305	87
***Runt*-related transcription factor 2**	RUNX2	QT00020517	860	5720	101
***SRY* -box 9**	SOX9	QT00001498	6662	3963	111
Glyceraldehyde -3-Phosphate	GAPDH	QT01192646	2597	1310	119

2.6 Histology examination

2.6.1 Cryosection of Tendon Samples and/or Constructs

Tissue samples were rinsed at room temperature in 0.1 M PBS and fixed with 5% sucrose. The tissue samples were transferred to embedding moulds and the moulds were filled with fresh OCT gel (Bright Instrument Company Limited, Huntington, UK). The moulds were rapidly submerged into liquid nitrogen (N_2), after which the samples and OCT gel were frozen. The blocks were wrapped in aluminium foil and store at -20°C until required. Five micron sections were cut using a Microtome (Frigocut 2800 Reichert-Jung, New York, USA) at -20°C and the sections were mounted on pre-cooled slides. The sections were then allowed to dry on the glass slides at room temperature and they were then stored at -20°C until needed.

For Sirius red staining, the slides were removed from the freezer, and allowed to defrost in room temperature, and air dried. The Sirius red staining procedure was the same as that described in **Section 2.3**, but without the NaOH dissolving step. After staining, the slides were washed in xylene twice and were dried before being sealed with DPX resin (VWR, UK) and cover slides (See **Appendix E** for sealing protocol).

2.6.2 Paraffin embedded sections of tendon sample and/or constructs

Tendon sample and/or constructs were fixed in 10% neutral buffered formalin (4 gram monobasic sodium phosphate, 6.5 gram diabasic sodium phosphate and 100ml 37% formaldehyde dissolved in 900ml of distilled water, PH 6.8) for 48 hours and went through

a series of grading alcohol dehydration (the samples in 70% ethanol and 80% ethanol for 24 hours each, 95% ethanol for 2 hours, and 100% ethanol for 30 mins) before being embedded by Leica EG 1120 paraffin dispenser (Leica, Germany). The tissue samples in paraffin blocks were sectioned into 5μm tissue sections by using a Leica RM2255 Microtome (Leica, Germany) and mounted on glass slides.

2.6.3 H&E staining of tendon sample and/or constructs sections

Standard H&E staining procedure was used. Briefly, the slides were dewaxed, rehydrated before stained with H&E standard protocol. For full reference of the dewaxing and rehydration protocol, see **Appendix D**.

The slides were immersed in haematoxylin (Jinqiao, Zhongshan, China) to stain the nuclei and washed under tap water for 5 mins. The excess dye was removed by immersing the slides in 0.5％ HCl and 70％ ethanol solution for 5 seconds，then washed under tap water for 15 mins. The slides were then immersed in distilled water for 5 seconds before dipped in Eosin solution (Jinqiao, Zhongshan, China) for 40 seconds, followed by another immersion in distilled water for 5 seconds. The slides were sealed with DPX resin and cover slides (See **Appendix E** for sealing protocol).

2.6.4 Immunohistochemistry of tendon sample and/or constructs sections

Standard immunohistochemistry procedure of the tendon sample and/or constructs sections was performed.

Briefly, the slides were subjected to dewaxing, rehydration, antigen retrieval and blocking protocols before the primary antibody was added. For full reference of those protocols mentioned, see **Appendix F**.

Rabbit anti **Human** COL-I polyclonal IgG (dilution 1:400) (Abcam, Hongkong, China) was added to each tissue section after they were blocked by 25µl 10% goat serum (dilution 1:20) (Jinqiao, Zhongshan, China) at room temperature for 30 mins. The slides were kept in a humidified tray at 4°C for overnight. After the slides were washed with 0.01M PBS for five mins, repeated 3 times, 25µl of goat anti rabbit IgG Biotinylated secondary antibody (Jinqiao, Zhongshan, China) were added to the tissue section and incubated at 37°C for 25 mins. The slides were washed with PBS at room temperature for 5 mins, repeated three times. 25µl of Avidin Biotin-Peroxidase Complex (Jinqiao, Zhongshan, China) was then added to the tissue sections and the slides were incubated at 37°C for 25 mins. The slides were then washed with PBS at room temperature for 5 mins, and this step was repeated three times. 20µl of DAB (3, 3'-diaminobenzidine) substrate (Jinqiao, Zhongshan, China) solution were then added to the tissue sections and incubated at room temperature for 15 mins. After washing the slides under tap water for 15 mins, the slides were immersed in haematoxylin to stain the nuclei, and then wash under tap water for 5 mins. The excess dye was removed by immersing the slides in 0.5% HCl and 70% ethanol solution for 5 seconds，then washed under tap water for 15 mins. The slides were sealed with DPX and cover slips (See **Appendix E** for sealing protocol).

Negative control experiments were performed with each group by substituting the primary antibody with 0.01M PBS.

2.6.4.1 Validation of the immunohistochemistry staining reflects the staining of COL-I

Control experiments were performed with exactly the same procedure but substituting the primary antibody with 0.01M PBS (PH 7.2-7.4) on tendon sections of the positive control group described in **Chapter 6**. The positive control group was known to be natural human hamstring tendon of which the main constituent was collagen type I. The results showed that brown coloured staining on the tissue sections could be observed by using rabbit anti human COL-I polyclonal IgG antibody **(Figure 2-6B)**. However, by substituting the primary antibody with 0.01M PBS, there was no brown coloured staining could be found on the tissue sections **(Figure 2-6A)**. This indicates that the collagen type I is localised by the brown colouration in the sections.

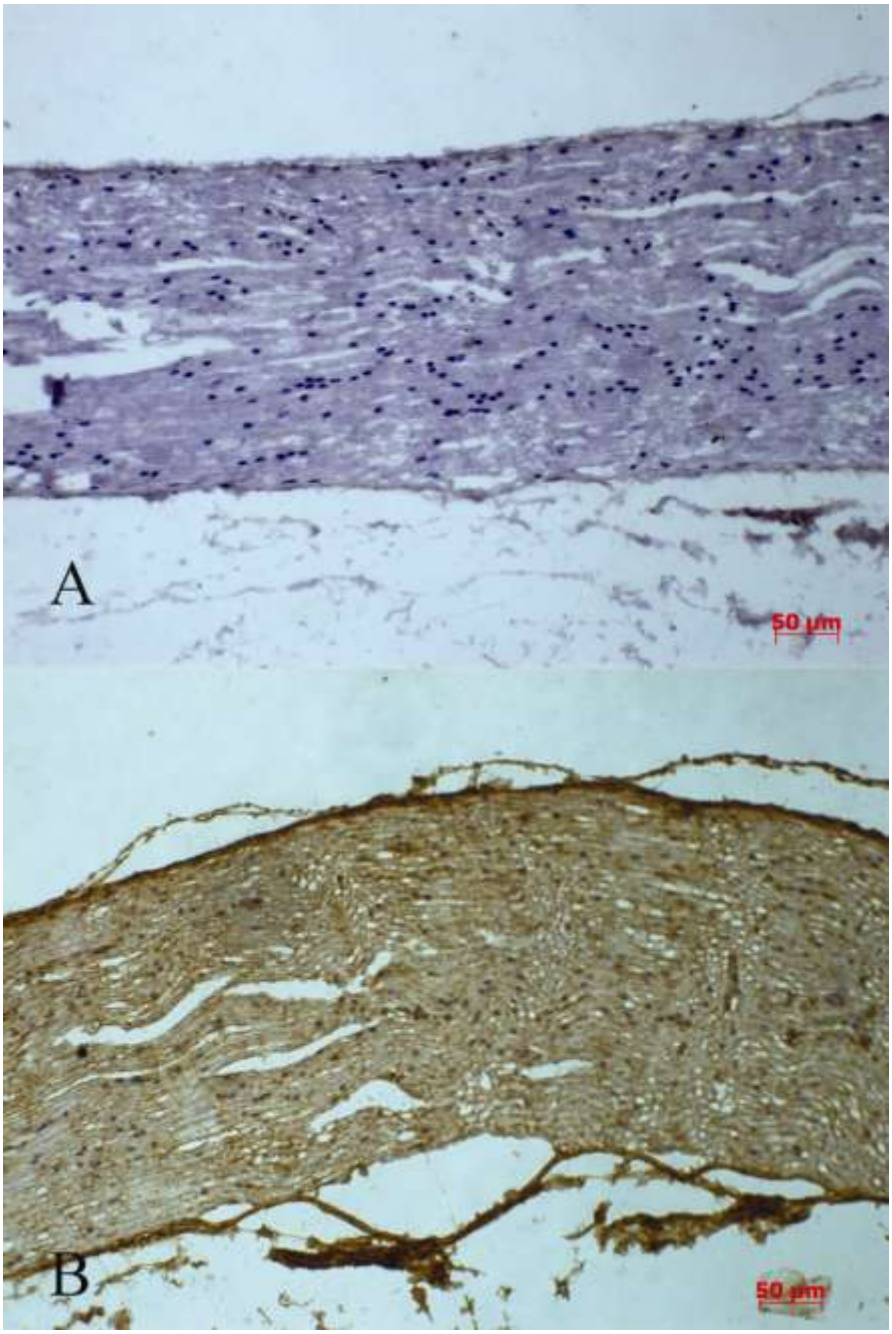

Figure 2-6: Immunohistochemical staining of COL-I on tissue sections of human hamstring tendon. (A) Negative control, in the absence of primary antibody, (B) in the presence of primary antibody (i.e., Rabbit anti Human COL-I polyclonal IgG, dilution 1:400), Magnification × 200, scale bar 50µm.

2.7 Mechanical testing

Mechanical testing was conducted using BOSE EnduraTec ELF 3200 DMA (Dynamic Mechanical Analysis) system (BOSE, Germany) **(Figure 2-7)**, on which the tendon/constructs samples were placed on specialized clamp as illustrated in **Figure 2-7**. Engineered constructs were examined and compared to normal human hamstring tendon samples (positive control) and degummed *Bombix* silk bunches without cells (negative control). In brief, tendon samples/constructs were kept in culture medium before they were mounted on the clamp. The gauge length was set at 15mm and the constructs were stretched to failure at a speed of 10mm/min. To account for end effects, force and extension data were collected only from samples that failed in the region away from the clamp (a minimum of 10% of the gauge length). The gauge length and cross-sectional dimensions were accurately measured for every sample using digital callipers. Six samples were used in each group and the average values of the 6 samples were used as data points (n=6). The values were expressed as means ± SEM.

Maximum tensile stress was computed by dividing the maximum force by the cross-sectional area.

$$\sigma_{Max} = \frac{F_M}{A}$$

Where

σ_{Max} is the maximum tensile stress

F_M is the maximum force exerted on the tendon samples/ constructs

A is the cross sectional area of the tendon samples/ constructs

Similarly, the **Young's modulus of elasticity** was computed by dividing the tensile stress by the tensile strain.

$$E = \text{tensile stress / tensile strain} = \frac{\sigma}{\varepsilon} = \frac{F/A_0}{\Delta L/L_0}$$

Where

E is the Young's modulus (modulus of elasticity)

F is the force applied to the object;

A_0 is the original cross-sectional area through which the force is applied;

ΔL is the amount by which the length of the tendon samples/ constructs changes;

L_0 is the original length of the tendon samples/ constructs.

Strain at break was calculated as the ratio of the change in the length of the tendon samples/ constructs to the original length at the point of failure.

$$\varepsilon = \frac{\Delta L}{L} = \frac{l-L}{L}$$

Where

ε is the strain at break

l is the final length of the tendon sample/construct at breaking point

L is the original length of the tendon sample/construct

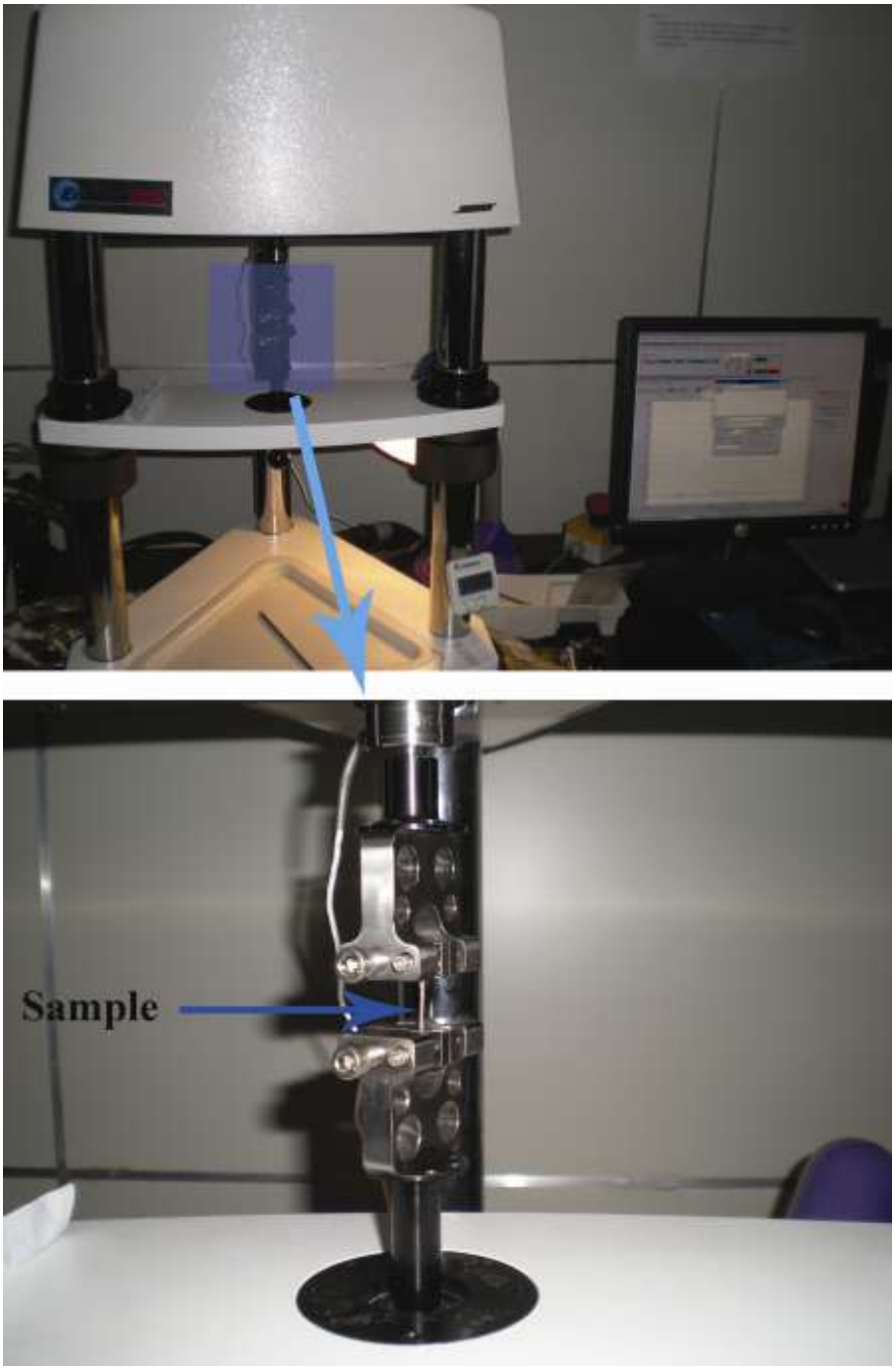

Figure 2-7: Photograph of BOSE EnduraTec ELF 3200 DMA system which was employed for mechanical testing of 3-D constructs and human hamstring tendon. Enlarged area indicates the specialized clamp where the tendon samples/constructs were placed for testing.

2.8 Statistical analyses

Analysis of variance was performed on datasets when appropriate using a significance of $p<0.05$. One-way analysis of variance (ANOVA) was used when multiple comparisons of the data were performed to determine if significant differences between the means existed. A Tukey multiple range post hoc test was used to compare paired groups of data. Statistical analyses package (GraphPad InStat®, v.3.06 for Windows, San-Diego, USA) and Microsoft Excel were used when appropriate. For this purpose, minimum number of replicate for each study was 6, unless otherwise stated.

CHAPTER THREE:

FACTORS REQUIRED FOR *IN VITRO* TENOCYTE EXPANSION IN LOW SERUM CULTURE CONDITIONS

Preliminary studies indicated that single application of human $PDGF_{BB}$, bFGF and IGF-1 in culture media could promote long term culture and enhance tenocytes **expansion** *in vitro*. Furthermore, IGF-1 and TGFβ-3 were found to be the most appropriate of growth factors in promoting long term **differentiation** and maintaining tenocytes survival *in vitro*. Based on these preliminary findings, the present and the subsequent Chapters report the findings of the optimal combination of growth factors which promote tenocytes **expansion** and **differentiation**, respectively.

3.1 EXPERIMENTAL DESIGN

The main objective of this part of the study was to identify possible beneficial growth factor combinations, and supplementation of culture media that could support tenocyte **expansion** *in vitro* for the purpose of tissue engineering.

To achieve this aim, a sequential fractional factorial experimental design and tiered analysis approach were employed to reduce the number of required trials. A tiered outcome analysis approach consisted of assessing: (1) cell number increase, (2) collagen synthesis and (3) messenger RNA expression of the tenocytes' phenotypic and differentiation markers (SCX, TNMD, COL-I and DCN). These parameters were investigated in order to establish the optimal combination growth factors required for the **expansion** phase.

A factorial design allows researchers to study the effect and interaction of each factor on the variable responses [195]. Factorial experiment usually consists of two or more factors, each with discrete possible "values" or "levels" [195]. Based on report by Montgomery, the number of assays can be calculated as $levels^{factors} \times levels^{factors}$ [196]. For example, in the

current study, the number of assays required to test and compare the effects of 4 (3) different concentrations of 4 treatments on various outcomes can be calculated using the following manner ($3^1 \times 4^3 = 192$) whereby one factor with 3 doses and 3 factors with 4 concentrations would provide an extensive number of assays to undertake. As indicated in **Table 3-1**, a sample number of **n** = 6 per assay per batch, given 3 main experimental approaches (AlamarBlue™, Sirius red and real time RT-PCR) carried out in triplicates would generate 10,368 ($3^1 \times 4^3 \times 6 \times 3 \times 3 = 10,368$) trials! As each variable has an exponential effect in trial numbers, the variables can either be eliminated or fixed during each experiment based on the selection criteria. This would reduce the trial number significantly by fixing one parameter at any one time and hence decrease the number of experiments from 10,368 ($3^1 \times \mathbf{4^3} \times 6 \times 3 \times 3$) to 2,592 ($3^1 \times \mathbf{4^2} \times 6 \times 3 \times 3$).

The sequential fractional factorial design and tiered outcome analysis approach used in this Chapter were as follows **(Figure 3-1)**.

1. Effect of FBS supporting tenocyte proliferation. Tenocytes were cultured in 96-well culture plates with media supplemented with different concentrations of FBS (0%, 1%, 5% and 10%) for AlamarBlue™ assays over 14 days of culture. The FBS concentration which promoted the highest cell number expansion was selected as the positive control group for proliferation assays. The other two groups consisting of lower FBS concentrations which resulted in least cell number expansion were selected as the base media for testing the effects of various growth factors and their combinations.

2. The effect of combinations of growth factors on tenocyte proliferation and collagen synthesis in low serum concentrations were assessed up to a 14-day period. The effects of three different growth factors ($PDGF_{BB}$, bFGF and IGF-1) at various concentrations were assessed on tenocytes' expansion *in vitro* within 1, 7 and 14 days using AlamarBlue™ assays **(Table 3-1)**.
3. Total collagen synthesis by tenocytes in response to various growth factors and concentrations was measure after 14 days of culture. To normalize the amount of collagen synthesis per cell number, the cell numbers were also assessed at this time point using AlamarBlue™ assays.
4. The treatment groups which resulted in a high tenocytes expansion and/or low collagen synthesis per cell, were selected for mRNA expression of the tenocytes markers (SCX, TNMD, COL-I and DCN) using real time RT-PCR. Any changes in the cell morphology in these groups were also noted.
5. All of the above experiments were conducted in triplicates in order to overcome any intra- or inter- assay variation.

For detailed methodology of each of these assays refer to **CHAPTER 2, Section 2.3, 2.4 and 2.5.**

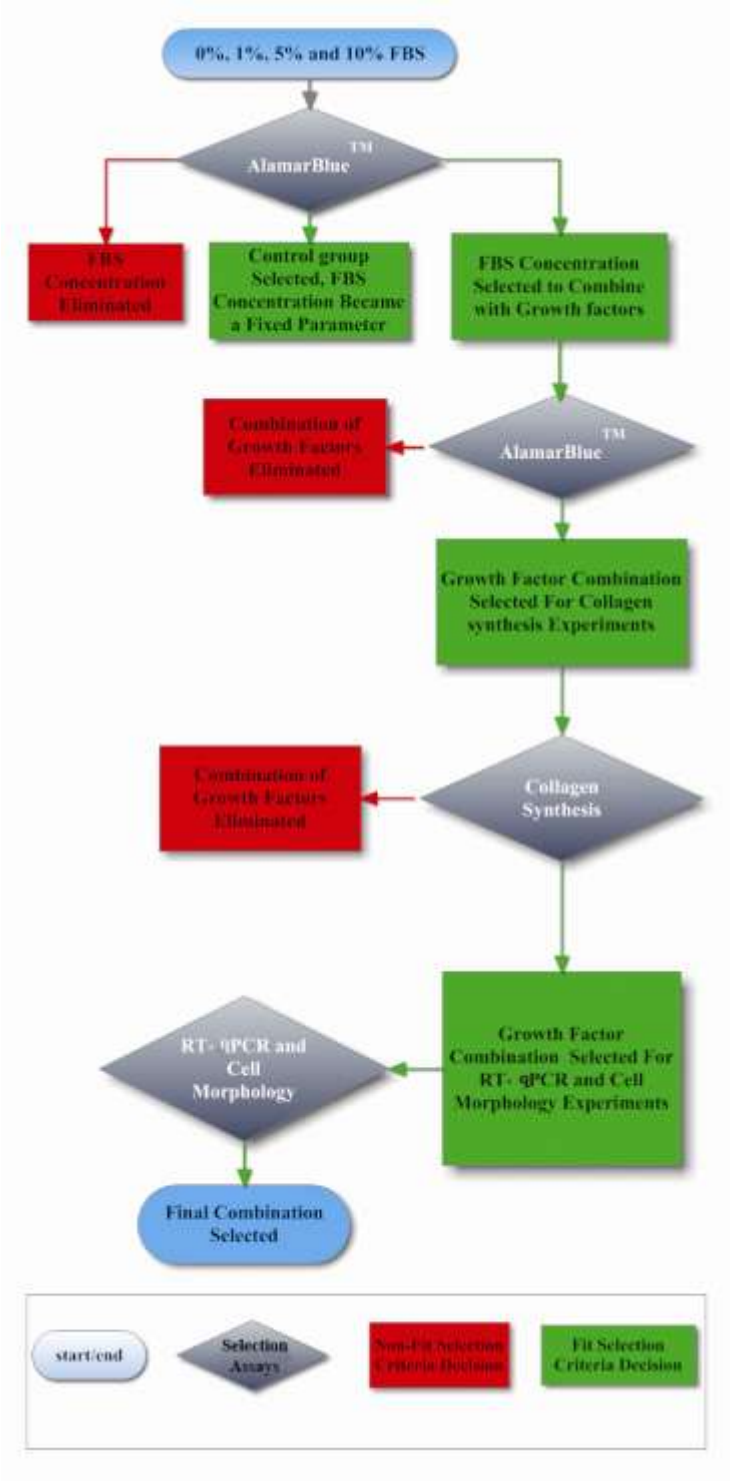

Figure 3-1: Flow chart for the fractional factorial design. For further details, refer to text.

Table 3-1: Parameters for experimental design

Fixed parameters	**Manipulated parameters**	**Levels**
1. Cells and passage	1. FBS	0%, 1%, 5% and 10%
2. Media change rate	2. $PDGF_{BB}$	0, 5, 10 and 50ng/ml
3. Growth environment	3. bFGF	0, 5, 10 and 50ng/ml
	4. IGF-1	0, 10 and 50ng/ml

3.2 RESULTS

3.2.1 Tenocyte proliferation

a) In cultures supplemented with FBS only, the tenocyte numbers at 0% and 1% FBS exhibited a significant decrease after day 1 and there were no surviving cells within 7 or 14 days compared to day 1, respectively. In higher FBS concentration, the tenocyte cell number increased by 4 and 6 fold on day 7, in 5% and 10% FBS, respectively. After 14 days in 5% and 10% FBS the tenocyte number maintained a nearly 4 fold increase which is significant ($p<0.05$) compared to the cell number on day 1 **(Figure 3-2)**.

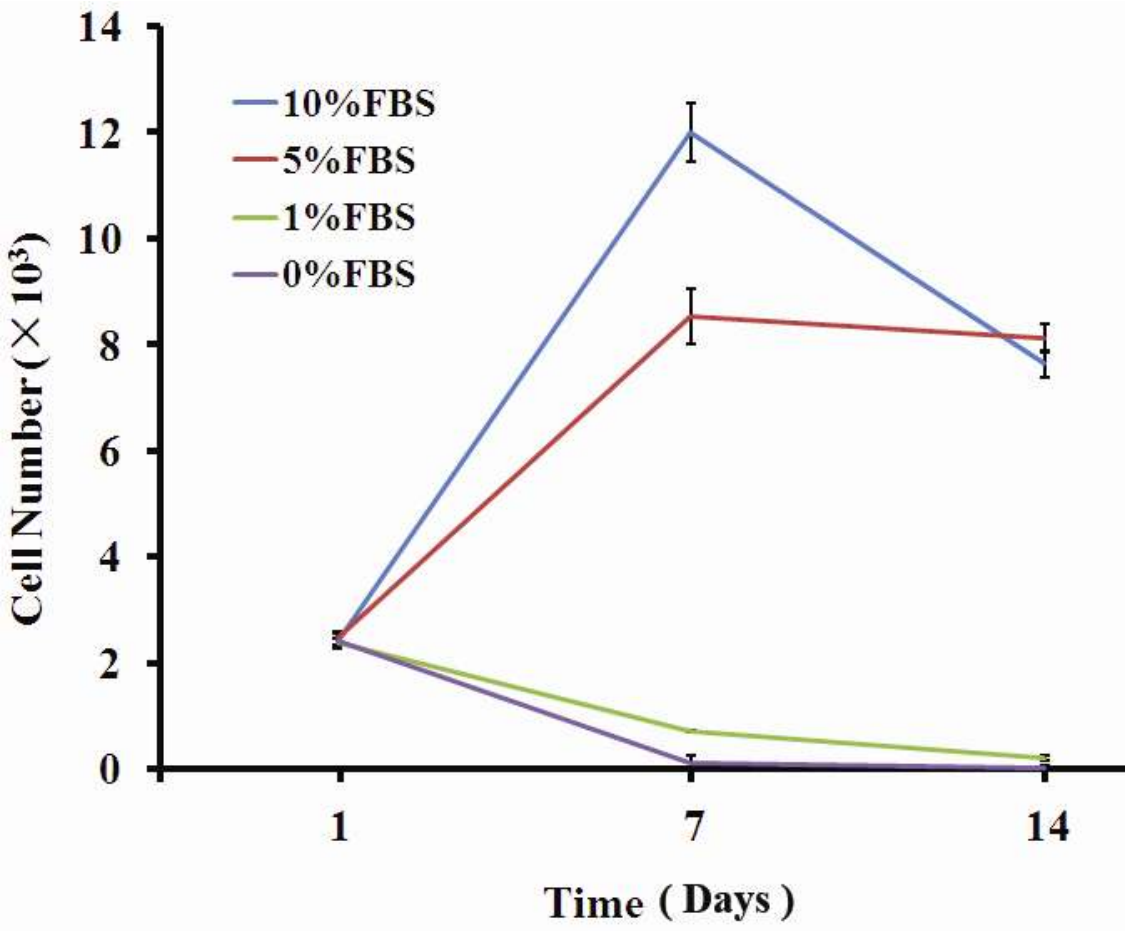

Figure 3-2: Tenocyte proliferation in 96 well plates after 14 days of culture in different concentrations of FBS as determined by AlamarBlue™ assay. Data were presented as means ± SEM.

b) As the cultures supplemented with 0% FBS and combinations of growth factors, did not significantly increase the cell number after 14 days of culture (Data not shown), all subsequent experiments using various growth factors were conducted in the presence of 1% FBS and compared to 10% FBS as a positive control. The addition of different concentrations of $PDGF_{BB}$, there was a significant dose-dependent increase in tenocyte cell number after 14 days in culture ($p<0.05$). The maximum cell number increase was observed when tenocytes were cultured for 7 days with

10% FBS or in the presence of 50ng/ml $PDGF_{BB}$ **(Figure 3-3)**. Furthermore, this increase in cell number in $PDGF_{BB}$-treated cultures was significantly higher ($p<0.05$) than the cell treated in the presence of 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF **(Figure 3-3)**.

c) After 14 days in culture, the number of tenocytes was not significantly different in cultures treated with either 50ng/ml $PDGF_{BB}$ or 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF compared to the control groups ($p>0.05$). Supplementation of $PDGF_{BB}$ with bFGF resulted in a reduced but more steady rate of cell number increase than the control group with the maximum cell number increase being observed only on day 14 **(Figure 3-3)**.

d) The 50ng/ml of bFGF or IGF-1 group showed neither significant cell number increase nor cell number decrease at day 14**.** The groups showing the highest cell number increase at day 14 were those cultures treated with 50ng/ml $PDGF_{BB}$ (~ 4 fold) and the cell number was significantly higher in these treated groups as compared to those without $PDGF_{BB}$ supplements ($p<0.05$). There was no significant increase in the tenocyte cell number when cultured in the presence of $PDGF_{BB}$ supplemented with IGF-1, bFGF or IGF-1 + bFGF ($p>0.05$) **(Figure 3-4)**.

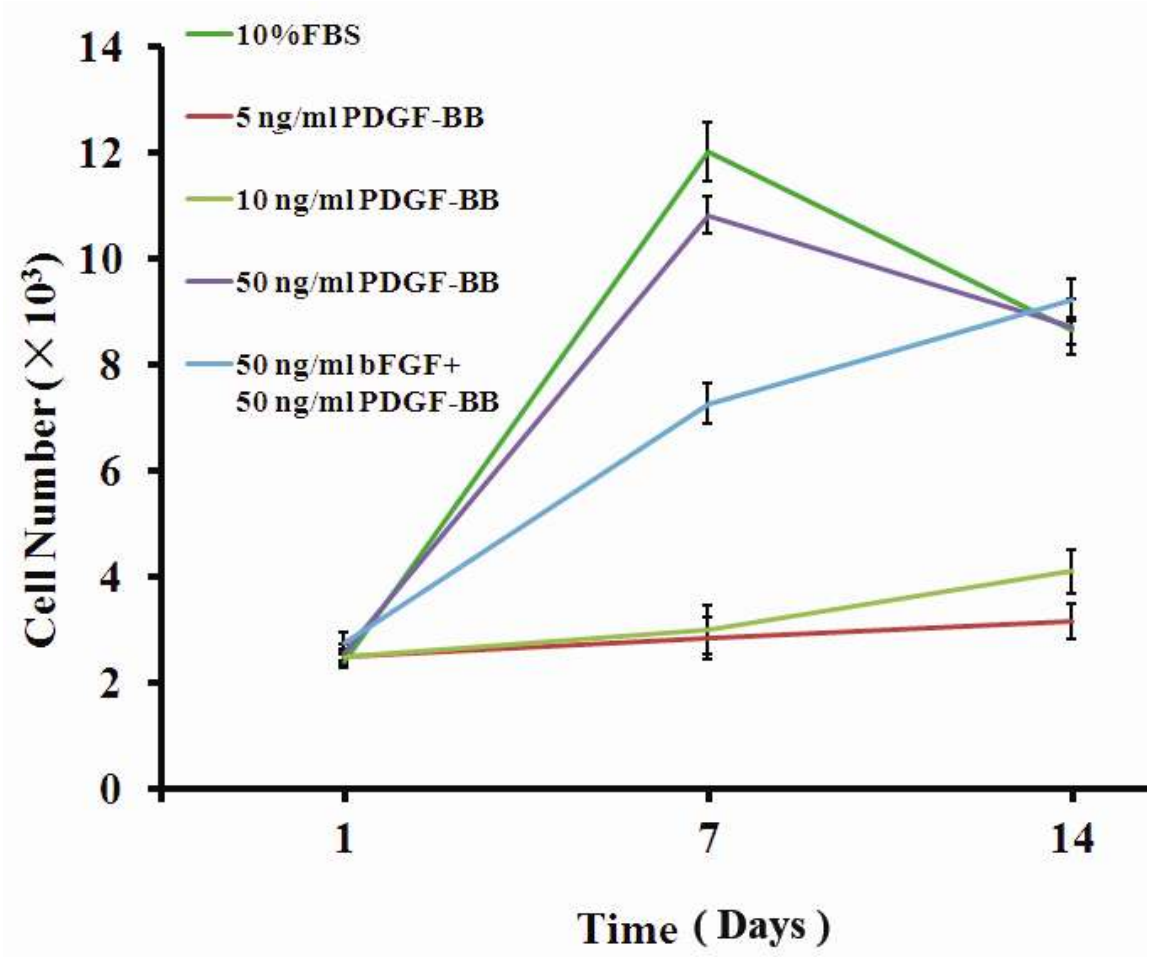

Figure 3-3: Tenocyte proliferation as determined by AlamarBlue™ assay over a 14-day culture period in the presence of different concentrations of $PDGF_{BB}$ supplemented with 1% FBS. After fractional factorial studies, the tenocytes proliferation in the presence of 50ng/ml bFGF and 50ng/ml $PDGF_{BB}$ was also determined (Blue line). 10% FBS was used as positive control group. Data were presented as means ± SEM.

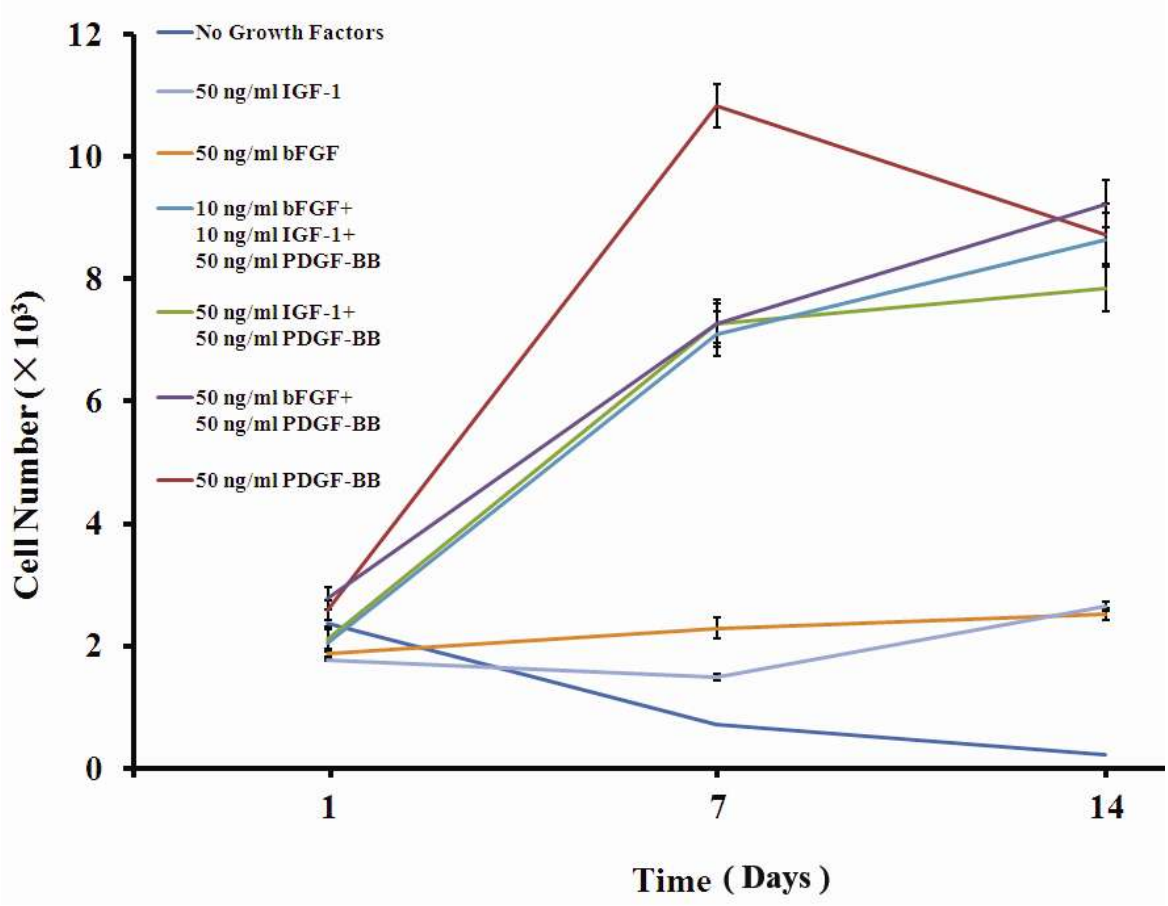

Figure 3-4: Tenocyte proliferation after 14 days of culture in 1% FBS supplemented with different combination of growth factors as determined by AlamarBlue™ assay. Data were presented as means ± SEM.

3.2.2 Collagen synthesis

Total collagen synthesis was analyzed at day 14 and data were normalized as described in **Chapter 2, Section 2.3**. The extent of collagen synthesis in cultures treated with $PDGF_{BB}$ was not significantly different compared to the 10% FBS control group. Treatment of tenocytes with bFGF showed a significant inhibitory effect toward tenocytes collagen formation as the group showed the lowest collagen synthesized among all the groups (§$p<0.05$). Co-supplementation of bFGF with $PDGF_{BB}$ showed a significantly lower

collagen synthesis compared to $PDGF_{BB}$ alone and to the control group (*$p<0.05$). Treatment of tenocytes with IGF-1 had no significant effect on the extent of collagen synthesis compared to the 10% FBS group and those treated with $PDGF_{BB}$. Co-supplementation of IGF-1 with $PDGF_{BB}$ showed no significant increase in collagen synthesized per cell compared to the 10% FBS group or the group with $PDGF_{BB}$ alone. Based on these findings, all subsequent real time RT-PCR analyses for the tenocyte markers were conducted using 1% FBS supplemented with either 50ng/ml $PDGF_{BB}$ alone, 50ng/ml bFGF alone or 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF **(Figure 3-5)**.

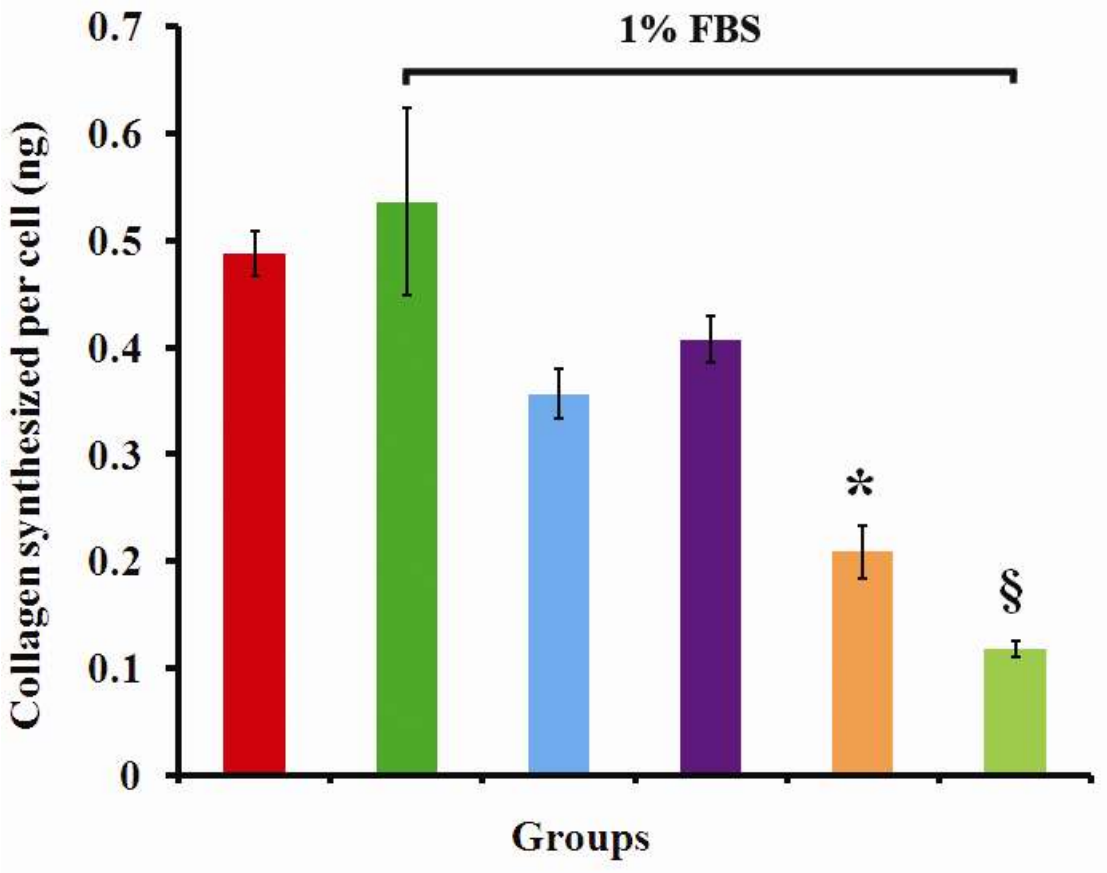

Figure 3-5: Collagen quantification per cell using Sirius red staining after 14 days in culture in the presence of various growth factor supplemented with 1% FBS. 10% FBS supplemented in α-MEM without growth factors was used as a positive control group. Data were presented as means ± SEM.
*** p<0.05: significant differences were noted when compared with 10% FBS or 50ng/ml $PDGF_{BB}$ (One-way ANOVA);**
§ p<0.05: in comparison with all the other treated groups (One-way ANOVA)

Red indicates the group treated with 10% FBS
Dark Green indicates the group treated with 50ng/ml $PDGF_{BB}$
Blue indicates the group treated with 50ng/ml IGF-1
Purple indicates the group treated with 50ng/ml IGF-1+50ng/ml $PDGF_{BB}$
Orange indicates the group treated with 50ng/ml bFGF+50ng/ml $PDGF_{BB}$
Light Green indicates the group treated with 50ng/ml bFGF

3.2.3 Messenger RNA expression of tenocyte phenotypic and differentiation markers

***SCX mRNA*-** The SCX expression in all growth factor-supplemented media was significantly reduced as compared to 10% FBS positive control ($p<0.05$, **Figure 3-6A**). The expression of SCX mRNA was reduced by 0.33 for bFGF, 0.18 for bFGF + $PDGF_{BB}$ and 0.17 for $PDGF_{BB}$ alone. There were no significant differences within the bFGF treatment groups in SCX mRNA expression ($p>0.05$).

***TNMD mRNA*-** The extent of TMND mRNA expression was significantly reduced in all treated cultures as compared to positive 10% FBS-treated groups **(Figure 3-6B)**. Compared to the control group (10% FBS), bFGF + $PDGF_{BB}$-treated cultures resulted in a 0.03 ratio decrease in TNMD expression whilst tenocytes cultured with $PDGF_{BB}$ alone or bFGF alone exhibited a 0.005 & 0.003 ratio reduction in TNMD expression, respectively. There were no significant differences in TNMD expression between each growth factor treatment ($p>0.05$).

***COL-I mRNA*-** Of all the growth factor-treated cultures, only those incubated with 50ng/ml $PDGF_{BB}$ showed a significant increase in COL-I expression compared to the control group (10% FBS) ($p<0.05$) **(Figure 3-6C)**. Other treatments had no or little effect on the expression of COL-I by tenocyte as compared to the controls.

***DCN mRNA*-** The DCN mRNA expression was significantly reduced in cultures supplemented with 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF where the reduction in the ratio of DCN expression was 0.26 **(Figure 3-6D)**. However, those tenocytes treated with $PDGF_{BB}$ or bFGF alone had a less marked reduction in ratio of DCN mRNA expression as compared to positive control (0.932 and 0.47, respectively).

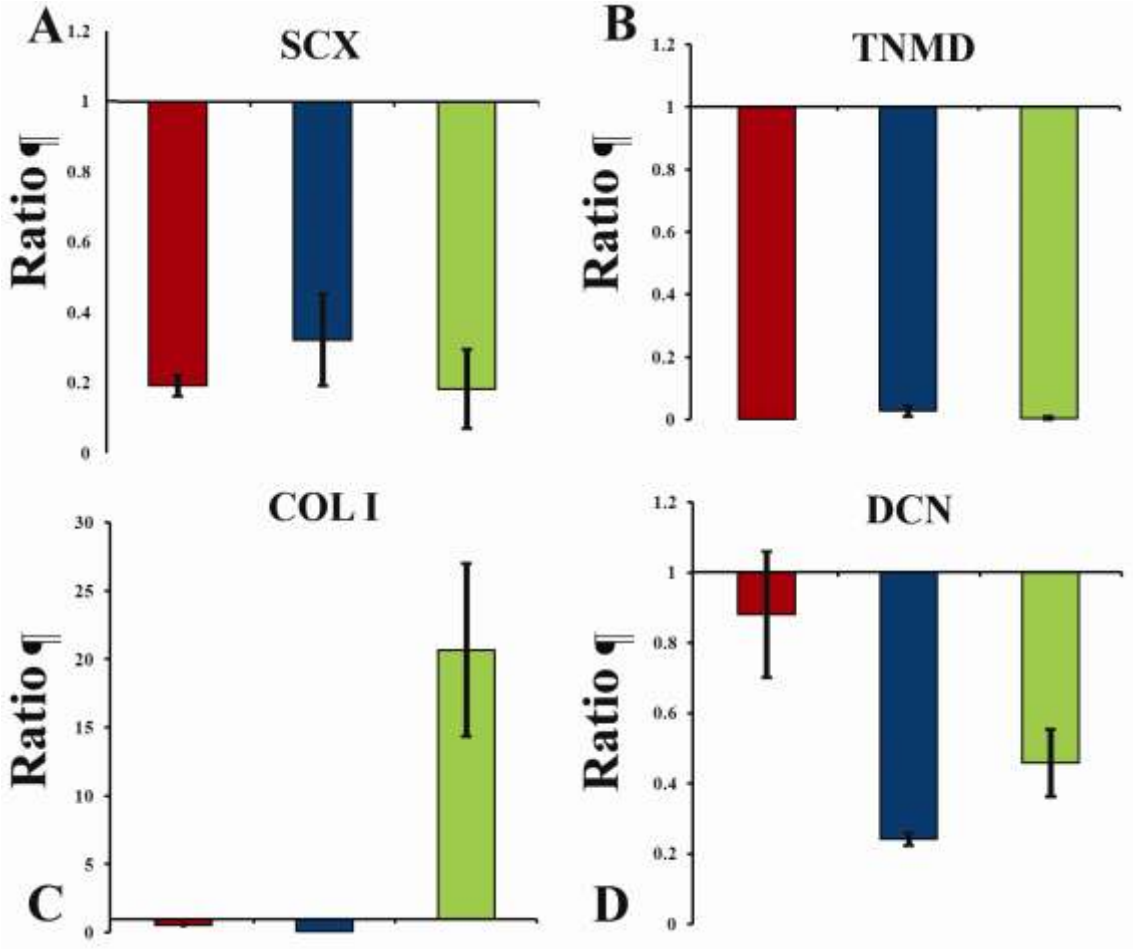

Figure 3-6: Messenger RNA expression levels of various tenocyte markers as determined by real time RT-PCR.
A: SCX, B: TNMD, C: COL-I, and D: DCN in tenocytes at the 14-day time point cultured in the presence of 1% FBS and various growth factors over a 14-day culture period.
¶: The data were presented as means ± SEM ratio of mRNA expression of different markers relative to that of the control group (10% FBS). GAPDH mRNA was used to normalize the variability in template loading.

Red indicates the group treated with 50ng/ml bFGF
Blue indicates the group treated with 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF
Green Indicates the group treated with 50ng/ml $PDGF_{BB}$

3.2.4 Sirius red staining of cell morphology

Next, the cell morphology of tenocytes treated with various combinations of growth factors supplemented with 1% FBS was determined. After 14 days in all treated cultures, tenocytes exhibited an elongated, spindle-shaped, fibroblast-like appearance. Those treated with 10% FBS had a more aggregated collagen layer which was comprised of thick, dense parallel collagen fibres with strong Sirius red staining **(Figure 3-7A)**.

Tenocytes treated with 1% FBS + 50ng/ml $PDGF_{BB}$ also showed a similar morphology to the control group, with parallel cell alignment and the collagen fibres stained with Sirius red, but the collagen layers were not evident in these cultures **(Figure 3-7B)**.

The 50ng/ml bFGF-treated cultures, tenocytes exhibited thin and random aligned collagen fibres where the Sirius red staining was far less intense than the 10% FBS group or the 50ng/ml $PDGF_{BB}$-treated group **(Figure 3-7C)**.

In cultures treated with 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF the morphology of tenocytes was similar to that observed with those incubated with bFGF alone **(Figure 3-7D)**.

None of the groups showed rounded shaped tenocytes which indicative of tenocytes phenotypic drift [63].

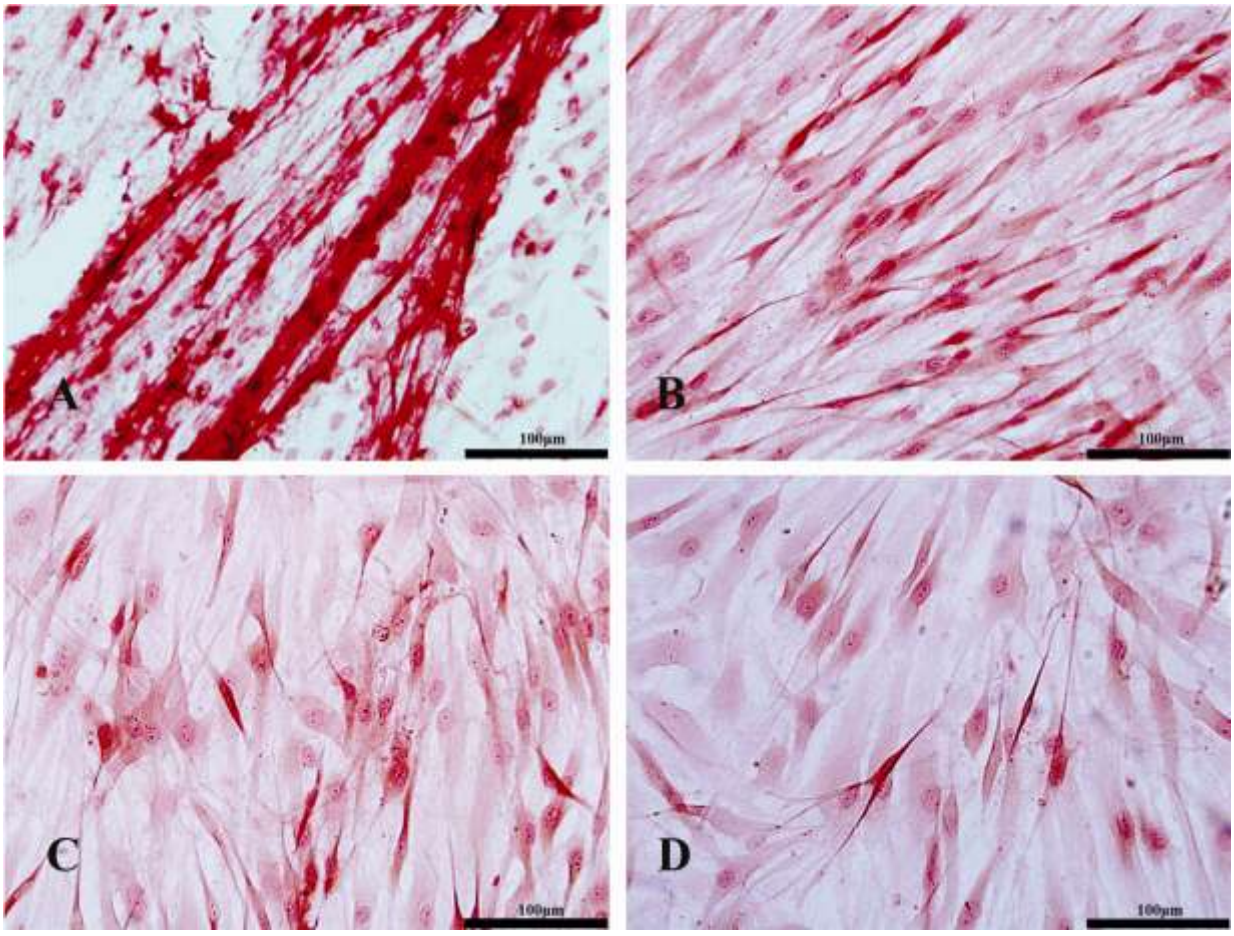

Figure 3-7: Sirius red staining of the tenocytes cultured 14 days in different culture conditions. (A) 10% FBS, (B) 1% FBS + 50ng/ml $PDGF_{BB}$, (C) 1% FBS + 50ng/ml bFGF and (D) 1% FBS + 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF (Magnification ×200, scale bar 100μm)

3.3 DISCUSSION

The results of this Chapter indicate that supplementation of culture media with combination of growth factors (50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF) in low FBS (1%) allows human tenocyte **expansion** *in vitro* with reduced extracellular matrix formation. This effect is

favourable to the first stage of tendon tissue engineering, when tenocyte **expansion**, rather than extracellular matrix formation is desired [65].

The present findings also revealed that, in the absence of FBS, the supplementation of culture media with above named growth factors ($PDGF_{BB}$ + bFGF), although maintained tenocyte survival, did not support the expansion of cell number over 14-day culture. However, with the addition of 1% FBS, the effect of $PDGF_{BB}$ was amplified dramatically **(Figure 3-3)**. Although the maximum cell number increase was lower than that of the 10% FBS, the cell number after 14 days in 1% FBS + 50ng/ml $PDGF_{BB}$ was similar to that with 10% FBS supplemented group. The treatment groups supplemented with 1% FBS and a combination of 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF, exhibited a steady cell proliferation (about 4 fold increase) after 14 days in culture compared to the day 1.

The results presented here demonstrate the ability of $PDGF_{BB}$ to act as a potent proliferation inducer of human tenocytes. This effect of $PDGF_{BB}$ on proliferation was dose dependent and evident at all concentrations employed. Furthermore, the findings suggest that $PDGF_{BB}$ is not only a potent stimulator of tenocyte proliferation *in vitro*, but it can also promote collagen mRNA expression as shown by real time RT-PCR. Interestingly, $PDGF_{BB}$ was capable of inhibiting tenocyte differentiation by down regulating SCX, TNMD and DCN mRNA expression. These results are in agreement with Banes *et al*., who also suggested that $PDGF_{BB}$ is a suitable inducer of avian tenocyte proliferation *in vitro* [197]. However, the role of $PDGF_{BB}$ as a regulator of tenocyte differentiation has not been previously reported.

In addition to the effects of $PDGF_{BB}$ on human tenocyte, the present study also addressed

the role of bFGF and IGF-1 on differentiation of human tenocyte *in vitro*. The findings suggest that bFGF and/or IGF-1 did not influence tenocyte proliferation under the condition employed here, whether or not supplemented with $PDGF_{BB}$. Data presented in this Chapter indicate that bFGF and IGF-1 alone, or in combination, do not promote proliferation of human tenocyte *in vitro* over a 14-day culture. Indeed, bFGF showed strong dose-dependent inhibition of human tenocyte collagen production and differentiation *in vitro*.

It was evident that in the presence of 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF, the expression of SCX and TNMD were significantly down regulated. It has been previously reported by Shukunami that TNMD expression is regulated by SCX [111], the results here are consistent with these previous findings as the TNMD showed corresponding decreases in the $PDGF_{BB}$ and bFGF treated group. It suggests that both $PDGF_{BB}$ and bFGF have significant effects on inhibiting tenocyte differentiation. However, the COL-I mRNA expression indicates that $PDGF_{BB}$ promoted tenocyte collagen synthesis and it can be reversed by the addition of bFGF. In addition, 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF also exhibited a significant inhibition in expression of DCN, a marker for extracellular matrix formation.

Overall, 50ng/ml bFGF + 50ng/ml $PDGF_{BB}$ in 1% FBS concentration showed strong inhibition in tenocyte differentiation as shown by mRNA expression of those markers.

Microscopically, thinner and more randomly-aligned collagen fibrils were observed in bFGF treated cultures (50ng/ml) compared to FBS (10%) alone, in which a thicker and more paralleled collagen fibril were noted **(Figure 3-7)**. Similar results were also obtained

when 50ng/ml of bFGF was supplemented with 50ng/ml $PDGF_{BB}$, resulting in a significant inhibition of collagen synthesis and microstructure alignment of collagen fibrils. It is worth noting, however, that the treatment of tenocytes with a combination of bFGF and $PDGF_{BB}$ did not affect the rate of cell proliferation *in vitro* **(Figure 3-3)**. The inhibitory effect of bFGF in collagen synthesis found in this study is consistent with that reported by Chan *et al.*, whose finding suggested that treatment of rat tenocytes with a high dose of bFGF (30 ng/ml) can significantly lower the cell density and gene expression levels (COL-I, COL-III, and fibronectin) compared to a lower concentration of bFGF (3 ng/ml) [166]. The inhibitory effects of bFGF on other cell types have also been reported. For example, Locklin *et al.*, have reported that bFGF can inhibit the differentiation of human bone marrow stromal cells in 10% FBS conditions as the osteogenesis was inhibited over 7 days of culture as measured by reduced phosphatase activity compared to the group without bFGF [190]. Others have shown that bFGF can dose dependently inhibit chondrocyte differentiation (indicated as collagen type II and aggrecan mRNA expression) in low/medium FBS (2.5%), as compared to the group without bFGF treatment [198].

Currently there are only a few research articles which report the effect of PDGF (all isoforms) on **human** tenocyte **expansion** and **differentiation** *in vitro*. Costa *et al.* showed that $PDGF_{BB}$ had significant effect in promoting rabbit tenocyte proliferation dose dependently in serum free condition over a 3-day period, a finding which is in agreement with that reported herein [188]. Furthermore, the findings presented here are consistent with that reported by Wong *et al.*, [75] who suggested that treating human tenocytes for 6 days with a combination of $PDGF_{BB}$ and dexamethasone can reverse the inhibitory effects

of dexamethasone alone (i.e. inhibiting tenocyte proliferation and collagen synthesis) [75]. Moreover, data reported here are in part in agreement with that published by Thomopoulos *et al.* who suggested that $PDGF_{BB}$ is capable of promoting canine tenocyte proliferation and collagen synthesis [199]. The findings reported in this Chapter are also in agreement with those documented by Yoshikawa *et al*., that *in vitro* addition of $PDGF_{BB}$ to rabbit tenocytes stimulates collagen synthesis over a 4-day period [178].

Overall, the present findings are in contrast with those reported by Costa *et al*., who showed that treatment of rabbit tenocytes with a combination of $PDGF_{BB}$ (50ng/ml), bFGF (5ng/ml) and IGF-1(100 ng/ml), maximizes their proliferation in serum free conditions *in vitro* within 3 days of culture [188]. The data reported herein are also in disagreement with that documented by Thomopoulos *et al*., whose findings suggested that bFGF promotes canine tenocyte collagen production after 24 hours treatment under serum free conditions [191]. There may be difference in cellular responses because of different species employed in each investigation. In the present study, human tenocytes were used whereas rabbit [188] or canine [191] cells were used by Costa *et al.* and Thomopoulos *et al.*, respectively. In addition, different culture periods were studied; the present study focused on a 14-day culture period only whereas others employed either a 3-day [188] or 24 hour [191] period.

Basic fibroblast growth factor (bFGF) has long been recognized as a potent mitogen that stimulates proliferation, migration and differentiation of cells of mesenchymal origin [183]. The biological response of cells to bFGF is mediated through specific, high affinity cell surface receptors (FGFR-1) that possess intrinsic tyrosine kinase activity and are phosphorylated upon binding of bFGF [189]. Xu *et al*. showed that high concentrations of

bFGF supplemented in culture medium have a significant effect in suppressing bone morphogenetic protein (BMP) signaling and supports long term undifferentiated proliferation of embryonic stem cells [192]. Moreover, BMP gene transfer could induce mesenchymal stem cells to differentiate into tenocytes [74, 200, 201]. Previous studies have demonstrated that bFGF can interrupt BMP signaling either by preventing the nuclear translocation of phosphorylated Smad1 [202] or by repressing Smad1 activity in the nucleus [203]. The involvement of BMP signaling pathway in the present study was not conducted and this requires further investigation in any future work.

Previous reports have shown that IGF-1 has a significant effect in promoting avian tendon fibroblast [152] and human primary placental fibroblasts [204] proliferation *in vitro* culture. The findings reported herein revealed that, in low serum concentrations, IGF-1 showed no significant effect in supporting tenocyte cell number **expansion** *in vitro* but it does maintain tenocyte survival and promotes **differentiation**. A possible explanation for this is that the experiments reported in this Chapter were conducted with low concentrations of FBS (1%). Abrahamsson *et al.* showed that IGF-1 promoted rabbit tenocyte proliferation and collagen synthesis dose dependently. In their report, markedly higher concentrations of IGF-1 (10-250ng/ml) were employed than that reported herein. Furthermore, these authors documented a collagen synthesis assay which was performed over a much shorter culture period than that presented here (3 day vs. 14 days) [156].

It would have been ideal if the culture conditions employed for the **expansion** of tenocyte *in vitro* were totally free of any exogenously added serum. However, this could not be achieved in total serum free medium, with or without all supplemented growth factors. As

such a low concentration of FBS (1%) had to be used to provide the optimal **expansion** of tenocytes *in vitro* for the present study. Further research is required to develop a FBS free culture medium for human tenocyte **expansion**. At the moment, this would be rather challenging as the initial tenocyte isolation step is carried out in the presence of FBS and further investigations on serum-free media would be required.

In the present study, in addition to $PDGF_{BB}$ and bFGF supplementation, the tenocyte culture medium was also supplemented with IGF-1. The role of the IGF-1 on tenocyte will be discussed in detail in **CHAPTER 4**.

3.4 SUMMARY

- The results of this Chapter illustrate that a combination of low serum (1% FBS), and high concentrations of $PDGF_{BB}$ and bFGF (50ng/ml for both) is the optimal condition as studied here that is required for promoting human tenocyte **expansion** *in vitro*, but at the same time limiting their **differentiation**.
- Of all the growth factors tested using the conditions employed here ($PDGF_{BB}$, bFGF and IGF-1), only IGF-1 was not selected for the **expansion** phase of the tenocyte as it did not meet the "**high expansion /low differentiation**" criteria.
- In the next Chapter, the effects of a number of growth factors (TGFβ-3 and IGF-1) will be investigated on **differentiation** of tenocytes *in vitro*. As such, it would be possible to create an *in vitro* **expansion** and subsequent **differentiation** phase to optimize the *in vitro* conditions required for tenocyte-based tendon tissue engineering.

CHAPTER FOUR:

FACTORS REQUIRED FOR *IN VITRO* TENOCYTE DIFFERENTIATION IN SERUM FREE CULTURE CONDITIONS

4.1 EXPERIMENTAL DESIGN

Preliminary studies (including the results outlined in **CHAPTER 3**) indicated that out of all the growth factors studied; only IGF-1, TGFβ-3 and a combination of the two could support and promote tenocyte **differentiation** in the lowest possible FBS concentrations. In order to optimize the generation of the tenocyte for the purpose of tissue engineering, the following selection criteria were defined in the study reported in this Chapter:

(1) To maintain cell survival without exogenously added serum for 14 days,

(2) To induce maximal collagen synthesis per cell during this period, and

(3) To facilitate optimal mRNA expression of differentiation markers (SCX, TNMD, COL-I and DCN) of tenocytes within 14 days.

Therefore, to achieve these objectives, a sequential fractional factorial experimental design was employed as described in **CHAPTER 3, Section 3.2**. The combination of parameters manipulated or fixed for this part of the study, is listed in **Table 4-1**.

Table 4-1: Parameters for experimental design

Fixed parameters	**Manipulated parameters**	**Concentrations**
1. Cells and passage	1. FBS	0%, 1%, 5% and 10%
2. Media change rate	2. TGFβ-3	0, 1 and 10ng/ml
3. Growth environment	3. IGF-1	0, 10 and 50ng/ml

The following outcome measures were assessed for each manipulated/fixed parameter prior to finalising the optimal conditions required for *in vitro* tenocyte **differentiation**:

1. In order to achieve the maximal tenocyte differentiation within 14 days, tenocytes were cultured in 48-well culture plates with media supplemented with different concentrations of FBS (0%, 1%, 5% and 10%) for 14 days. Cell proliferation and the extent of collagen synthesis *in vitro* by tenocytes were assessed using AlamarBlue™ assay and Sirius red staining, respectively (as described in detail in **CHAPTER 2, Section 2.2, 2.3**). The FBS concentration supplemented in culture media that promoted the highest proliferation and collagen synthesized per tenocyte were selected as positive control group for any subsequent experiments. Serum free culture media were selected to ascertain the effects of various growth factors on tenocyte differentiation and to compare their *in vitro* effect with positive control group.
2. The effect of various concentrations of IGF-1, TGFβ-3 and a combination of the two, in serum free media, was investigated on cell proliferation and collagen synthesis over a 14-day culture period as described above.
3. The treatment groups which demonstrated the higher collagen synthesized per tenocyte and maintained most optimal cell survival over 14 days, were selected for subsequent real time RT-PCR experiments to examine tenocyte differentiation markers (SCX, TNMD, COL-I and DCN). In this manner, one was able to select the most suitable growth factor(s) and concentrations that provided the optimal

conditions for *in vitro* tenocyte **differentiation**. Any changes in the cell morphology in these groups were also noted.

4. All of the above experiments were conducted in triplicates in order to overcome any intra- or inter- assay variation.

4.2 RESULTS

4.2.1 Tenocyte proliferation

In order to determine whether increasing concentrations of FBS over a fixed period of time had any effect on tenocyte proliferation, 0, 1, 5 and 10% FBS-supplemented culture media were used for various time points (1, 7 and 14 days). As indicated in **Figure 4-1**, the media supplemented with the lowest concentrations of FBS (0 and 1%) resulted in a significant decrease ($p<0.05$) in cell proliferation after 7 days compared to day 1. Within 14 days, few or no viable cells were detectable in low (1% FBS) or serum free tenocyte cultures.

On the other hand, after 7 days in culture, in the presence of 5 or 10% FBS, the tenocyte cell numbers increased significantly by 278% and 324%, respectively ($p<0.05$), as compared to day 1-treated cultures. Due to limited surface area available in 48 well culture plates and also due to rapid expansion of tenocytes after 14 days in culture, their cell numbers on day 14 in 5 and 10% FBS were markedly reduced compared to day 7. Nonetheless, the cell numbers in 5% and 10% cultures remained significantly higher than their respective tenocyte number on day 1 ($p<0.05$) **(Figure 4-1).**

Based on the above findings, it was decided that 10% FBS treatment will be used as positive controls for determining tenocyte differentiation whilst 0% FBS was selected for establishing the direct effect of growth factors on this parameter.

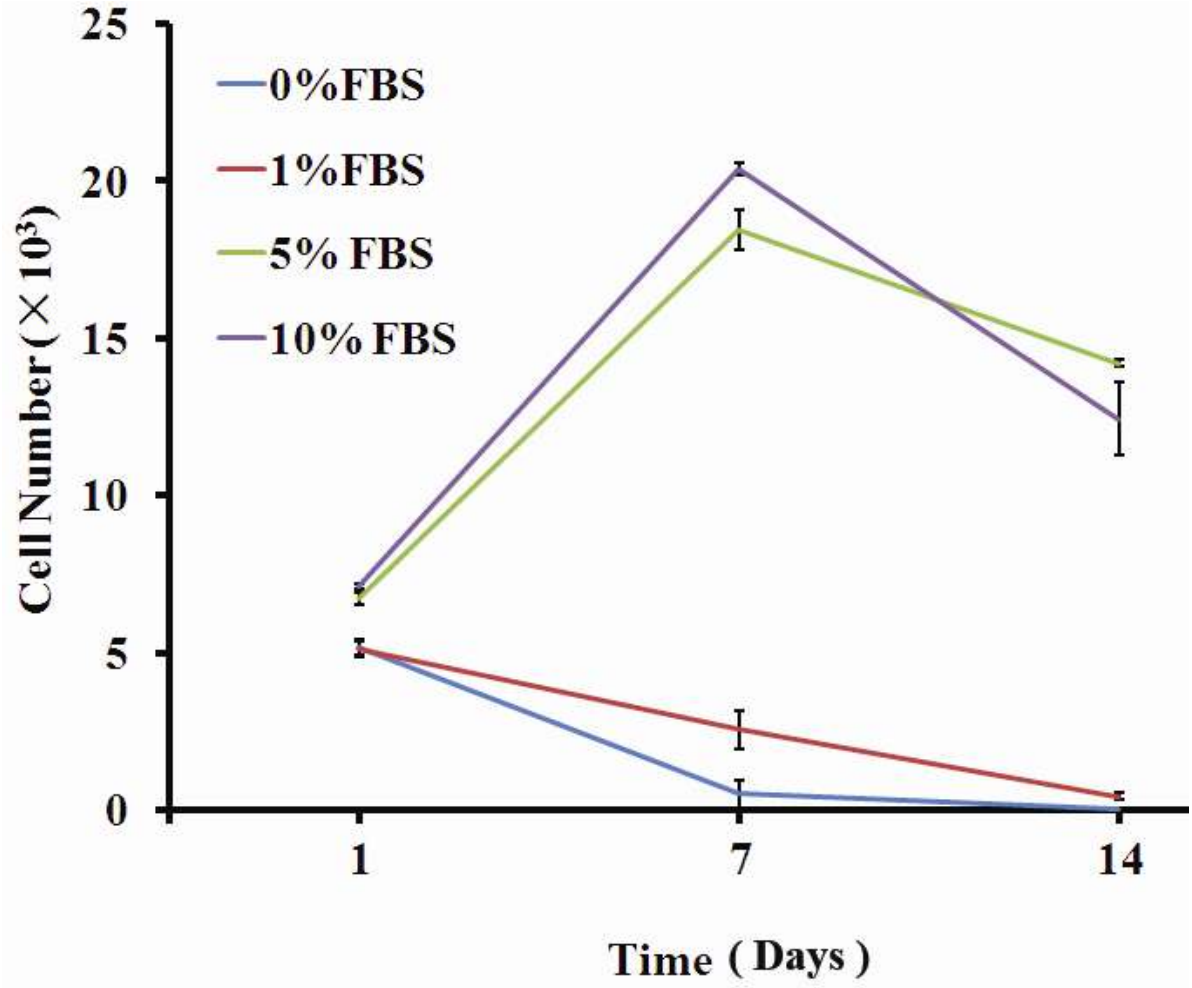

Figure 4-1: Tenocyte proliferation in 48 well plates after 14 days of culture determined by AlamarBlue™ assay, in the presence of various concentrations of FBS. Data were presented as means ± SEM.

As indicated in **Figure 4-2 and Figure 4-3**, treatment of tenocytes in serum free media supplemented with TGFβ-3 (1 and 10ng/ml), IGF-1 (10 and 50ng/ml) and a combination of the two growth factors (TGFβ-3 at 1ng/ml + IGF-1 at 10ng/ml or TGFβ-3 at 10ng/ml + IGF-1 at 50ng/ml) for 14 days, resulted in measurable changes in tenocyte cell number compared to day 1 and positive control cultures. Although the cell number in culture media supplemented with 0% FBS and combinations of TGFβ-3 and IGF-1 had significantly increased compared to serum free media (with no other added growth factors) ($p<0.05$)**(Figure 4-1, Blue line)**, none of the treatment groups exhibited comparable cell number increase as that observed for the positive 10% FBS treated control group.

This suggested that TGFβ-3, IGF-1 and the combination of the two were capable of maintaining tenocyte cell survival in serum free media for 14 days of culture compared to 0% FBS where no viable tenocytes were detectable.

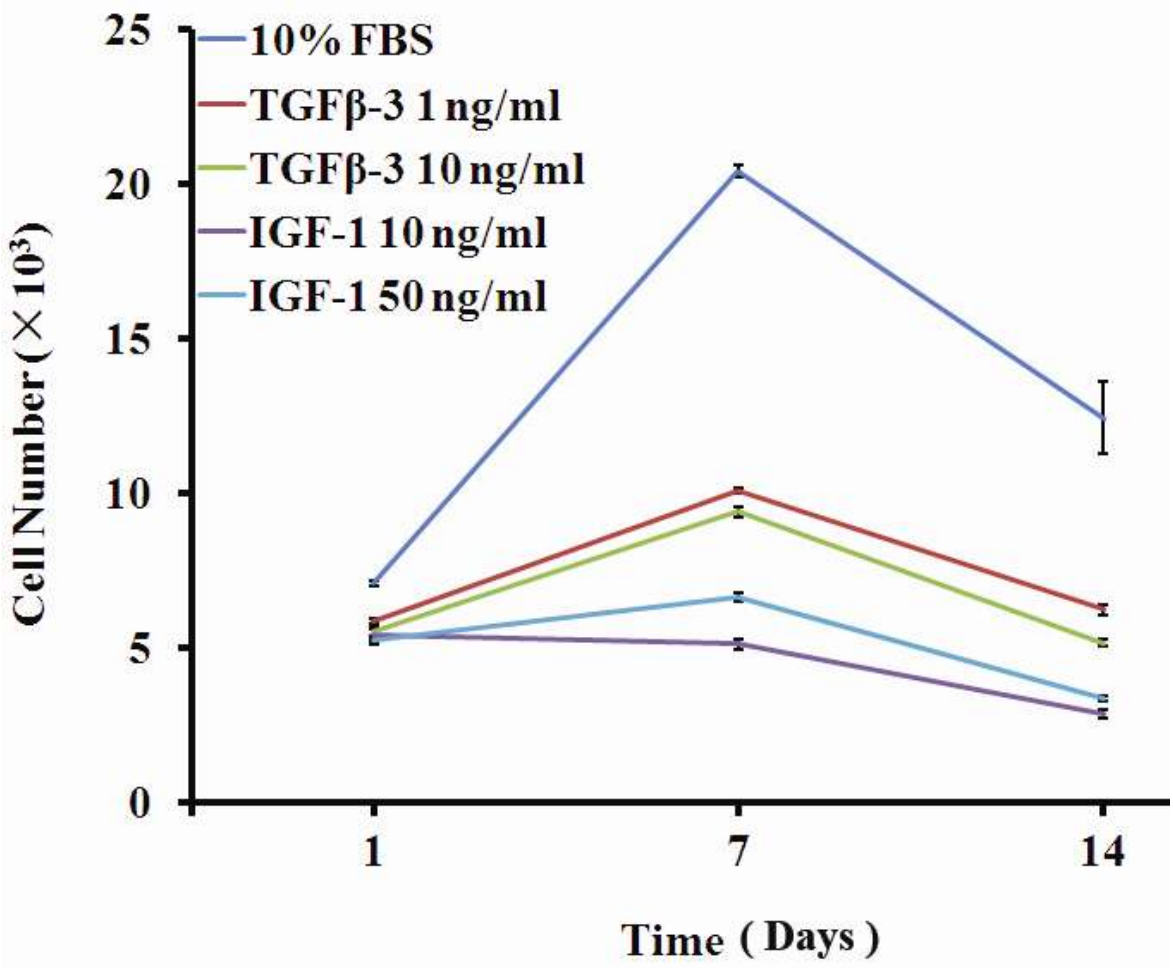

Figure 4-2: Tenocyte proliferation after 14 days of culture in 0% FBS supplemented with different concentrations of TGFβ-3 and IGF-1 as determined by AlamarBlue™ assay. 10% FBS supplemented in α-MEM without growth factors was used as control group. Data were presented as means ± SEM.

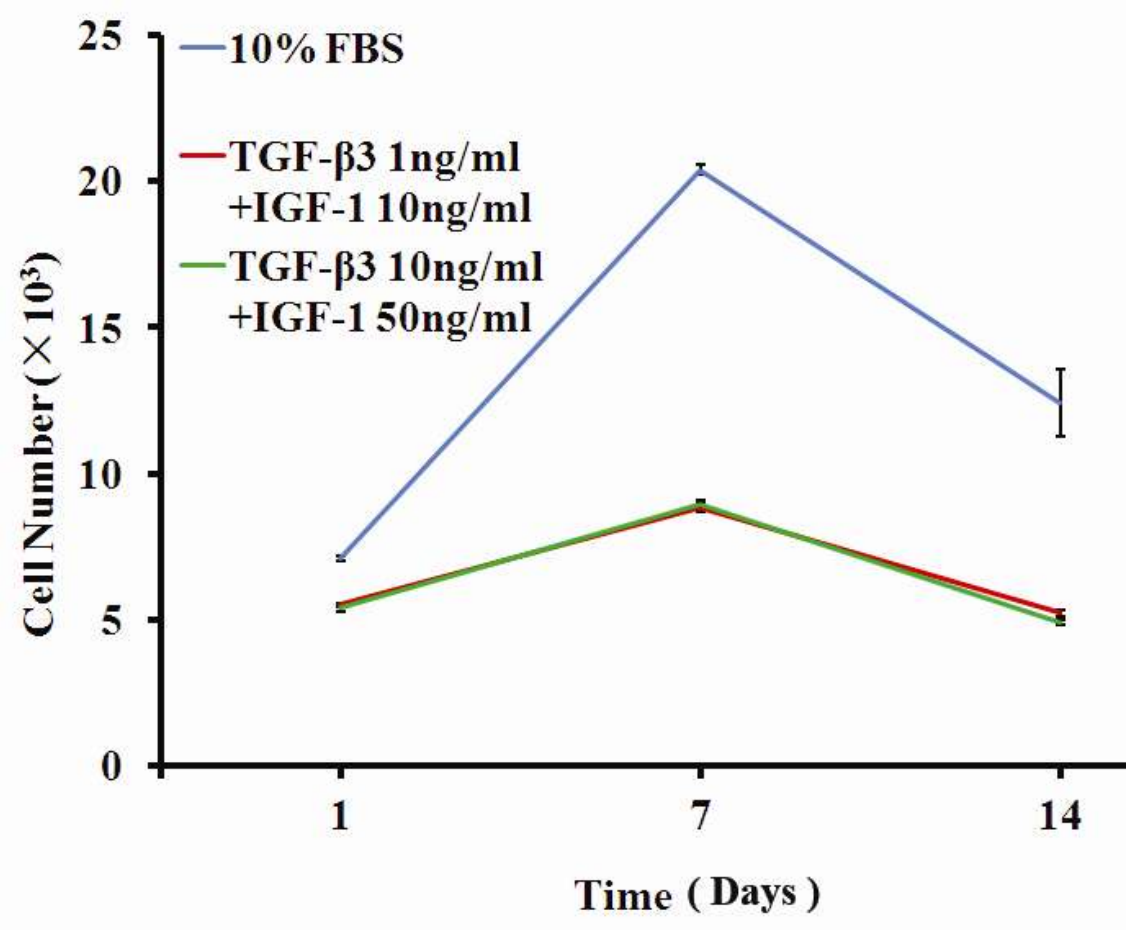

Figure 4-3: Tenocyte proliferation after 14 days of culture in 0% FBS supplemented with different combinations of TGFβ-3 and IGF-1 as determined by AlamarBlue™ assay. 10% FBS supplemented in α-MEM without growth factors was used as control group. Data were presented as means ± SEM.

4.2.2 Collagen synthesis

In conjunction with the cell survival results, collagen synthesis measurements by tenocytes were determined using Sirius red staining (as described previously in **CHAPTER 2, Section 2.3**), for TGFβ-3, IGF-1 and a combination of these two factors after 14 days in cultures. As indicated in **Figure 4-4,** the most optimal treatment which resulted in maximal collagen synthesis in serum free media was determined to be the combination treatment of

tenocytes with 10ng/ml TGFβ-3 + 50ng/ml IGF-1. Although this treatment group resulted in a significant increase in collagen synthesis in tenocytes compared to other treatment groups (*****$p<0.05$), this increase was still significantly lower than that noted for 10% FBS treated tenocyte cultures (§$p<0.05$) **(Figure 4-4)**. Given these results, the combination treatment of tenocytes with TGFβ-3 (10ng/ml) and IGF-1 (50ng/ml) was chosen for the mRNA expression of tenocyte differentiation markers (see below).

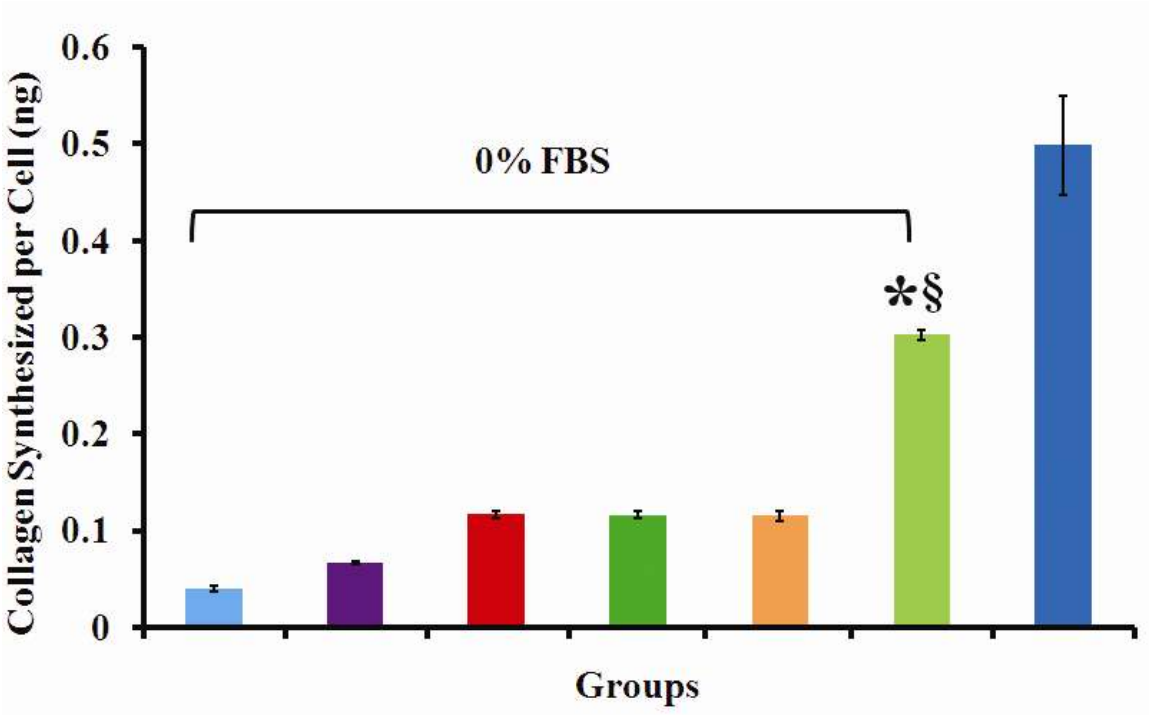

Figure 4-4: Collagen quantification of the tenocytes by Sirius red staining after 14 days of culture in different culture media supplements. Collagen synthesized per cell was calculated as indicated in text. 10% FBS supplemented in α-MEM without growth factors was used as control group to compare with the groups with 0% FBS supplemented with different combinations of growth factors. Data were presented as means ± SEM.

*** p<0.05: in comparison with all the groups supplemented with 0% FBS (One-way ANOVA)**

§ p<0.05: in comparison with positive control group which supplemented with 10% FBS (One-way ANOVA)

Light Blue indicates the group treated with 10ng/ml IGF-1
Purple indicates the group treated with 50ng/ml IGF-1
Red indicates the group treated with 1ng/ml TGFβ-3
Orange indicates the group treated with 1ng/ml TGFβ-3 + 10ng/ml IGF-1
Light Green indicates the group treated with 10ng/ml TGFβ-3 + 50ng/ml IGF-1
Dark Blue indicates the group treated with 10% FBS

4.2.3 Messenger RNA expression of tenocyte phenotypic and differentiation markers

To demonstrate the specific phenotypic and differentiation markers of the tenocytes cultured in 0% FBS supplemented with IGF-1 and TGFβ-3, mRNA expression of human SCX, COL-I, TNMD and DCN were measured by real-time RT-PCR after 14 days in culture. All the data were normalised with respect to the in-house GAPDH controls and results of the treated groups were expressed as ratios to the mRNA expression of these tenocyte markers in positive control cultures (10% FBS). Furthermore, in order to ascertain whether the combination treatment of IGF-1 (50ng/ml) + TGFβ-3 (10ng/ml) were more effective than their sole treatment, the mRNA expression of SCX, COL-I, TNMD and DCN were compared to that noted for cultures treated with 50ng/ml IGF-1 or 10ng/ml TGFβ-3 alone.

As indicated in **Figure 4-5**, except for DCN expression, all other tenocyte mRNA expressions were significantly up-regulated in TGFβ-3 + IGF-1 combination treatment as compared to their respective control groups **(Figure 4-5A-C)**.

The expression SCX was significantly increased in tenocytes treated with TGFβ-3 (by 132 fold) and a combination of TGFβ-3 and IGF-1 (by 140 fold), as compared to positive 10% FBS treated groups ($p<0.05$) (**Figure 4-5A**). Similarly, TNMD mRNA expression was significantly up-regulated in TGFβ-3 (by 535 fold) and TGFβ-3 + IGF-1 (by 605 fold), as compared to control groups ($p<0.05$) **(Figure 4-5B)**. Moreover, COL-I mRNA expression was markedly increased in all treatment groups compared to controls where the maximum increase was only noted in cultures treated with the combination of TGFβ-3 + IGF-1 where there was a 5.2 fold increase in the mRNA expression of this marker **(Figure 4-5C)**.

Although DCN mRNA expression was elevated in TGFβ-3 + IGF-1 treated cultures, this increase was not comparable to that observed for IGF-1 or TGFβ-3 alone treated cultures **(Figure 4-5D)**.

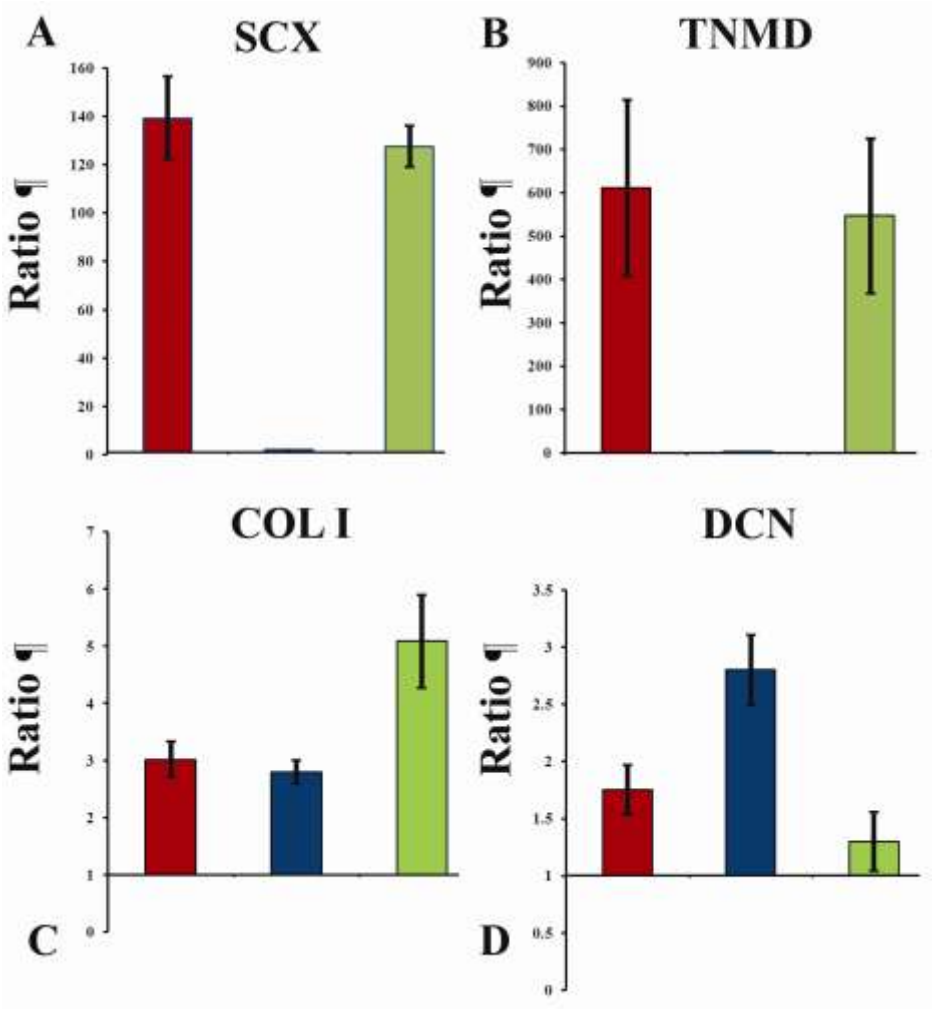

Figure 4-5: Messenger RNA expression levels of various tenocyte markers as determined by real time RT-PCR.
A: SCX, B: TNMD, C: COL-I, and D: DCN in tenocytes cultured in the presence of various growth factors over a 14-day culture period.
¶: The data were presented as means ± SEM ratio of mRNA expression of different markers to that of the control group (10% FBS). GAPDH mRNA was used to normalize the variability in template loading.

Red indicates the group treated with 10mg/ml TGFβ-3
Blue indicates the group treated with 50ng/ml IGF-1
Green indicates the group treated with 10mg/ml TGFβ-3 + 50ng/ml IGF-1

4.2.4 Sirius red staining of cell morphology

In order to determine the cell morphology of tenocytes cultured *in vitro* treated with 10ng/ml TGFβ-3, 50ng/ml IGF-1 or the combination of the two, as well as the 10% FBS (positive control group), cells grown on coverslips in 48-well plates for 14 days were stained with Sirius red for collagen synthesis and their morphologies were examined under a light microscopy (at ×200 magnification). Cells cultured in all of the treatment groups exhibited elongated, spindle-shaped, fibroblast-like appearances and no tenocyte phenotypic drift was noted in any of the treatment groups [63]. The positive control (10% FBS) treated groups exhibited more aggregated collagen layers which comprised of thick, dense parallel collagen fibrils as was evident by strong Sirius red staining **(Figure 4-6A)**. Similar collagen staining and cell morphology was noted in tenocyte cultures incubated in the absence of serum but in the presence of TGFβ-3 (10ng/ml) + IGF-1 (50ng/ml) **(Figure 4-6D)**. The cell alignment and the collagen fibrils morphology in these cultures was similar to that observed for the positive controls **(Figure 4-6A vs. D)**. Tenocytes treated with TGFβ-3 **(Figure 4-6B)** or IGF-1 **(Figure 4-6C)** alone did not exhibit this pattern of morphology and the Sirius red staining was far less prominent than positive control or combination treatment group.

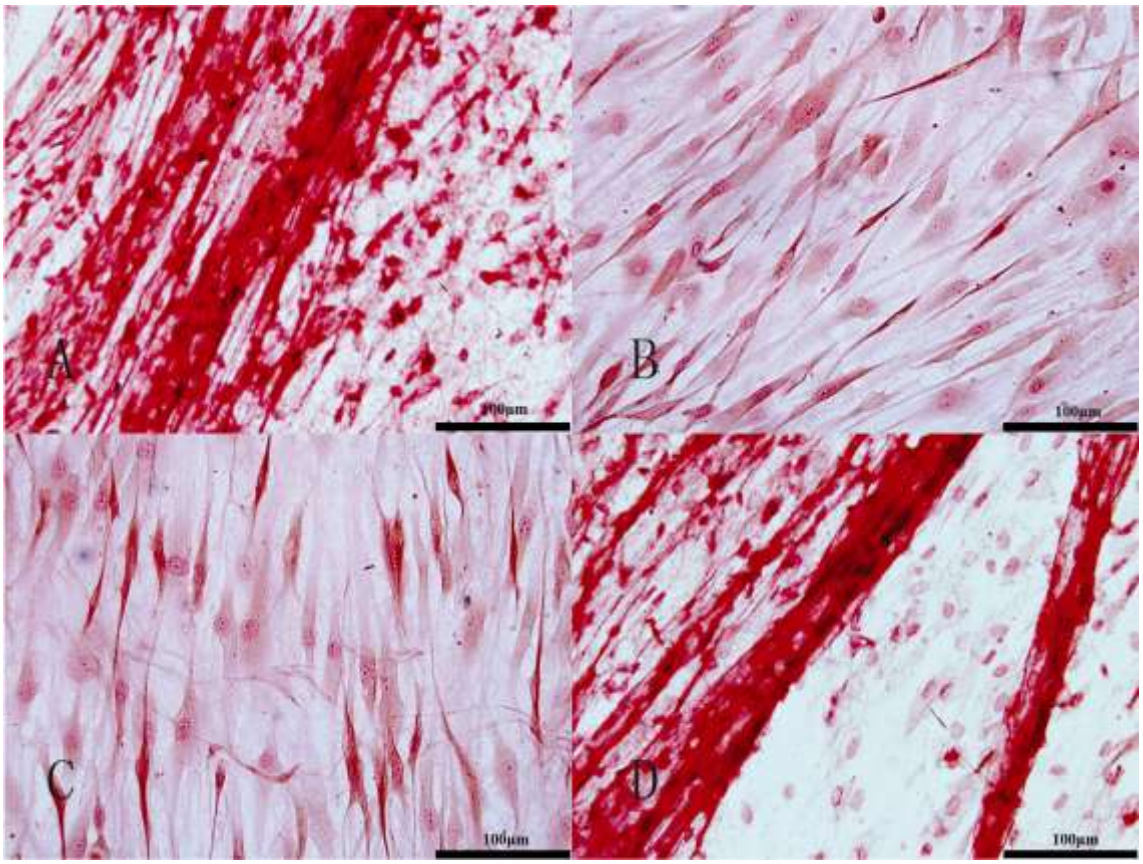

Figure 4-6: Sirius red staining of the tenocytes cultured 14 days with different culture media. (A) 10% FBS, (B) 0% FBS + 10ng/ml TGFβ-3, (C) 0% FBS + 50ng/ml IGF-1 and (D) 0% FBS + 10ng/ml TGFβ-3 + 50ng/ml IGF-1 (Magnification ×200, scale bar 100µm)

4.3 DISCUSSION

In the previous Chapter, it was demonstrated that it is possible to **expand** human tenocytes *in vitro* with 1% FBS supplemented with 50ng/ml $PDFG_{BB}$ and 50ng/ml bFGF, in a manner comparable to that observed with tenocytes cultured in 10% FBS alone. The present Chapter has demonstrated that the **differentiation** of tenocytes can be induced by treating cultures with a combination of 50ng/ml IGF-1 and 10ng/ml TGFβ-3 **in the**

absence of FBS. This work signifies, for the first time, that *in vitro* **human** tenocyte survival and **differentiation** can be achieved over a 14-day culture period, without the addition of any exogenous FBS. These findings could facilitate the use of this methodology for potential tendon tissue engineering.

To date, there has been only one other research group who had employed a similar experimental approach as that described in this Chapter. Costa *et al*. have demonstrated that IGF-1 alone or in combination with $PDGF_{BB}$ could be used to expand rabbit tenocytes *in vitro* [188]. Their results suggested that these two growth factors were capable of supporting **expansion** of rabbit tenocytes whilst the findings in this Chapter demonstrated that a combination of IGF-1 and TGFβ-3 were capable of supporting ***only*** the **differentiation** stage of human tenocytes in serum-free culture conditions. Such discrepancies could be explained by (1) the different species used in these studies [rabbit vs. human in the present investigation], (2) concentration of growth factors [IGF-1 100ng/ml vs. 50ng/ml], (3) different growth factors used in combination with IGF-1 [$PDGF_{BB}$ vs.TGFβ-3] and (4) duration of culture period [3 days vs. 14 days]. None of these investigators had reported on the expression of other tenocyte differentiation markers such as (SCX, TNMD, IGF-1 and DCN). Nonetheless, the present results do indeed substantiates their findings further despite the differences in the experimental approach employed.

The importance of TGFβ in tendon biology has been previously reported by a number of authors. For instance, Chang *et al.* have shown that TGFβ-1 mRNA expression is markedly increased in rabbit tendon injuries animal models [186]. Furthermore, it was reported by

Ngo *et al*. that an up-regulation of TGFβ receptors (I, II and III) mRNA expression in the rabbit tendon injury model [205]. In a different study, Pryce and his colleagues reported that TGFβ signalling plays a major role in murine tendon differentiation by increased expression of SCX in organ culture assays and they also reported that inactivating TGFβ receptors (II and III) can result in the loss of tendons and ligaments in the limb, trunk, tail and head [206]. Klein *et al*. have shown (1 or 5ng/ml) TGFβ-(1, 2 and 3) was capable of inducing collagen synthesis dose dependently by rabbit tenocytes *in vitro* over a 3-day period [207]. Such dose dependent effects of TGFβ observed by Klein *et al*. were not noted in the present Chapter with regards to collagen protein synthesis.

Shukunami and his colleagues documented that murine TNMD expression was positively regulated by SCX [111], a finding which is consistent with that reported in this Chapter. In the present investigation reported herein, it was demonstrated that TGFβ-3 can significantly up-regulate SCX mRNA expression (by 100 fold) in human tenocytes and that this expression was most prominent in TGFβ-3 treated groups as compared to positive controls. In support of findings by Shukunami *et al*., newly differentiated tenocytes in the present study also exhibited increased expression of TNMD mRNA (by 400 fold) in TGFβ-3 treated cultures.

As for the involvement of IGF and its family in tendon biology, IGF-1 (mRNA and protein) has been documented to be expressed in normal uninjured avian epitendon extracts [152]. These authors demonstrated that IGF-1 can stimulate avian tenocyte mitogenic activity (DNA synthesis) and collagen synthesis *in vitro* [152]. They also reported that this stimulatory effect of IGF-1 could be inhibited by treating the avian epitendons with anti-

IGF-1 antibodies indicating that this growth factor was the responsible mediator for the extracts' mitogenic activity [152]. The present findings are partly in agreement with that of Abrahamson and his colleagues [156] who reported that IGF-1 is a potent stimulator of rabbit tenocyte differentiation as determined by the increased collagen synthesis reported herein. This increased collagen synthesis in response to IGF-1 was also mirrored by the elevated mRNA expression of COL-I. In contrast, the proliferation results of human tenocytes in response to IGF-1 in this Chapter are not in agreement with those reported by Abrahamson *et al*., who documented that IGF-1 could also promote rabbit tenocyte proliferation [156]. This discrepancy could have been due to a number of factors, namely species type and concentrations of IGF-1. In the report by Abrahamson *et al*., rabbit tenocytes were exposed to higher doses of IGF-1 (10-250ng/ml) [156].

It is worth noting that, the combination treatment of tenocytes with IGF-1 and TGFβ-3 resulted in a significantly more COL-I mRNA expression compared to either treatment alone, suggesting that although IGF-1 or TGFβ-3 are suitable inducers of COL-I expression, the response is significantly enhanced when human tenocytes are exposed to **both** growth factors.

In the present study, one anomaly which cannot be explained is the increased expression of DCN by human tenocytes treated with 50ng/ml IGF-1. This was unexpected and was not observed in cultures treated with IGF-1 and TGFβ-3. Although DCN is thought to be involved in the stabilization and alignment of collagen fibrils during fibrillogenesis [118-124], its elevated mRNA expression in response to IGF-1 warrants further research which is out of scope of this report.

Another anomaly is the elevated mRNA expression of the COL-I which was not consistent with the results of the collagen synthesis. Here, it was revealed that mRNA expression of COL-I in combination treatment of human tenocytes with IGF-1 and TGFβ-3 was 5 fold higher than that observed in 10% FBS cultures, although the total collagen protein synthesis was markedly lower than that in positive control cultures (10% FBS). This could be due to an increase in degradation of the protein or the reduced turnover of collagen mRNA.

In conclusion, the results so far have suggested that the combination of 10ng/ml TGFβ-3 and 50ng/ml IGF-1 without the presence of serum, not only can maintain cell survival over a 14-day period but also can induced tenocyte **differentiation** in a defined culture condition. These findings could be used for the second stage of tendon tissue engineering where cell **differentiation** is desirable after the cell **expansion** phase has been completed.

4.4 SUMMARY

- The results herein have shown that the combination of 50ng/ml IGF-1 and 10ng/ml TGFβ-3, without the presence of serum, not only maintained cell survival over a 14-day culture period but also induced tenocyte **differentiation** *in vitro* for tendon tissue engineering purposes.
- One of the clear benefits of the culture conditions used in this study is the capacity to promoted tenocyte differentiation and survival over a longer period (14 days) than has been reported previously in serum free conditions. This is of obvious benefit for future work on producing tenocytes for clinical use..
- In conjunction with the data from the previous Chapter where it was indicated that 50ng/ml $PDGF_{BB}$ and 50ng/ml bFGF supplemented in low serum in culture medium can promote 2-D tenocyte **expansion** *in vitro* without **differentiation**, the present results could be successfully employed for any subsequent 2-D and 3-D experiments in the following Chapters.
- Next Chapter will focus on the findings of the **sequential** application of the optimized human tenocyte culture conditions described so far in 2-D and 3-D *in vitro* culture system.

CHAPTER FIVE:

IN VITRO 2-D AND 3-D TENOCYTE CULTURE WITH SEQUENTIAL APPLICATION OF GROWTH FACTORS

In previous Chapters, the optimal culture conditions for tenocyte **expansion** and **differentiation** were investigated. The findings suggested that, in order to maximize the tenocyte cell number with minimal concentrations of FBS, combination treatment of 1% FBS + 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF was the optimal culture condition required for this purpose (see **CHAPTER 3** for details). In order to achieve the optimal tenocyte **differentiation** post **expansion** phase, out of a number of growth factors and concentrations, TGFβ-3 (10ng/ml) and IGF-1 (50ng/ml) were found to be the most suitable growth factors (see **CHAPTER 4** for details). The present Chapter is to ascertain whether the findings established in **CHAPTER 3** and **4** could be implemented for 2-D or 3-D human tenocyte cultures *in vitro*. Furthermore, the mechanical properties of the 3-D construct will also be investigated in this Chapter.

5.1 EXPERIMENTAL DESIGN

5.1.1 Two Dimensional Culture of Tenocytes

Tenocytes ($5\times10^3/cm^2$) were seeded in 48 well plates and 10 cm^2 culture dishes. The cells were cultured with α-MEM media supplemented with 1% FBS + 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF for 14 days **(expansion phase)** and for a further 14 days in serum free α-MEM media supplemented with 10ng/ml TGFβ-3 + 50ng/ml IGF-1 **(differentiation phase)**. Tenocytes cultured with α-MEM + 10% FBS were used as positive controls whilst those supplemented with 0% and 1% FBS were considered as negative control groups. The treatment and control groups were cultured for a total of 28 days, during which the culture media were replenished twice per week. AlamarBlue™ assays were performed on

tenocytes cultured in 48 well plates after various time points; within 1, 7, 14 days **(expansion phase)** and within 21 and 28 days in culture **(differentiation phase)**. Six replicates were used for each treatment per time point and the means ± SEM were evaluated. In order to determine any morphological changes in the tenocytes during this culture period, three replicates for treatment and positive control groups were set up and observed in $10cm^2$ petri-dishes. Evidence of any significant changes at any time point was photographed using DMC-LX2 digital camera (Panasonic, Japan). As there were no or little changes noted during the **expansion phase**, (1, 7 and 14 days), macroscopic alterations in tenocytes were only noted after 21 and 28 days in culture, i.e. during the **differentiation phase**.

5.1.2 Three Dimensional Culture of Tenocytes

In order to establish a 3-D culture condition for human tenocytes for tendon tissue engineering, some findings using degummed silk scaffold from a colleague undertaking a DPhil research programme* in the NDORM department were used for this section.

As degummed (Sericin free) *Bombix* silk has been shown to promote tenocyte attachment *in vitro* with minimal inflammatory reaction *in vivo**, in the present study, 300 single stranded degummed *Bombix* silk fibres with a total surface area of $2cm^2$ were employed. Silk strands were grouped by tying both ends using an extra silk strand **(Figure 5-1)** and were placed in 15ml centrifuge tubes. Tenocytes in α-MEM were added at 1×10^4 total cells to every tube. The treatment group consisted of tenocytes with degummed silk incubated with 10ml of the **expansion** media (1% FBS + 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF) for 14 days after which it was replaced with the **differentiation** media (0% FBS + 10ng/ml

* SILK AS A BIOMATERIAL FOR TENDON REGENERATION. By Xiao Wang, DPhil Candidate

TGFβ-3 + 50ng/ml IGF-1) and cultured for a further 14 days. The culture media were replenished twice per week as described in **Section 5.1.1**. For control tubes, degummed silk were either cultured in the presence of tenocytes in 10% FBS or in the absence of cells. Six replicas were used in each treatment and the culture media ± growth factors were replenished twice per week. The *constructs* were examined using DMC-LX2 digital camera (Panasonic, Japan) after 7, 14, 21 and 28 days in culture. In order to determine and compare the structure of engineered constructs, human hamstring tendons were selected as positive control group and each were subjected to (1) H&E histological examination, (2) COL-I expression using immunohistochemistry and (3) scanning electron microscopy assays (for detailed methodologies, refer to **CHAPTER 2, Section 2.6 and 2.5**, respectively and **Appendices D-F**) after 7, 14, 21 and 28 days in culture. The mechanical properties of the constructs were determined after 28 days in culture and compared with human hamstring tendons in a method as described in **CHAPTER 2, Section 2.7**.

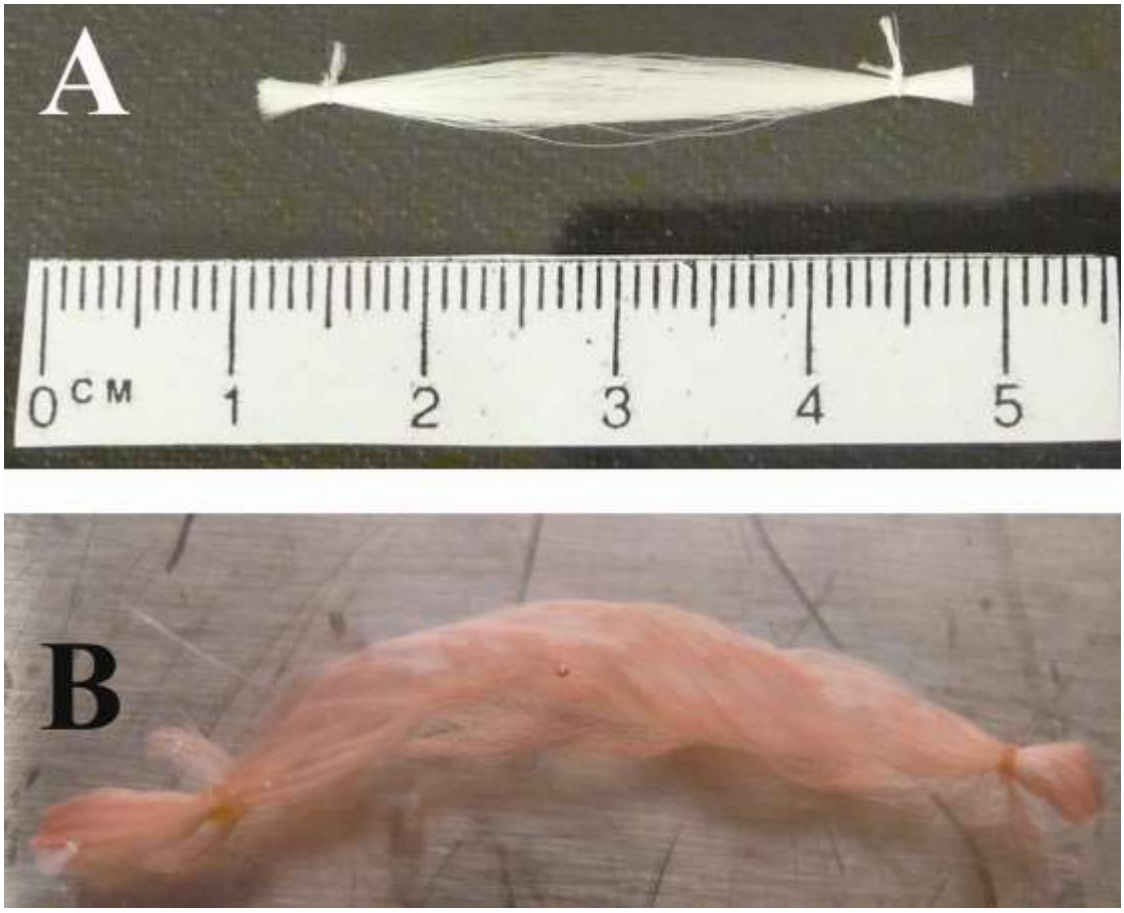

Figure 5-1: Photo micrograph of dried (A) and α-MEM-soaked (B) degummed *Bombix* Silk bunch used for the 3-D culture conditions.

5.2 RESULTS

5.2.1 Two Dimensional Tenocyte Construction

Tenocytes cultured continuously with α-MEM supplemented with 10% FBS (control group) in 48 well plates for 28 days showed a rapid cell number increase from day 1 to day 7 which was approximately 5 fold increase compared to the cell number observed on day 1. After 14 days in culture, the tenocyte numbers showed a significant decrease ($p<0.05$) compared to day 7 but with a 3.5 fold increase in cell number compare to day 1 ($p<0.05$).

There were no significant changes in tenocyte cell number after 14 days and compared to 21 and 28 days in culture ($p>0.05$) **(Figure 5-2, Green line)**.

On the other hand, tenocytes incubated with the **expansion** media (1% FBS + 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF) exhibited a slower but steadier increase in cell number from day 1 to day 14 in culture **(Figure 5-2, Purple line)**. In contrast to the positive controls, the tenocyte cell numbers were significantly decreased ($p<0.05$) after 14 days followed by a further reduction within 21 and 28 days. In 10% FBS-treated cultures, this significant decline in cell numbers was not noted at the same time points **(Figure 5-2, Purple line vs. Green line)**. Tenocytes cultured in 1% FBS or serum free media were not capable of expansion after 14 and 7 days incubation, respectively.

In treated 48-well plates containing **expansion** and **differentiation** media, a collagen-like tissue was observed after 28 days which warranted further investigation. To ascertain the nature of this collagen-like "construct", tenocytes were re-cultured in $10cm^2$ petri-dishes in a similar fashion described for 48 well plates. The presence of a collagen-like ring and mass was noted after 21 and 28 days culture, respectively in the treatment group **(Figure 5-3 A and B)**. This observation was not noted in cell cultures in the presence of 10% FBS after 21 or 28 days **(Figure 5-3)**. Interestingly, culturing of tenocytes for significantly longer period (up to 45 days) in 10% FBS did not promote the formation of such collagen-like masses.

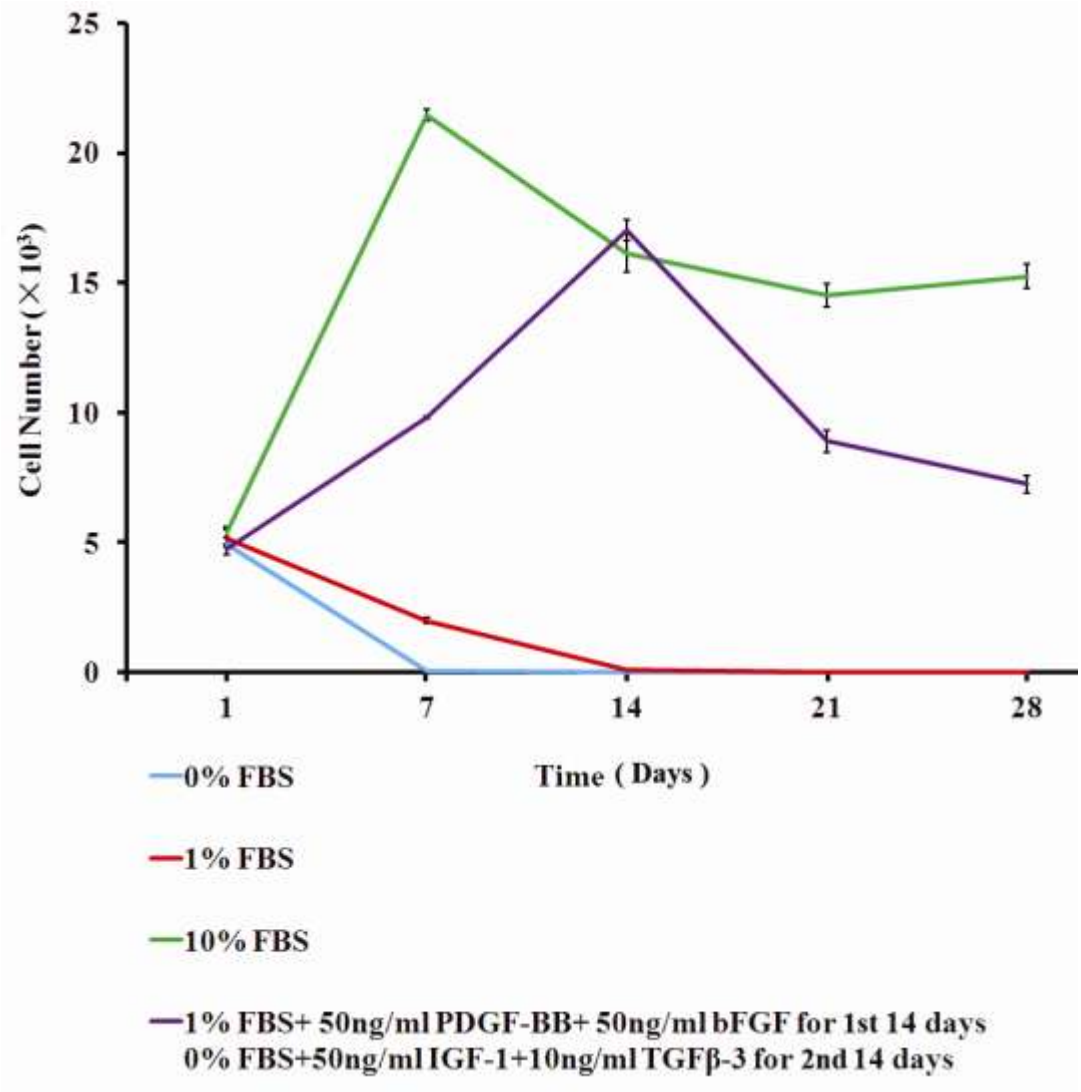

Figure 5-2: Cell proliferation as determined by AlamarBlue™ assay of tenocytes cultured for 28 days in α-MEM supplemented with various concentrations of FBS (0%, 1%, 10%) and a combination of 1% FBS supplemented with 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF for the first 14 days followed by a further 14-day incubation with 10ng/ml TGFβ-3 and 50ng/ml IGF-1, in the absence of FBS (Purple line). Data were presented as means ± SEM.

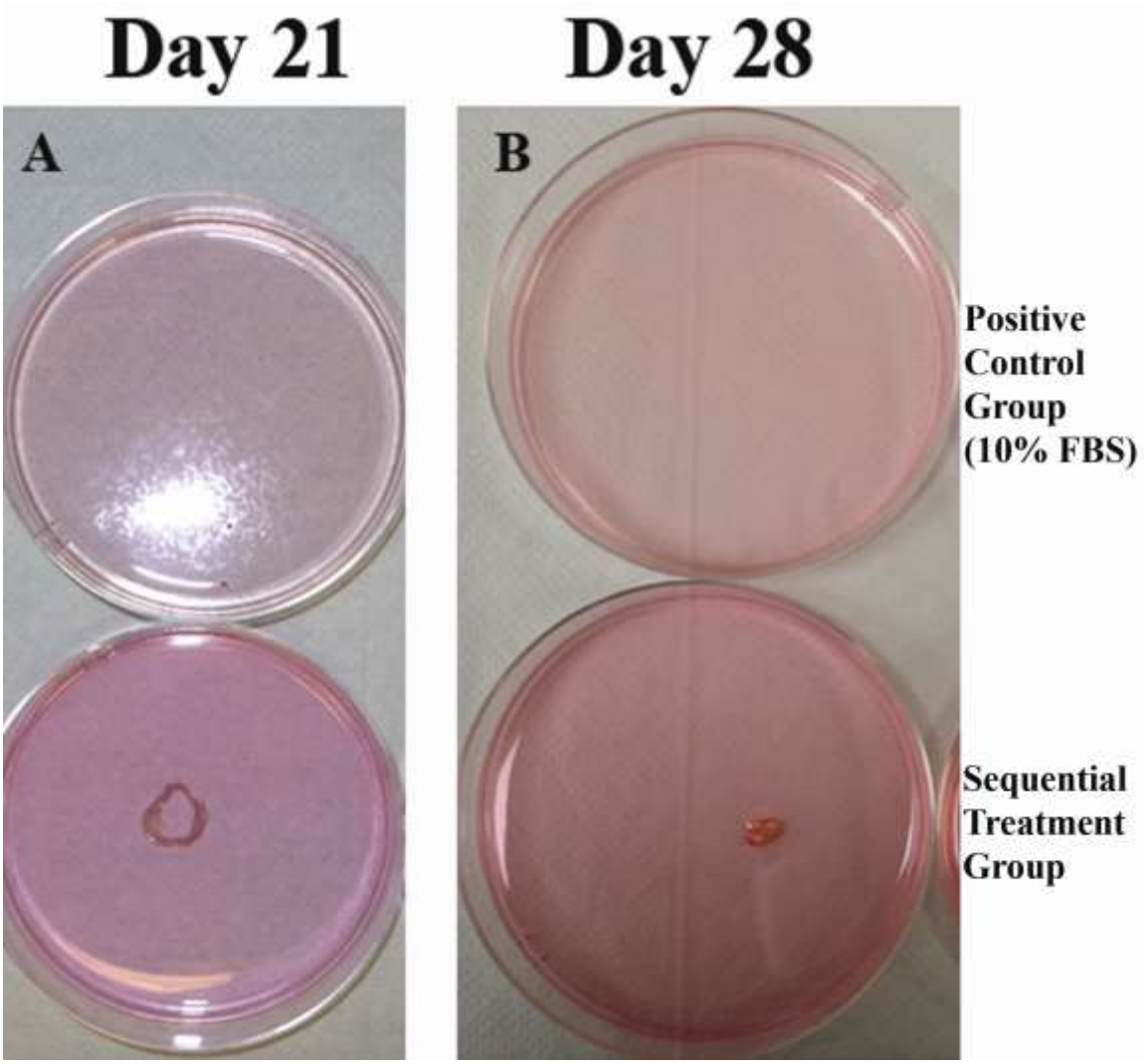

Figure 5-3: Tenocytes cultured in 10 cm culture dishes with α-MEM supplemented with 10% FBS (above panel) and sequential application of the growth factors (1% FBS + 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF for 14 days and for a further 14 days in serum free α-MEM media supplemented with 10ng/ml TGFβ-3 + 50ng/ml IGF-1, panel below) for (A) 21 days and (B) 28 days.

In order to determine whether collagen was present in such mass, cryosectioning of the "constructs" formed after 21 and 28 days were undertaken and sections were stained using Sirius red (for detailed methodology, refer to **CHAPTER 2, Section 2.3**). Human

hamstring tendon was employed as a positive control for this observational study. As indicated in **Figure 5-4**, "constructs" were enriched with collagen as indicated by dark red staining (**Figure 5-4A and B**). The extent of staining was comparable to that observed for human hamstring tendon **(Figure 5-4C)**.

Although no definitive evidence has been provided, the collagen-enriched mass formed in two dimensional cultures in response to **expansion** and **differentiation** media exposure, did not exhibit structured alignment as one would expect with healthy tendons.

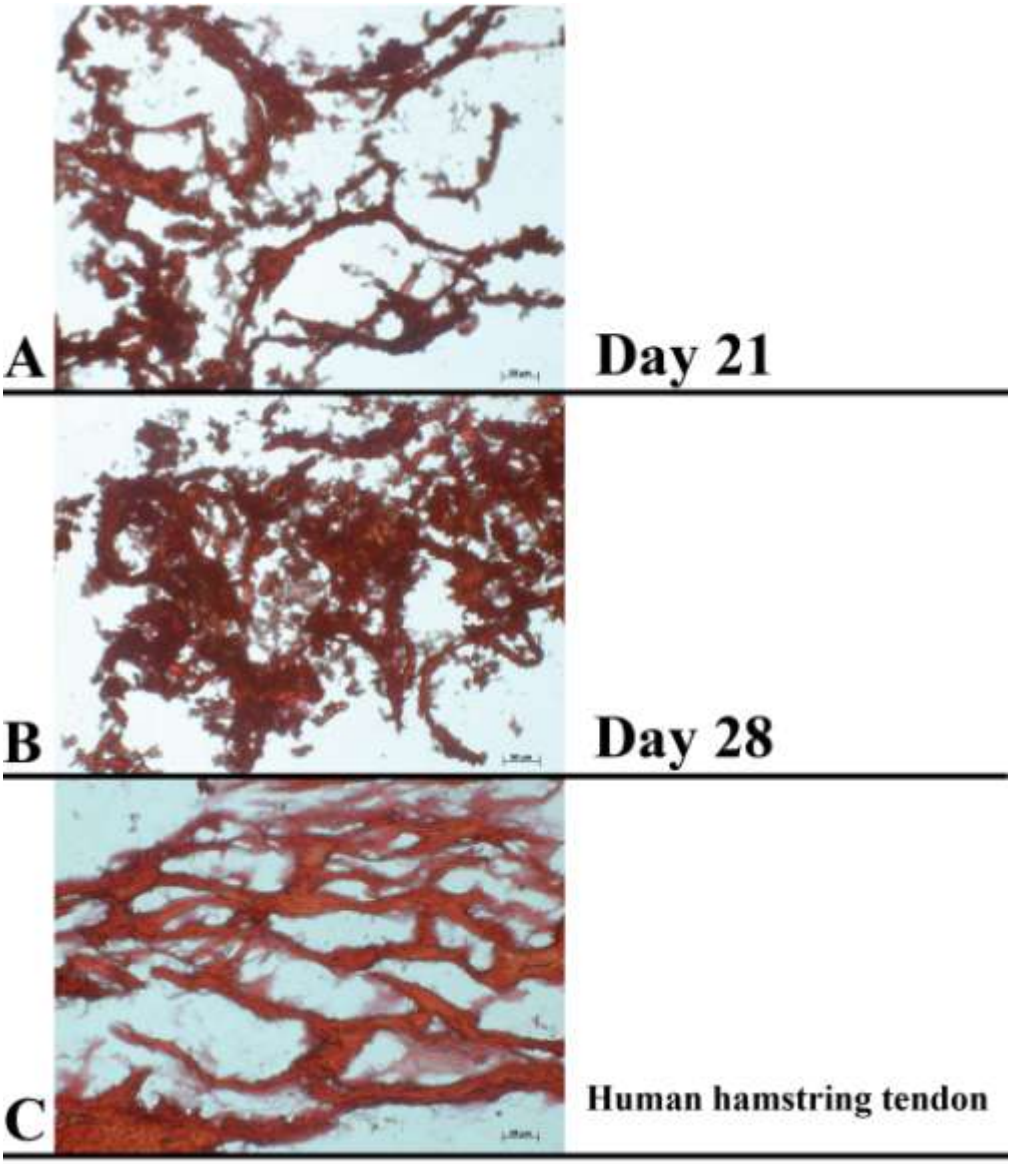

Figure 5-4: Sirius red staining of the 5µm cryosections of the construct formed in 2-D culture on (A) day 21 and (B) day 28. Human hamstring tendon (C) was used as positive control. (Magnification ×200, scale bar 50µm)

5.2.2 Three Dimensional Tenocyte Construction

The morphology of human tenocytes cultured on degummed silk, as a scaffold, in the presence of **expansion** and **differentiation** media for different culture period were

macroscopically similar to that noted in 10% FBS supplemented media **(Figure 5-5)**. Macroscopically, on days 7 and 14, the control group showed markedly increased collagen formation on the silk strands compared to the experimental group, as the experimental group exhibited a "looser structure". However, in 21 and 28 day cultures, the appearance of tenocyte-enriched silk constructs in the treated group was more compact and the silk strands appeared to be more bound together as compared to the control group (10% FBS) **(Figure 5-5 H vs. G)**.

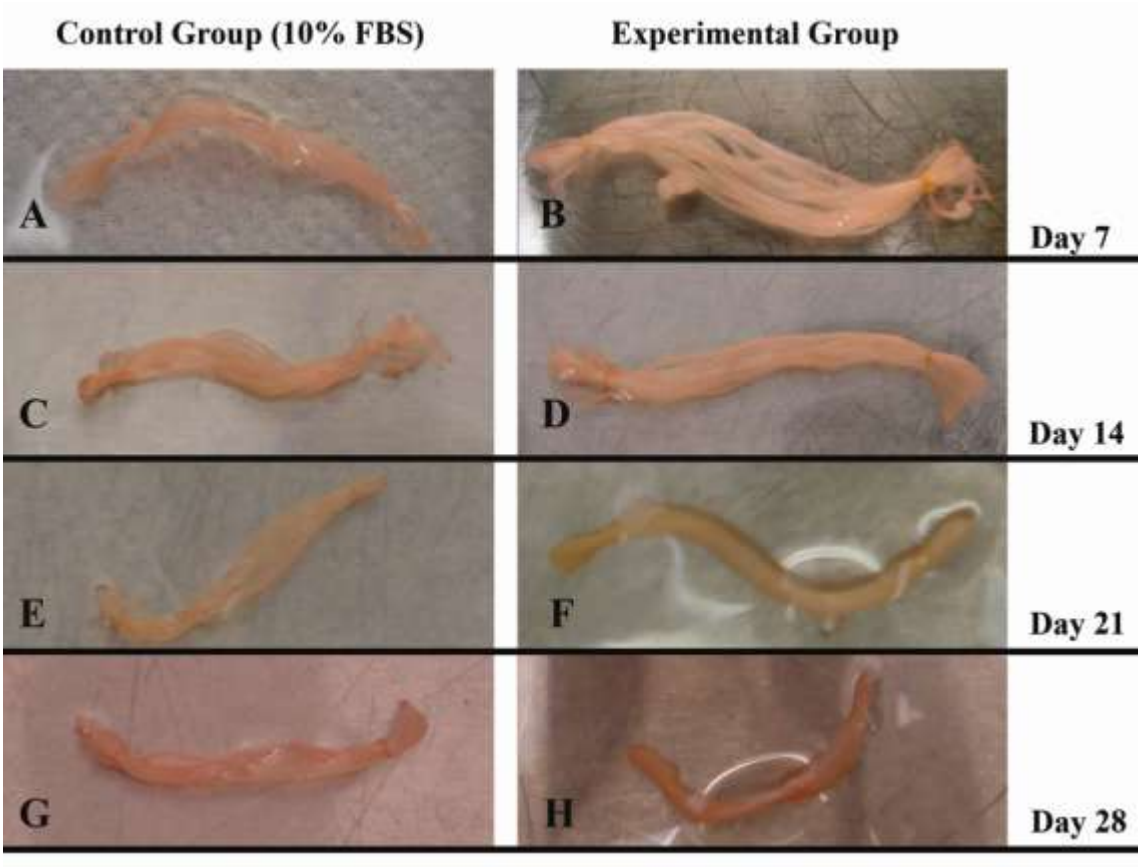

Figure 5-5: Tenocytes were cultured with degummed *Bombix* silk for up to 28 days. Left panel (A, C, E and G) and right panel (B, D, F and H) represent tenocytes enriched silk cultured in the presence of 10% FBS or sequential culture media (expansion + differentiation phases), respectively. Different time points were investigated for each treatment and this included 7-day (A and B), 14-day (C and D), 21-day (E and F) and 28-day (G and H) post incubation.

To evaluate these macroscopic observations, the constructs were examined microscopically by H&E staining (for detailed methodology, refer to **CHAPTER 2, Section 2.6.3**), and their collagen type I content was assessed immunohistochemically (for detailed methodology, refer to **CHAPTER 2, Section 2.6.4**).

The H&E staining of the "3-D constructs" indeed revealed that in 21 and 28 days cultures of the experimental group, the cellular pattern was similar to that observed with healthy hamstring tendon and that noted for cultures in 10% FBS **(Figure 5-6)**.

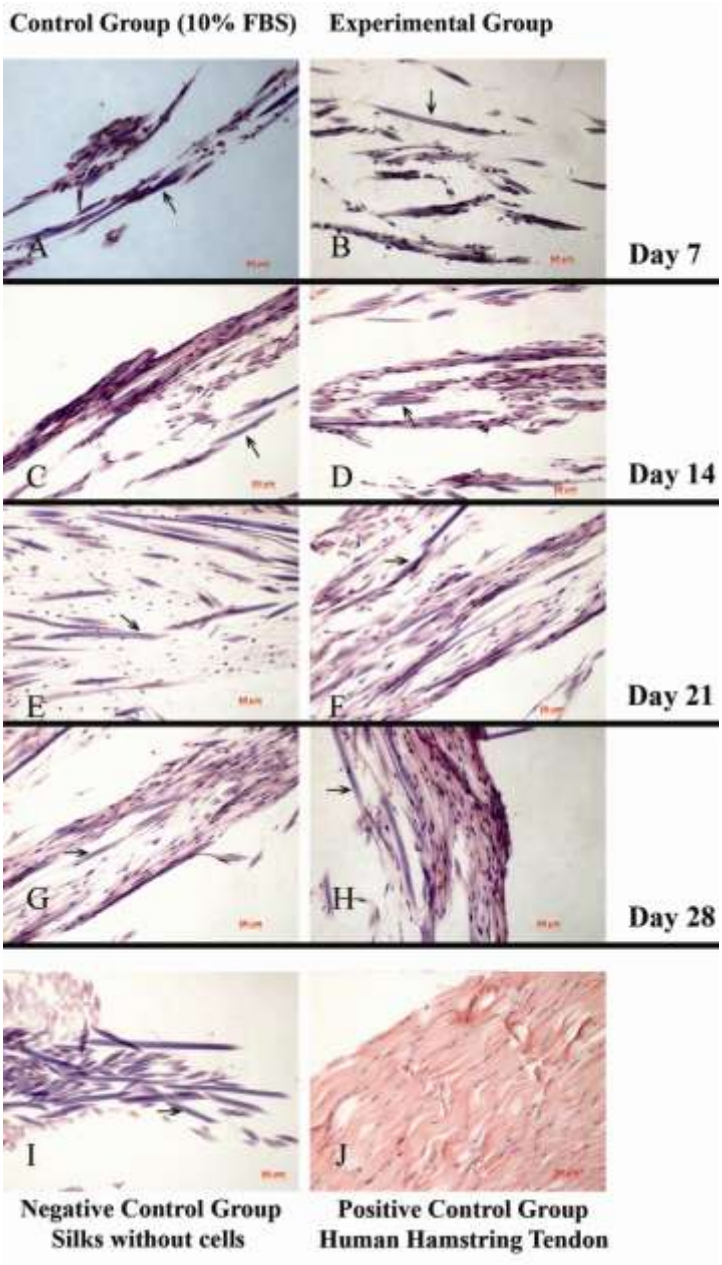

Figure 5-6: H&E staining of the paraffin embedded sections of the 3-D cultured constructs at different time points. α-MEM supplemented with 10% FBS were used as control groups (left panel) and the microstructures of the constructs were examined on day 7(A), 14(C), 21(E) and 28(G). Sequential application of the growth factors to the culture media were used as experimental groups (right panel) and the microstructures of the constructs were examined on day 7(B), 14(D), 21(F) and 28(H). Degummed *Bombix* Silks cultured without cells were used as negative control (I). Human hamstring tendon was used as positive control (J) (Magnification ×200, scale bar 50μm). Arrows indicate degummed *Bombix* silks.

Similar results were also obtained when the presence of collagen type I was detected immunochemically with these "3-D constructs" **(Figure 5-7)**. The data presented in **CHAPTER 3** where it was noted that differentiation of the tenocyte was regulated by 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF (**expansion** phase, 1-14 days) was indeed confirmed in "3-D constructs" assays. As indicated in treated tenocytes cultured with 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF for 14 days, **the absence of tenocyte differentiation and collagen synthesis** was clearly evident in **(Figure 5-6 D and 5-7 D)**.

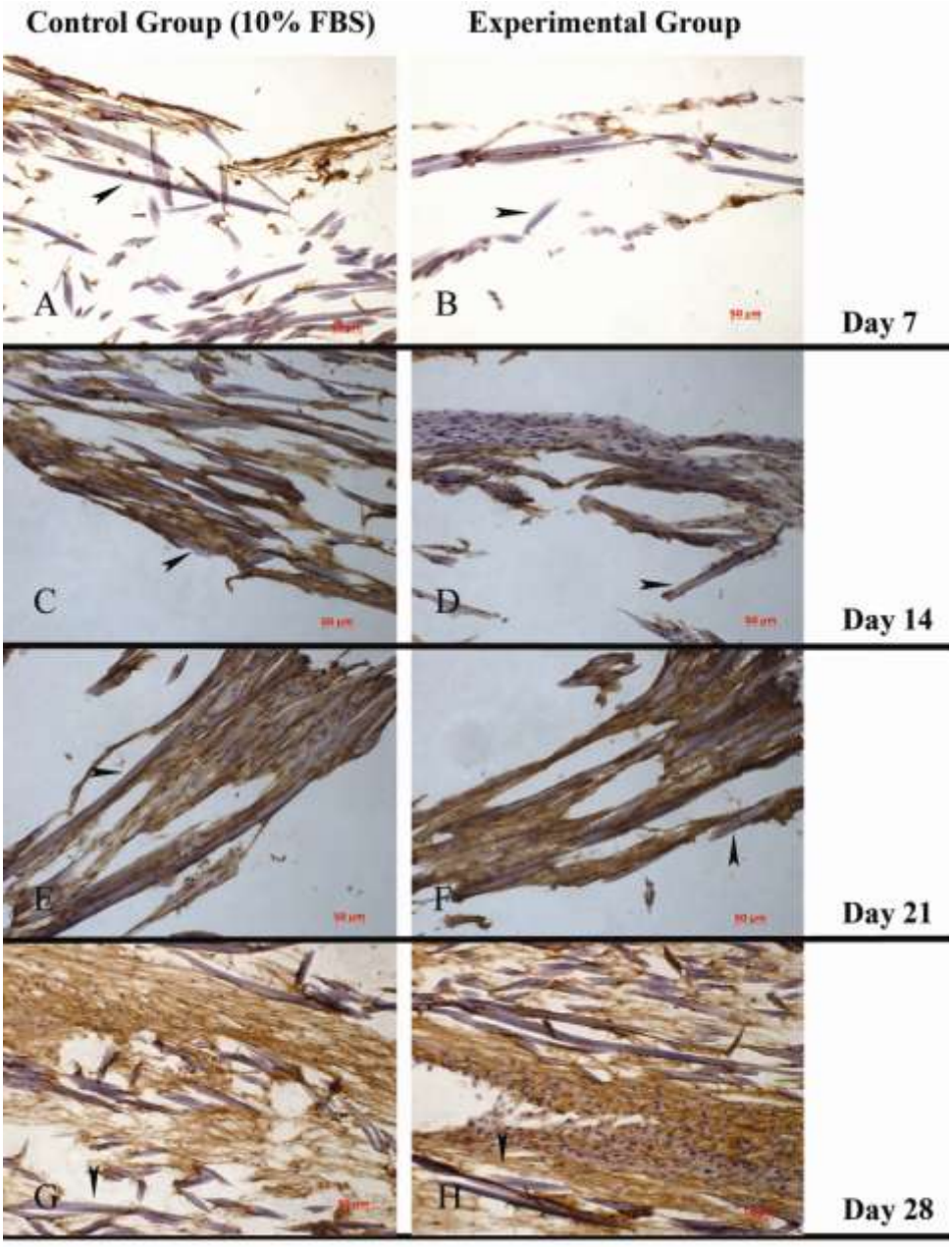

Figure 5-7: Immunohistochemical staining for human COL-I staining using the paraffin embedded sections of the 3-D cultured constructs at different time points. α-MEM supplemented with 10% FBS were used as control group (left panel) and the microstructures of the constructs were examined on day 7(A), 14(C), 21(E) and 28(G). Sequential application of the growth factors to the culture media were used as experimental groups (right panel) and the microstructures of the constructs were examined on day 7(B), 14(D), 21(F) and 28(H). (Magnification × 200, scale bar 50µm). Arrow heads indicate degummed *Bombix* silks.

To ascertain the tendon-like structure formed on degummed silk, in "**expansion**/**differentiation**" culture media, scanning electron microscopy was conducted on these constructs and compared with human hamstring tendons (for detailed methodology, refer to **CHAPTER 2, Section 2.5**). Initially, the samples were examined macroscopically as indicated in **Figure 5-8**. For day 1 to day 14, both groups of silks showed similar patterns of cell adherence and attachment as the loose strands of silk became more aggregated and began to attach to each other. The control group showed more collagen synthesis than the experimental group (**Figure 5-8, A B C D**). On day 21, the constructs of the experimental group showed a significant difference in appearance to the control group as much thinker and denser collagen was coated around the silk bundle and the loose silk strands became combined as one piece (**Figure 5-8 F**). The control group also showed increased collagen formation on the silk but still loose silk strands could be observed (**Figure 5-8 E**).

On day 28, the experimental group showed even denser collagen formation than on day 21 (**Figure 5-8 H**) and the control group also had thicker and denser collagen formation on the silk (**Figure 5-8 G**). There were no changes observedwith the negative control group after 28 days (**Figure 5-8 J**). After 28 days of culture, both experimental and control groups showed similar general appearance which resembled the natural tendon.

The ultra-structure of the tendon-like constructs around the degummed silk after 28 days in treated groups was similar to that observed with healthy human hamstring tendon **(Figure 5-9H vs. I, respectively)**.

Once again, results obtained in **CHAPTER 3** and **4** where the **expansion** and the **differentiation** phases of the tenocytes were determined *in vitro*, can be reconfirmed by the ultra-structure of the "tendon constructs". As indicated in **Figure 5-9D**, the cell differentiation on silk in the presence of 1% FBS + 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF is markedly less pronounced than that in 10% FBS **(Figure 5-9C)**. Similarly, findings in **CHAPTER 4** and the 2-D culture results reported in this Chapter **(Section 5.2.1)** are further substantiated and illustrated in **Figure 5-9F**, where the differentiation of the tenocytes surrounding degummed silk strands is more pronounced in the presence of 0% FBS + 10ng/ml TGFβ-3 + 50ng/ml IGF-1 culture media after 21 days (which represents 7 days after the initiation of the **differentiation** phase).

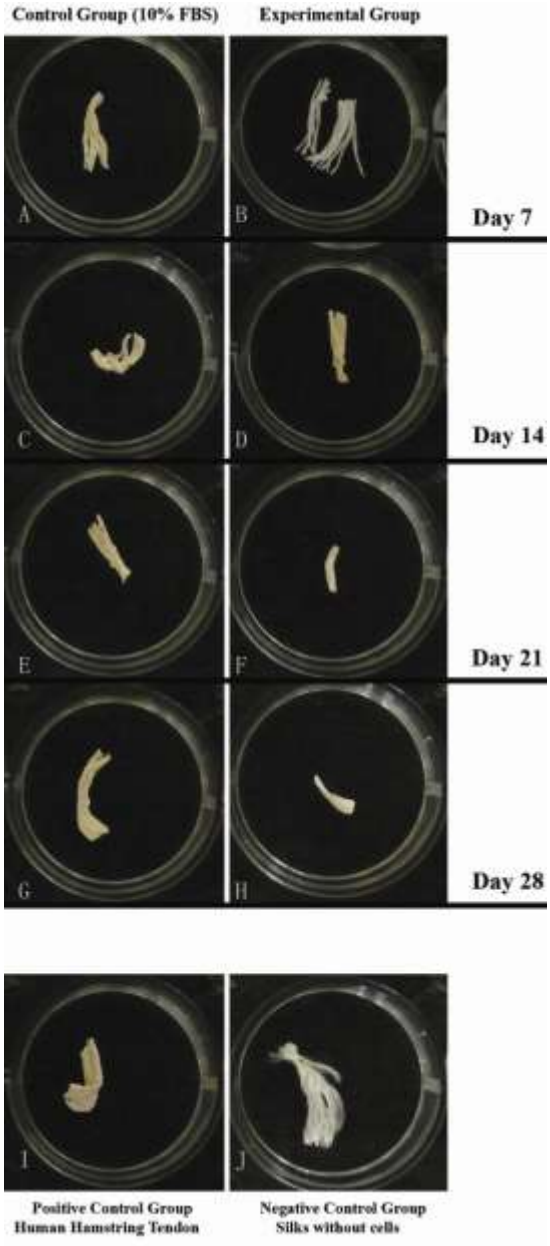

Figure 5-8: Macroscopic comparison of the 3-D cultured constructs at different time points. Samples were fixed in 4% glutaraldehyde and going through a series of dehydration before processing for scanning electron microscopy. α-MEM supplemented with 10% FBS were used as the control group (left panel) whereas sequential application of the growth factors in the culture media were used as the experimental group (right panel). As indicated, the constructs were examined on days 7(A & B), 14(C & D), 21(E & F) and 28(G & H). Human hamstring tendon was used as the positive control (I) whereas degummed *Bombix* silks cultured without cells were used as the negative control (J).

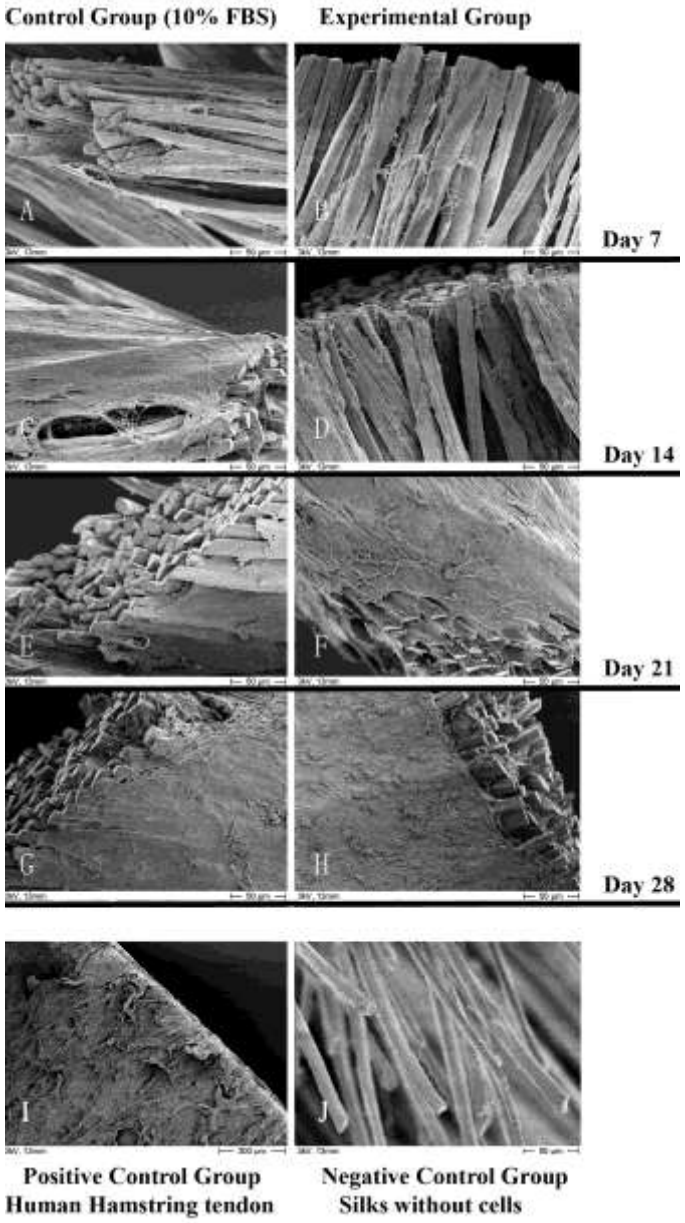

Figure 5-9: Scanning electron microscopy (SEM) pictures of the engineered constructs at different time points. α-MEM supplemented with 10% FBS were used as control groups (left) and the microstructures of the constructs were examined on day 7(A), 14(C), 21(E) and 28(G). Sequential application of the growth factors to the culture media were used as experimental groups (right) and the microstructures of the constructs were examined on day 7(B), 14(D), 21(F) and 28(H). Degummed *Bombix* Silks cultured without cells were used as negative control (J) (Magnification ×500, scale bar 50μm). Human hamstring tendon was used as positive control (I) (Magnification ×100, scale bar 300μm)

5.2.3 Mechanical properties of the 3-D construct

The mechanical properties of the 3-D constructs were examined using the BOSE EnduraTec ELF 3200 DMA system, as described in detail in **CHAPTER 2, Section 2.7**. The results indicated that the ultimate tensile stress (defined as the maximum stress a material can withstand when subjected to tension, compression or shearing) for 3-D degummed silks enriched with tenocytes and growth factors was similar to that observed with control 3-D silk constructs containing tenocytes cultured with 10% FBS (**Figure 5-10A**, Blue bar vs. Red bar). Furthermore, the results have indicated that long term cultures of silks samples in 10% FBS does not affect the tensile stress as illustrated in (**Figure 5-10A**, Green bar). Interestingly, the tenocytes 3-D cultures in the presence of growth factors (**Figure 5-10A**, Blue bar), revealed a significantly higher ultimate tensile stress compared to human hamstring tendon (*$p<0.05$, One-way ANOVA) (**Figure 5-10A**, Orange bar). Similar results were also obtained when examining the Young's modulus (defined as a measure of the stiffness of an isotropic elastic material) of the 3-D constructs and the human hamstring tendon **(Figure 5-10B)**.

Moreover, the data presented in **Figure 5-10C**, also suggests that the breaking point strain (defined as the change in the metric properties of a continuous body in the displacement from an initial placement to a final placement) was remarkably similar for all experimental (3-D silk constructs with tenocytes ± growth factors), control samples (i.e. 3-D constructs in the absence of tenocytes) and human hamstring tendon.

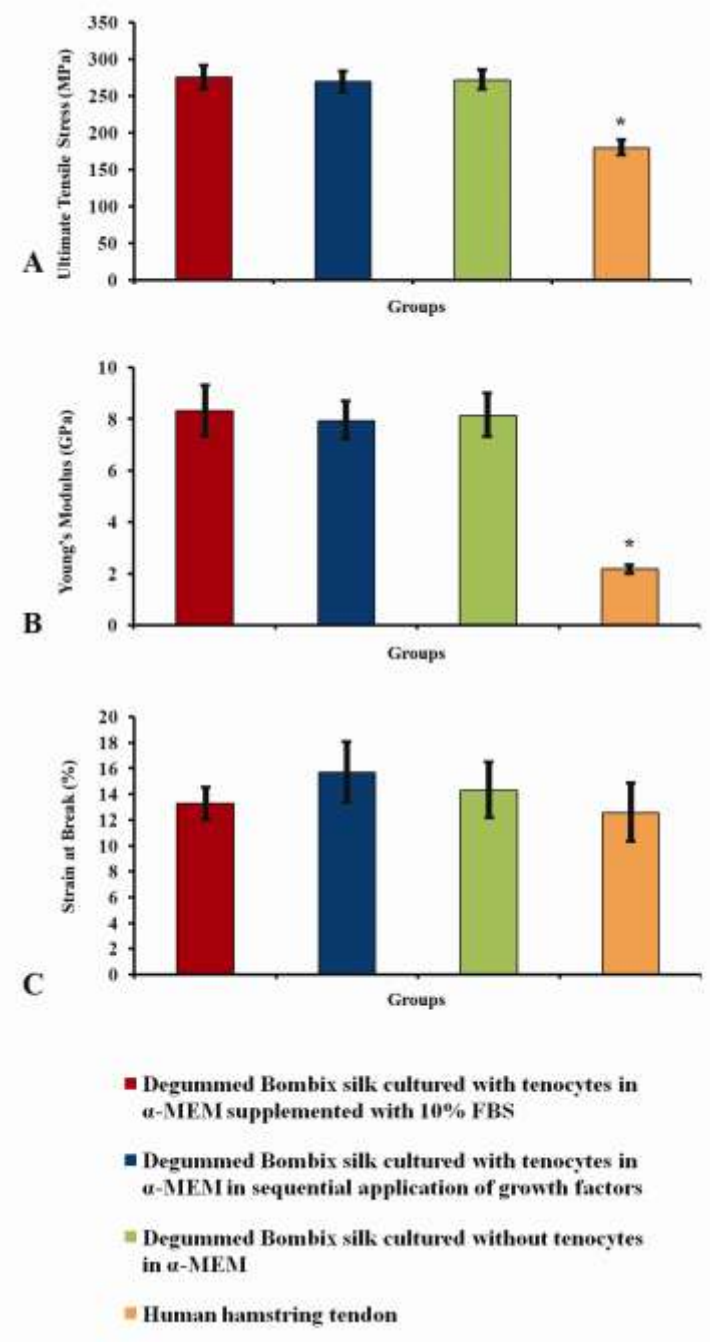

Figure 5-10: Mechanical properties of the degummed *Bombix* silk cultured with tenocytes in different culture media. Degummed *Bombix* silk cultured with sequential application of growth factors were used as experimental group; cultured with α-MEM supplemented with 10% FBS were used as control group; cultured without tenocytes were used as negative control group; human hamstring tendons were used as positive control group. (A) Ultimate Tensile Stress, (B) Young's Modulus, (C) Strain at Break; * $p<0.05$: in comparison to other groups, One-way ANOVA.

5.3 DISCUSSION

The main objective of this Chapter was to ascertain whether the data obtained so far for the **expansion** and **differentiation** phases can be further extrapolated from 2-D to 3-D culture conditions. The results have indeed indicated that the culture conditions for **expansion** phase (i.e. 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF for 14 days) followed by a further 14-day incubation in **differentiation** media (i.e. 10ng/ml TGFβ-3 + 50ng/ml IGF-1) was sufficient to allow human tendon regeneration in 2-D and 3-D (in the absence and presence of silk scaffold, respectively). The advantage of this novel method to 10% FBS supplemented culture was that the process could be separated into two isolated stages where each stage has a specific function (i.e. **expansion** or **differentiation**), and hence one is able to regulate and control each stage if necessary.

The results of both 2-D and 3-D culture showed that, instead of conventional tissue culture methods which use FBS as a tenocyte proliferation and differentiation stimulator, the experiment procedure can be divided into two isolated steps by utilizing specific combination of growth factors.

The results presented in this Chapter not only showed that this method is superior to the conventional FBS method, but also confirmed the findings in **Chapter 3** and **4** by SEM, H&E staining and COL-I immunohistochemistry. These results showed that (1) the tenocyte **differentiation** in the cell **expansion** stage was indeed inhibited and (2) the less differentiated tenocytes which were expanded in the cell **expansion** stage can regain their differentiation potential after the combination treatment of TGFβ-3 and IGF-1.

Furthermore, this specific sequential treatment of tenocytes, i.e. first 14 days in 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF followed by a further 14 days incubation in 10ng/ml TGFβ-3 + 50ng/ml IGF-1, was the most suitable treatment combination. This was confirmed as the long term culture of tenocytes in 10cm plates for **28 days** with the **expansion** media alone (1% FBS + 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF), or the **differentiation** media alone (0% FBS + 10ng/ml TGFβ-3 + 50ng/ml IGF-1) did not promote the collagen formation which had been observed when ***sequential*** culture conditions were performed (as illustrated in **Figure 5-3**).

The accelerated collagen formation during the ***sequential*** culture conditions of tenocyte could be due to the synergistic effects of the $PDGF_{BB}$, TGFβ-3 and IGF-1 which has not been documented previously. Furthermore, the findings of this study have suggested that bFGF can inhibit tenocyte differentiation and collagen synthesis and as such its removal promoted the differentiation of tenocytes in the second phase in the **sequential** culturing. The inhibitory effects of bFGF on tenocyteshave already been discussed in detail in **Chapter 3**.

PDGF has been shown to induce synthesis of other factors (IGF-1) [175] and is one of the initial signals in the cascade of events in fibroblast proliferation and differentiation, collagen deposition and angiogenesis [176, 177]. Because PDGF has been shown to up-regulate the expressions of IGF-1 and IGF-1 receptors in porcine dermal fibroblast, it is possible that PDGF may have exerted at least some of its biological effects during the differentiation phase of the tenocytes indirectly through the actions of IGF-1 [175]. However, in order to elucidate on this suggestion, further experiments are warranted.

During the **expansion** phase of tenocytes in 2-D cultures, a significant increase in cell proliferation was noted as initially expected **(Figure 5-2)**. However, this cell number increase was markedly reduced after the first 14 days, when tenocytes were cultured with the **differentiation** media (0% FBS + 10ng/ml TGFβ-3 + 50ng/ml IGF-1). It is believed that this significant decline in cell number could have been attributed to enhanced collagen formation observed during this phase **(Figure 5-2, Purple line)**.

There could be three plausible explanations for such decline in tenocyte number: (1) the effect of TGFβ-3 and IGF-1 on cell differentiation was immediate and that the decrease in cell number began immediately after the **differentiation** phase had commenced; (2) a significant decline in tenocyte proliferation may have also been due to the formation of thick collagen in the 2-D cultures which may have prevented the nutrient reaching the proliferating cells; (3) the formation of thick collagen may have prevented the AlamarBlue^{TM} to be incorporated with the dividing cells and may have provided a "false" negative data. However, in order to verify any of the above proposals, further investigations are required to validate these suggestions.

While the histology examination of the 2-D construct verified that the main constituent of the construct was collagen, the microstructure and poor mechanical properties made it unsuitable to be called or used as "tendon construct". It is very unlikely that tenocytes cultured without scaffold in 2-D culture are suitable for any future clinical applications. There has been several tendon tissue engineering investigators who employed tenocytes as well as mesenchymal stem cells (MSCs), co-cultured with different scaffold, such as acellularized tendon allograft [79], polyglycolic acids [46], umbilical veins [42], small

intestine sub mucosa [72] and hyaluronan hybrid polymer fibre [80] and chitosan micro channel scaffolds [81]. Their data also showed promising micro-structural resemblances to natural tendon. However, one of the limitations to their findings was that they employed 5-10% FBS in the tenocyte- or MSC-culture media which would severely restrict their future clinical applications in reconstructive surgery. Furthermore, the mechanical properties of their engineered constructs were not satisfactory for any future tendon reconstruction.

In addition, these investigators employed the use of synthetic materials such as hyaluronan hybrid polymer fibre, polyglycolic acids and chitosan micro channel scaffolds as they are easy to produce. However, some of these synthetic materials may biodegrade which could initiate host foreign body responses making them unsuitable for long term reconstructive surgery. For instance, Cao *et al.* reported *in vitro* tendon tissue engineering by using polyglycolic acid as scaffold [46]. In their report, the microstructure of the construct showed high degree of resemblance to the natural tendon, but the mechanical properties of the construct was not satisfactory. Also, the synthetic polymers, such as polyglycolic acid, are usually acidic and toxic to surrounding cells and host tissues when degradation products accumulate at the transplantation site [46].

The use of acellularized tissues as scaffolds such as tendon allograft, umbilical veins and small intestine sub mucosa are less likely to provoke immune responses to the host than untreated tissues, as the antigenicity and immunogenicity of the tissue is lowered by removing the antigenic cells. However, the availability of those acellularized materials restricted their extensive applications.

Silk has been proven to be safe, strong, absorbable and resemble the hierarchical structure of the tendon [48, 82-85]. Degummed *Bombix* silks promise advantages compared to acellularized tissues or synthetic materials. Besides its easy accessibility owing to its abundance in nature, silk has long been used as surgical suture made it an ideal scaffold for tendon tissue engineering. The findings from another research project within the lab have shown that degummed *Bombix* silks had a much better cell adherence and less cytotoxity than undegummed silks*. By employing degummed *Bombix* silks as scaffold and co-culturing them with tenocytes in 3-D manner, it has been possible to successfully culture tendon like construct which resembled natural tendon's microstructure with satisfactory mechanical properties. The data presented in this Chapter indicated that the ultimate tensile stress, Young's modulus of the constructs were superior compared to that of the human hamstring tendon. One of the interesting findings reported in this Chapter is that, the mechanical properties (ultimate tensile stress, Young's modulus) of the silk scaffolds were not significantly affected by culturing with or without cells. The collagen formation on the scaffolds cultured with cells did not contribute to any significant increase in the mechanical properties of the constructs. One reason for this is that the *Bombix* silk itself is high in ultimate tensile stress and Young's modulus which represents the stiffness of the material, and this property could "mask" any potential differences that may have occurred due to culture. Although, previous studies by Abousleiman *et al.* also showed that tendon like structures could be generated using decellularized human umbilical veins enriched with MSCs [42], the mechanical properties of their constructs were not compared with that of natural tendon and as such it would be difficult to interpret their findings in detail.

* SILK AS A BIOMATERIAL FOR TENDON REGENERATION. By Xiao Wang, DPhil Candidate

One of the limitations of the 3-D construct designed during this study is the lack of "anchoring" regions in the constructs in order to facilitate the attachment of the tendon construct to the bone. The main function of tendons is to transmit force from the soft tissue to the bone; thus, failure to attach the engineered construct to the bone may make the engineered tendon dysfunctional. Several studies have tried to overcome this limitation by undertaking co-culturing of osteoblasts, fibroblasts [208, 209] and chondrocytes [210] together in order to create a "natural" anchorage system. Others, have employed alternative approaches, i.e. by using screws, staples, sutures [211, 212] and soft tissue plates [212] to overcome this technically and clinically challenging problem. Although, it would have been desirable to design a 3-D tendon construct which could anchor to bone either by natural approach or other alternative means, it is not within the scope of this study to undertake such investigation.

5.4 SUMMARY

- The results have shown that a novel approach for tendon tissue engineering and that tendon-like 3-D constructs can be generated by dividing the culture procedure into two specific and defined stages.
- This was achieved by **sequential** application of growth factors, each of which has specific purpose during the **expansion** and **differentiation** phases. It was demonstrated that both 2-D and 3-D culturing of the tenocytes in this manner have a more cell differentiation capabilities compared to conventional 10% FBS supplemented culture media.
- The microstructure, histology and immunohistochemistry analyses of the 3-D constructs verified the previous results in **Chapters 3** and **4** which had shown that not only the cell **expansion** and **differentiation** phases were precisely controlled but also the collagen synthesized by tenocyte was collagen type I which is one of the major components of natural tendon.
- By using degummed *Bombix* silk as scaffold, the engineered 3-D constructs cultured under defined condition showed a similar structure compared to the 10% FBS control group and both groups showed superior mechanical properties compared to human hamstring tendon.
- In order to determine whether the 2-D tenocytes generated during the **sequential** culture conditions *in vitro* also exhibit similar properties *in vivo*, tenocytes generated in this manner will be implanted in *Balb/c* nude mice for 30 days. The findings of this part of the thesis are given in the next Chapter.

CHAPTER SIX:

IN VIVO TRANSPLANTATION OF TENOCYTES CULTURED UNDER DEFINED CULTURE CONDITION

In previous Chapters, it was determined that it is practically possible to expand and differentiate human tenocytes *in vitro* using **sequential** culturing methodology with the aid of 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF for **expansion** phase and 10ng/ml TGFβ-3 + 50ng/ml IGF-1 for **differentiation** phase. Furthermore, it was demonstrated that this approach can be employed in 2-D and 3-D culture conditions. The next natural investigation to be undertaken would be its employment in *in vivo* model. This is the basis of the current Chapter. To address this experimental approach, the following paragraph will briefly outline some of the current relevant publications regarding *in vivo* tendon regeneration.

After ligament and tendon injuries, the original complex structures and mechanical properties are not fully restored. Tissue engineering provides an alternative approach to tendon healing. Orthopaedic surgery has benefited from tissue engineering by cell transplantation in many areas, including repair of long bone defects [213] and cartilage defects [214]. Ligaments and tendons, with impaired ability to fully heal, lead themselves well to tissue engineering applications. There has been reported successful BMSC and tenocyte transplantation in rabbit tendon defects that promoted tendon healing and neotendon formation [215]. Moreover, isolated murine tenocytes have been shown to be capable of regenerating tendon-like structures after extended expansion *in vitro* (in the absence of growth factors but in the presence of 10% FBS) and subsequent implantation in nude mice [62]. One main concern of BMSC and tenocyte transplantation is undesired ectopic osteogenesis, which was described in studies investigating construct of collagen gel and BMSC and tenocytes in patellar tendon defects [216-220]. The previous Chapters of this thesis showed that tenocytes cultured in serum free α-MEM supplemented with TGFβ-

3 and IGF-1 could up-regulate the mRNA expression of tenocyte phenotypic and differentiation markers significantly more than those cultured with 10% FBS. In addition, the tenocytes cultured in 3-D (with silk) in the presence of TGFβ-3 and IGF-1, were capable of inducing tendon structure formation *in vitro*. However, for any future clinical applications, the *in vivo* differentiation capability of the tenocytes cultured with defined culture media warrants further investigation. Thus, it is hypothesized that the transplantation of generated tenocytes *in vitro* will induce neotendon formation *in vivo* without undesired osteogenesis.

6.1 EXPERIMENTAL DESIGN

6.1.1 Tenocyte isolation and culture

Human tenocytes were isolated as described in **Chapter 2, Section 2.1**. Following expansion to passage 3, the cells were divided in two groups and seeded in 10cm culture plates. One group was fed with α-MEM supplemented with 10% FBS for 21 days. The other group was fed with α-MEM supplemented with 1% FBS + 50ng/ml $PDGF_{BB}$ + 50ng/ml bFGF for 14 days. These cells were cultured for a further 7 days in serum free α-MEM supplemented with 10ng/ml TGFβ-3 + 50ng/ml IGF-1. The reason for not culturing for 14 days, as had been optimized in previous Chapter, was to allow the **differentiation** phase to be completed *in vivo* and not before implantation. Figure 5-3 clearly showed fully differentiated tenocytes (collagen formation) after culturing with differentiation medium for 14 days. It would be highly unlikely to be able to assess the differentiation ability of the tenocytes *in vivo* by implantation of such a collagen mass into the mice.

At the end of the culture period *in vitro*, the monolayer on the plates were washed with sterile PBS before being scraped by cell scrapers, counted with haemocytometer and the cell number was adjusted to 2×10^5 cells for each group. The cells were then centrifuged at 1500 rpm for 5 mins, and the cell pellet was removed for implantation using a purpose built "sterile hook" as indicated in **Figure 6-1**.

Figure 6-1: Purpose built "sterile hook"

6.1.2 Animal model preparation

The animal experiments were performed at the animal facility of the Tianjin Medical University, Tianjin, China. All of the experiments procedures used conformed to the Animal Protection Laws in China and the University regulations regarding ethical issues with handling laboratory animals.

Balb/c nude mice with an average age of 6 weeks were used for the *in vivo* study. Sixty male mice were divided into 4 groups (15 per group): (1) 10% FBS group; (2) **sequential** growth factors treatment group (see **Section 6.1.1**); (3) positive control group (human hamstring tendon) and (4) sham operated group. Mice were anesthetized using isofluoran inhalation and under sterile conditions, the right quadriceps muscles were dissected via blunt dissection and the cell pellets from groups (1) and (2) were inserted. For the sham operated group, 0.5 ml of α-MEM was injected into the dissection sites. For the positive

control group, human hamstring tendon was cut into equal size as that of the pellet and inserted into the dissection site. The dissection sites were sutured by absorbable suture to make sure that no leakage could occur. On days 10, 20 and 30 post-implantation, five mice from each group were sacrificed and the tissue samples were harvested and analysed as detailed below **(Figure 6-2)**.

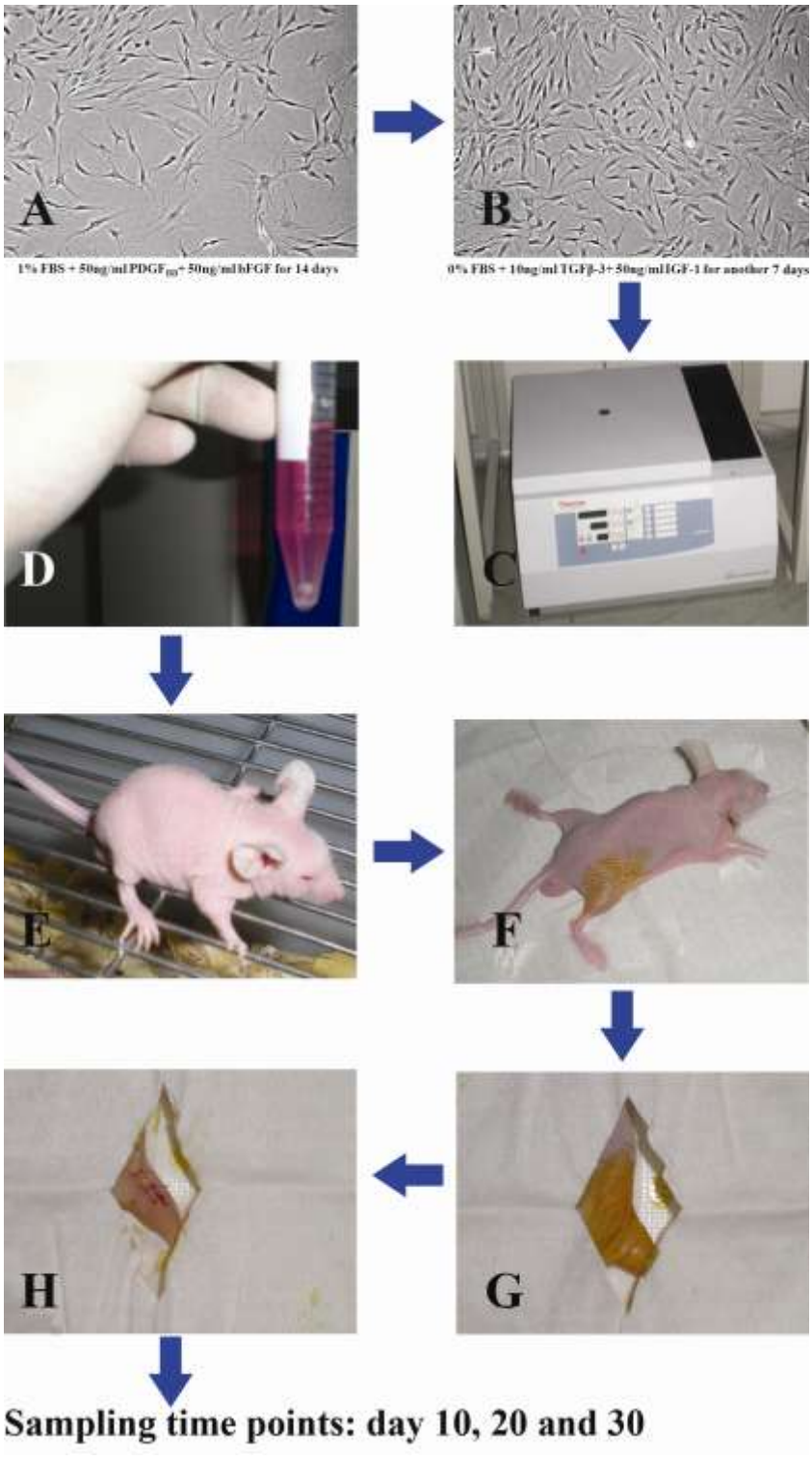

Figure 6-2: A summarized flow chart of the method of tenocytes culture and subsequent implantation in immune-deficient *Balb/c* nude mice.

6.1.3 Histology examination

The tissue sections obtained at different time points were stained with H&E (as described in **Chapter 2, Section 2.6.3**) and immunohistochemically for human COL-I (as described in **Chapter 2, Section 2.6.4**). Tissue sample sections were examined for ectopic bone and cartilage formation. Immunohistochemistry was performed using rabbit anti **human** COL-I polyclonal antibody to verify that the major component of the neotendon formation is human COL-I (Abcam, Hongkong, China) and that it was synthesized by the **human** tenocytes and not from the nude mice itself.

6.1.4 Molecular analysis

After 30 days of implantation, neotendon samples formed at the site of implantation was collected and homogenized to undertake RNA extraction and real time RT- PCR as previously described in **Chapter 2, Section 2.4**. Primers for human tendon and extracellular matrix markers were purchased from Qiagen, UK. These included COL-I, SCX, DCN and TNMD. The human osteogenesis marker RUNX2 and chondrogenesis marker SOX9 and COL-II were also analyzed to examine if there were any evidence of osteogenesis and/or chondrogenesis after implantation. The details of methodology for real time RT-PCR and quantification of mRNA expression are given in **Chapter 2, Section 2.4**.

6.2 RESULTS

6.2.1 H&E Staining

Figure 6-3 illustrates the formation of **human** tendon in nude mice with time and that the structured morphology of tendon is evident in mice which had the implantation for 30 days compared to 10 days. No tendon structures could be clearly observed in both treated and control groups on day 10 **(Figure 6-3A, B)**. However, on day 20, rippled and parallel tendon fibrils within the mice muscle fibres could be observed in both groups. The newly formed tendon-like fibres in these groups were more dense and compact than that formed within 10 days post-implantation **(Figure 6-3C, D)**. Compared to other time points studied, both control and experimental groups exhibited a structure that resembled tendon within 30 days. Moreover, these results indicated that in the experimental group whereby tenocytes were expanded and differentiated *in vitro* sequentially, the morphology of the human tenocytes was very similar to that of 10% FBS supplemented cultures within 30 days post implantation **(Figure 6-3E, F)**. No ectopic bone and/or cartilage formation was evident at any of the time points/ groups studied.

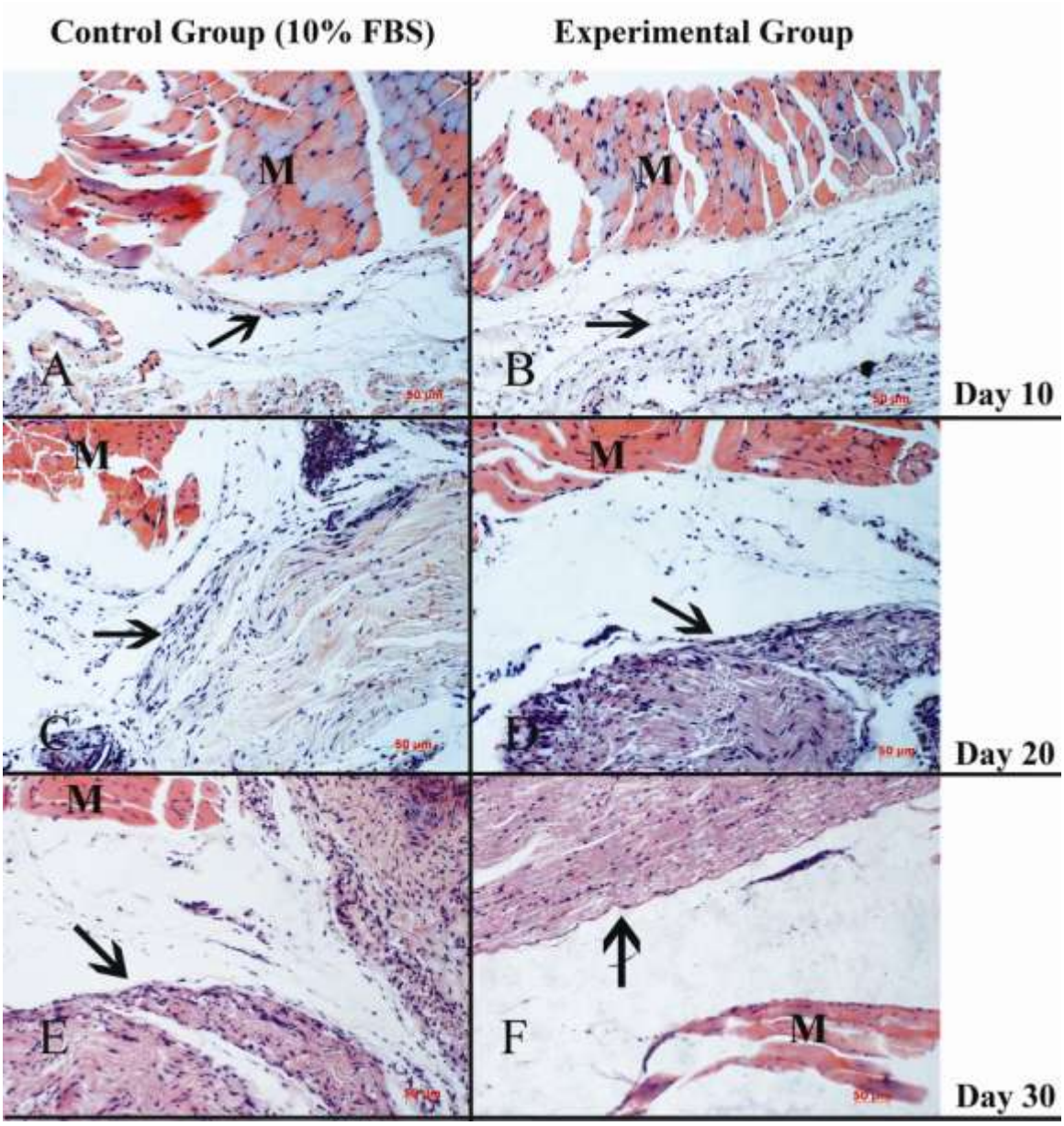

Figure 6-3: H&E staining of the 5µm paraffin sections of the neotendon samples after 10 to 30 days implantation of human tenocytes in *Balb/c* nude mice. Left panel indicates the control group whereas the right panel illustrates the sections from the sequentially-treated groups (see text for full details). Magnification ×200，scale bar 50µm. Arrows indicate tendons; M indicates muscles.

6.2.2 Immunohistochemistry for Human COL-I

In order to determine that the tendon formed in nude mice were generated from implanted **human** tenocytes, immunohistochemical analysis of human COL-I was undertaken on the murine tissue. All of the newly formed tendon sections were positively stained for human COL-I and that these newly formed tendon fibres were indeed synthesized by the **human** tenocytes that had been implanted. The positive control group showed positive staining of the COL-I antibody verified that not only the collagen type I was major component of the natural human tendon, but also the collagen fibres synthesized by the tenocytes was collagen type I **(Figure 6-4H)**. There was no indication of COL-I staining in sham operated negative control groups **(Figure 6-4G)**.

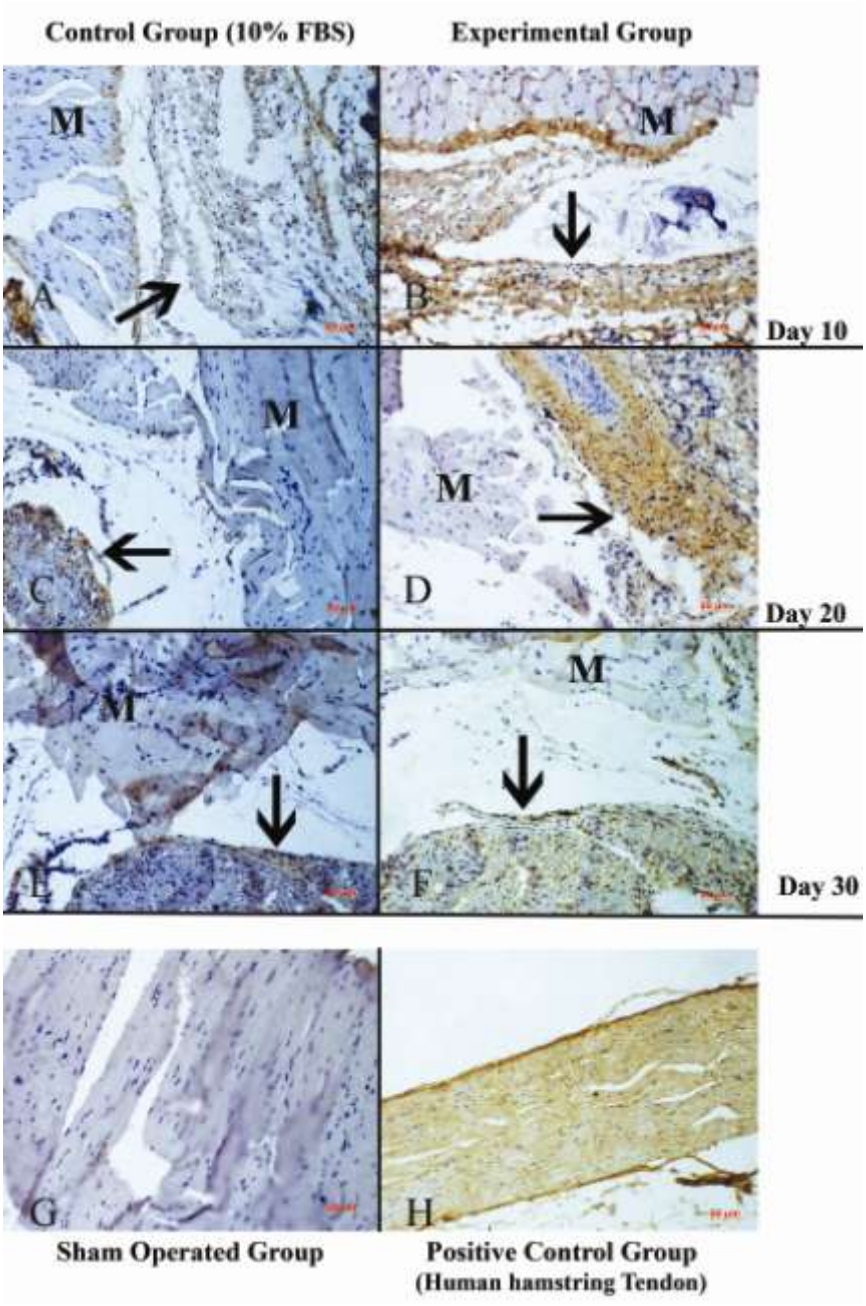

Figure 6-4: COL-I immunohistochemistry staining of the 5µm paraffin sections of the neotendon samples formed after human tenocytes were implanted from 10 to 30 days in *Balb/c* nude mice. Left panel indicates the control group (10% FBS treated) whereas the right panel illustrates the experimental group (sequential application of growth factors). (G) Samples obtained from sham operated animals. (H) Positive control samples obtained from 30 days post-implantation of human hamstring tendon in nude mice. Magnification ×200， scale bar 50µm. Arrows indicate tendons; M indicates muscles.

6.2.3 Molecular analysis

To ascertain further the specificity of the human tendon formed in the nude mice, the mRNA expression of tendon specific markers were examined in samples extracted from the mice 30 days post-implantation. The data for this part of investigation is illustrated in **Figure 6-5A & B** where the mRNA expression of SCX, TNMD, COL-I and DCN were clearly up-regulated in experimental group (sequential growth factor treatment). All data were expressed as ratio of specific markers in experimental to that of the control 10% FBS treated group. The results indicated that the SCX, TNMD, COL-I and DCN showed 40.3, 413, 4.3 and 2.5 fold increase in expression than that in control respectively which was all significant as determined using One-way ANOVA. The mRNA expression of the COL-II, SOX9 and RUNX2, which are known to be specific for chondrogenesis and osteogenesis were not up-regulated **(Figure 6-5B)**.

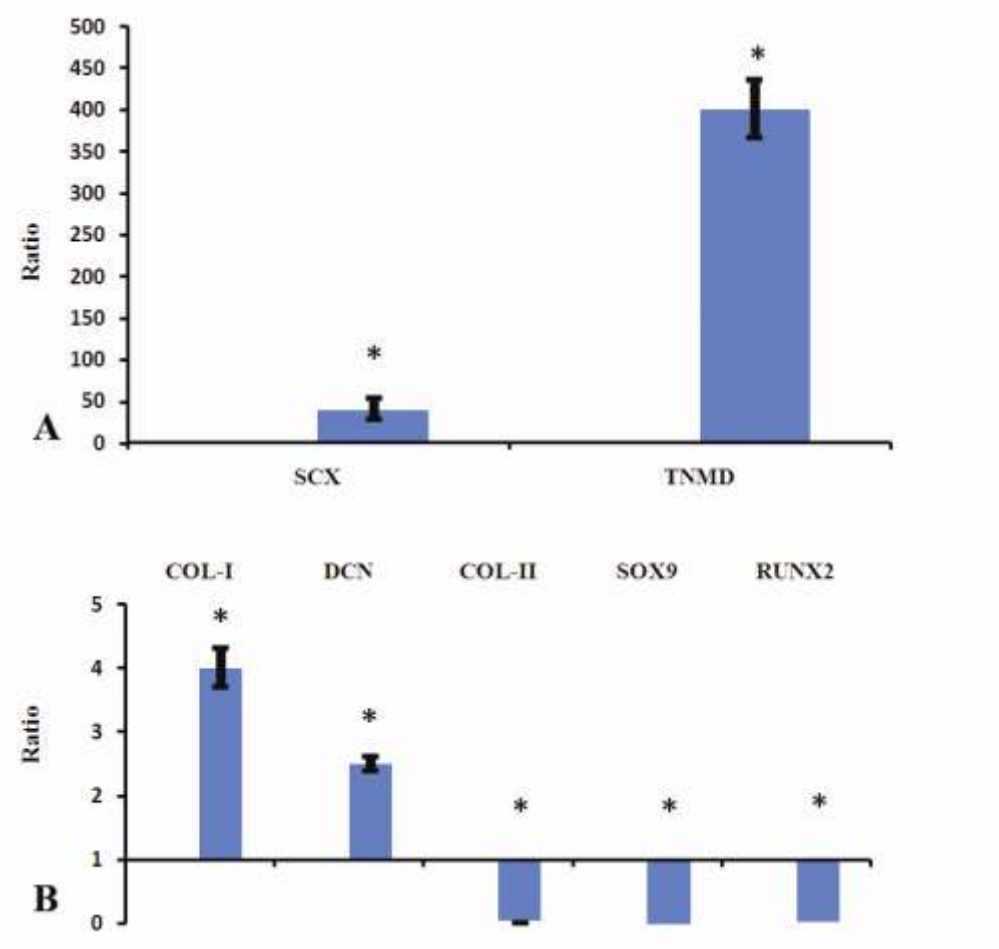

Figure 6-5: Messenger RNA expression ratios of the encoding human SCX and TNMD (A) and COL-I, DCN, COL-II, SOX9 and RUNX2 (B) in neotendon tissues formed 30 days post-implantation in *Balb/c* nude mice. GAPDH mRNA was used to normalize the variability in template loading. Data were presented as means ± SEM of the ratio of mRNA expression in experimental group compared to that of 10%FBS.
*** $p<0.05$: in comparison with the control group using One-way ANOVA.**

6.3 DISCUSSION

In this study, it was demonstrated that human tenocytes cultured under defined condition were capable of **differentiating** into human tendon *in vivo*. The data presented herein has demonstrated that not only the formation of human tendon constructs was possible in serum free culture condition *in vitro* but also that this approach was further expandable *in vivo*, a finding which had not been reported before. Therefore, the tenocytes expanded described herein have the potential to maintain their phenotype in serum free culture condition making them ideal for tissue engineering for reconstructive tendon surgeries without the inflammatory complications associated with FBS addition. There have been several studies showing that tenocytes and mesenchymal stem cells (MSCs) transplantation was a viable method for tendon regeneration [62, 216, 217, 220]. However, the culture method for the tenocytes has limited its future clinical applications for the use of 10% FBS in the culture medium. Moreover, the undefined ingredients in the FBS could stimulate tenocyte differentiation in an uncontrolled manner which may lead to undesired osteogenesis and chondrogenesis. Bi *et al.*'s study identified a stem/progenitor cell population within tenocytes and has the universal characteristics of stem cells, including multipotency toward tendon, bone and lipid differentiation potentials [62]. Bi *et al.*'s findings could justify the bone and cartilage formations which were described in those studies [216, 217, 220]. Dressler *et al.* and Chen *et al.* reported that, tendon healing could be improved by implanting autologous mesenchymal stem cells [216] or tenocytes [220] into tendon defect rabbit models, but unwanted osteogenesis was observed in those studies. Both studies used 10% FBS in their culture mediums and the time periods for the studies

lasted 12 weeks and 8 weeks, respectively. However, the findings reported in this Chapter, the control group of which tenocytes were cultured in 10% FBS did not show ectopic bone formation in histology examination, which was not consistent with above findings. The reasons could be that, firstly, the time period for experiments described in this Chapter lasted 4 weeks which is much shorter than those studies; secondly, the implantation sites for those studies were artificial defects made on patellar tendons [216] and rotator cuff tendons [220] where there were possible surgery induced bone exposures in close proximity to the cell implantation sites. The exposed bone surfaces may trigger unknown cell signalling cascade of the implanted cells which may lead to osteogenesis observed in those studies. Awad *et al.* [218, 219] also found that implantation of rabbit mesenchymal stem cells/collagen gel composite to the patellar tendon defect significantly improves healing but the bone formation was also observed in those experiments. The findings in the present study interestingly indicated a decrease of mRNA expression in osteogenic (RUNX2) and chondrogenic (COL-II and SOX9) markers post-implantation of tenocytes treated with TGFβ-3 and IGF-1 compared to the control group (10% FBS) suggesting no ectopic bone or cartilage formation had taken place. These results substantiate previous studies from another perspective which showed multipotency of the tenocytes after culturing with 10% FBS [216, 217, 220]. Although the focus of the study was not to fully define these findings, one explanation could be that unknown factors in FBS may have some effect that could have contributed to osteogenesis observed in previous studies [216, 217, 220].

Okamoto *et al*. studied the effect of TGFβ-1 in tenocyte differentiation in an autologous tenocyte transplantation of tendon defect rabbit models [221]. The results showed that, the TGFβ-1 treated group showed significant superiority in biomechanical properties and histological appearances than the untreated group. Nevertheless, this experiment was conducted with the addition of 10% FBS in the experiments group as well as the control group. Whereas similar results were achieved in this Chapter by using the combination of TGFβ-3 and IGF-1 in serum free conditions to stimulate the tenocyte **differentiation** before the implantation. Wolfman *et al.* demonstrated that, ectopic tendon and ligament formation can be induced in rats by injecting growth and differentiation factors 5, 6, and 7, which are members of the TGF-beta gene family [74]. Oka *et al.*'s study indicated that TGFβ signalling-mediated SCX expression is required for tendonogenesis in the developing skeletal muscle [222]. Pryce *et al.* showed that TGFβ signalling plays a major role in the formation of tendon tissues [206]. All these findings support those reported herein suggesting that TGFβ-3 does indeed have the capability to induce tenocyte differentiation *in vitro* and tendon formation *in vivo.* Similar to the findings in this thesis, Chen *et al.* showed that both TGFβ and IGF-1 had significantly higher expression after tendon injuries in chicken model implicating that both TGFβ and IGF-1 play critical roles in tendon regeneration [223]. There have been only limited studies on the effects of IGF-I on tenocytes in neotendon formation *in vivo*. For example, Schnabel *et al.* have been the only group who recently reported that IGF-I gene-enhanced mesenchymal stem cells improve structural healing in equine tendon [224].

In previous Chapters, it was indicated that, in 2-D monolayer culture, without the use of scaffold, the irregular and random collagen alignment could not represent the microstructure of the natural tendon. However, in this Chapter, tendon like structure formation was observed after the tenocytes implantation *in vivo* without scaffold. The newly formed tendon structure found in this study resembled the microstructure of natural tendon. The findings indicated that under *in vivo* environments, the mechanical stimulation could play a pivotal role in tenocyte differentiation, as the tenocytes were implanted in the quadriceps of the mice and were subjected to constant mechanical stimulation. There are several studies indicating that, mechanical stimulation is beneficial to tendon tissue engineering *in vitro* with various scaffolds [42, 47, 72, 225]. Although the previous researches have indicated the involvement of mechanical stimulation in tendon tissue engineering process *in vitro*, this observation has been further complicated by the inconsistent loading forces applied in different studies.

One of the limitations of this study is that the role of mechanical environment on tendon formation post implantation was not fully elucidated as it was not feasible to definitively suggest mechanical loading of the limbs *in vivo* contributed to the accelerated tendon formation. This would have to be further investigated in more detailed structured study.

The histological and microstructure results reported in this study showed a promising indication of the therapeutic potential of tenocyte based therapy for tendon reconstruction; lack of biomechanical testing of the newly formed tendons limited this study. Given the small sizes of the tendon samples, it was difficult to evaluate the mechanical properties of those newly formed tendons; however, the results of this study could be useful for further

transplantation of the engineered construct *in vivo*, and the mechanical properties of the constructs after implantation could be assessed. Another limitation to this study is that, due to the small sizes of the quadriceps of the *Balb/c* nude mice, it was not practical to implant the engineered 3-D constructs using *Bombix* silk as scaffold described in the previous Chapter. The 3-D construct could be implanted by using larger animals in future studies, for example in arabbit tendon-defect model.

6.4 SUMMARY

- The results in this Chapter further confirmed that tenocytes maintained *in vitro* in defined **sequential** culture condition can **differentiate** to neotendon *in vivo*.
- The nature of the tendon formed was confirmed immunohistochemically and phenotypically.
- There were no indications of undesired osteogenesis and chondrogenesis *in vivo*.
- The mRNA expressions of the osteogenesis and chondrogenesis markers of the experimental group were significantly down regulated compared to the control group.
- Further research interest would be the evaluation of the clinical potential of the engineered 3-D tendon constructs under *in vivo* condition.

CHAPTER SEVEN:

CONCLUSION AND FUTURE WORK

7.1 CONCLUSION

The results of these Chapters clearly demonstrate that:

- It is possible to **expand** tenocytes without differentiation *in vitro* with low serum concentrations (1%);
- Tenocyte **differentiation** and survival can be achieved by supplementing the culture media with TGFβ-3 and IGF-1 in the absence of any additional serum;
- By combining the conditions optimized for **expansion** and **differentiation** phases **sequentially**, it is possible to observe accelerated collagen formation in 2-D tenocyte cultures which were significantly superior to that conventionally used (i.e. 10% FBS-treated);
- This approach can successfully be achieved when using 3-D degummed *Bombix* silk as a scaffold. The engineered constructs has the overall appearance and microstructure to that observed with human hamstring tendon;
- The *in vivo* study indicates that, tenocytes cultured with **sequential** application of growth factor can maintain cell differentiation specifically towards tendon formation. The findings suggest that the tendency for any osteogenesis and chondrogenesis processes were minimal.

Overall, these findings suggest that, tendon like constructs can be generated by culturing human tenocytes with degummed *Bombix* silk with sequential application of growth factors and minimal FBS usage. This application has significant clinical benefit in tendon reconstructive surgery.

7.2 FUTURE WORK

Future work is likely to focus on:

1. Further evaluating the engineered constructs by transplanting the constructs into tendon defect animal models (e.g. rabbit model [218-220]).
2. Examining the mechanical properties of constructs formed *in vivo.*
3. The use of alternative scaffold to investigate differences (if any) in biological responses.
4. The cell signalling mechanisms involved in promoting tenocyte differentiation by TGFβ-3 and IGF-1.
5. The use of alternative growth factors to promote **expansion** and **differentiation** phases in a shorter culture period (e.g. VEGF).
6. Develop defined serum-free medium to eliminate the use of fetal calf serum.

Appendix A: **RNA EXTRACTION PROTOCOL**

1. Harvest cells
2. Cells are lysed directly in the cell-culture dish.
3. Completely aspirate the cell-culture medium, and wash the cells with PBS.
4. Disrupt the cells by adding 350µl Buffer RLT to the cell culture dish.
5. Homogenize the lysate by passing the lysate at least 5 times through a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe.
6. Add 350µl of 70% ethanol to the homogenized lysate, and mix well by pipetting.
7. Transfer up to 700µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 seconds at 10,000 rpm.
8. Discard the flow-through and reuse the collection tube.
9. If the sample volume exceeds 700µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.
10. Add 700µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 seconds at 10,000 rpm to wash the spin column membrane.
11. Discard the flow-through and reuse the collection tube.
12. After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.
13. Add 500µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 10,000 rpm to wash the spin column membrane. Discard the flow-through.

14. Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of 100% ethanol as indicated on the bottle to obtain a working solution.
15. Add 500µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at 10,000 rpm to wash the spin column membrane.
16. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.
17. After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.
18. Place the RNeasy spin column in a new 2 ml collection tube and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min. Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column.
19. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 50µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at 10,000 rpm to elute the RNA.

Appendix B: **REVERSE TRANSCRIPTION OF COMPLIMENTARY DNA PROTOCOL**

1. Reaction assembly, place components on ice, mix and then briefly centrifuge to collect contents to the bottom of the tube before using.

COMPONENT	**VOLUME**
qScript cDNA Supermix	4µl
RNA template	6µl
RNase free water	10µl
Total Volume	20µl

2. Combine reagent in 0.2 ml micro-tubes on ice
3. After sealing each reaction, vortex gently to mix the contents.
4. Centrifuge briefly to collect components at the bottom of the reaction tube.
5. Incubate: 5 mins at 25°C, 30 mins at 42°C, 5 mins at 85°C, and hold at 4.
6. After completion of cDNA synthesis, cDNA can be diluted and stored at -20°C.

Appendix C: **QUANTITATIVE REAL TIME REVERSE TRANSCRIPTION PCR PROTOCOL**

1. Reaction assembly

COMPONENT	**VOLUME FOR 50µl REACTION**
PerfeCta SYBR Green SuperMix	25µl
Forward Primer	5µl
Reverse Primer	5µl
Nuclease-free water	5µl
cDNA Template	10µl
Final Volume	50µl

2. After sealing each reaction, vortex gently to mix the contents.
3. Centrifuge briefly to collect components at the bottom of the reaction tube.
4. incubate complete reaction mix in Rotor gene RG-3000 real-time thermal detection system
5. Cycling conditions were: holding at 95ºC for 20 seconds, and then cycle at 95 ºC for 3 seconds, 60 ºC for 20 seconds, and this cycle was repeated 40 times

Appendix D: **PROTOCOL FOR DEWAXING TISSUE SECTIONS FOR STANDARD H&E STAINING**

1. The tissue sections were mounted on slides.
2. The slides were dewaxed by immersing the slides in xylene for 15 mins.
3. Immersing the slides in 100% ethanol for 5 mins, twice, 95% ethanol for 5 mins, and 80% ethanol for 5 mins.
4. The slides were washed under tap water for 5 mins.

Appendix E: **PROTOCOL FOR SEALING SLIDES**

1. The slides were immersed in 80% ethanol for 2 mins, 95% and 100% ethanol twice for 5 mins each, in xylene twice for 5 mins each.
2. The slides were then dried before sealed with DPX by cover slides

Appendix F: **PROTOCOLS FOR DEWAXING, REHYDRATION, ANTIGEN RETRIEVAL AND BLOCKING OF THE TISSUE SECTIONS FOR IMMUNOHISTOCHEMISTRY**

1. Human tendon samples and/or constructs sections are mounted on pre-treated slides.
2. The slides are dewaxed by immersing the slides in xylene for 15 mins.
3. The tissue sections are rehydrated by immersing the slides in 100% ethanol for 5 mins, twice, 95% ethanol for 5 mins, 80% ethanol for 5 mins, and then the slides are washed under tap water for 5 mins.
4. The slides are immersed in 3% peroxides solution for 30 mins, then washed under tap water for 15 mins.
5. After the slides are dipped in distilled water for 5 seconds, the tissue sections are washed with 0.01M PBS for 5 mins and this step is repeated three times.
6. Immerse the slides in citric buffer solution before antigen retrieval is performed.
7. The antigen retrieval is performed by heating up the samples in a microwave, medium power (500 Watt) for 5 mins.
8. The citric buffer solution is allowed reach room temperature and the microwave heating process is repeated one more time.
9. After the citric buffer solution is cooled to room temperature, the slides are removed from the solution and washed with 0.01M PBS for 5 mins, repeated three times.

10. The tissue sections are blocked by 5µl 10% goat serum (dilution 1:20) at room temperature for 30 mins before the primary antibody is added.

Appendix G: **COURSE LIST**

1. Excel fundamental
2. PowerPoint fundamental
3. Poster production workshop
4. SPSS introduction
5. Animal handling and welfare
6. Transfer report and thesis writing
7. Presentation skills
8. Advanced Light Microscopy lecture
9. Data Analysis

Appendix H: **PUBLICATIONS**

Publications

1. **Yiwei Qiu**, Xiao Wang, Raj Rout, Andrew J. Carr, Zhidao Xia. *$PDGF_{BB}$ and bFGF Promote Proliferation and Inhibit Differentiation of Human Tenocytes in Low Serum Culture In vitro.* (European Cells and Materials Vol. 18. Suppl. 2, 2009 , page 86)
2. **Yiwei Qiu**, Xiao Wang, Raj Rout, Andrew J. Carr, Zhidao Xia. *Long Term Human Tenocytes survival and Differentiation under Defined Culture Medium Condition.* (Poster presentation, CLSS-UK programme, 2009)
3. Xiao Wang, **Yiwei Qiu**, James T. Triffitt, Andrew J. Carr, Zhidao Xia, Afsie Sabokbar. *Effect of Platelet-Rich Plasma on Human Tenocyte Proliferation and Differentiation In vitro and In vivo.* (Poster presentation, ASBMR programme, 2010)
4. Xiao Wang, **Yiwei Qiu**, Afsie Sabokbar, James T. Triffitt, Andrew J. Carr, Zhidao Xia. *Improved Human Tenocyte Proliferation and Differentiation In vitro by Optimized Silk Degumming.* (Oral presentation, TREMIS programme, 2010, **50 Best Abstracts Awards**)
5. Xiao Wang, **Yiwei Qiu**, Andrew J. Carr, Zhidao Xia. *Attachment and Proliferation of Human Tenocytes /Macrophages on Silks with Different Treatments.* (European Cells and Materials, Vol. 18. Suppl. 2, 2009 , page 109)

6. Xiao Wang, **Yiwei Qiu**, Andrew J. Carr, Zhidao Xia. *Effect of Degummed Silk on Macrophage and Human Tenocytes Attachment and Proliferation.* (Poster presentation, CLSS-UK programme, 2009)
7. Yaonan Zhang, Xiao Wang, **Yiwei Qiu**, Jill Cornish, Andrew Carr, Zhidao Xia. *Dose-dependent effect of Indomethacin and Lactoferrin on normal Human Tenocytes in vitro*. (Poster presentation, TCES programme, 2009)

Unpublished:

1. **Yiwei Qiu**, Xiao Wang, Raj Rout, Andrew J. Carr, Zhidao Xia. *$PDGF_{BB}$ and bFGF Promote Proliferation and Inhibit Differentiation of Human Tenocytes in Low Serum Culture in vitro.* (delayed submission due to patent application procedure)
2. **Yiwei Qiu**, Xiao Wang, Raj Rout, Yaonan Zhang, Andrew J. Carr, Zhidao Xia. *Combination of IGF-1 and TGFβ-3 promote human tenocytes differentiation in serum free conditions in vitro.* (delayed submission due to patent application procedure)
3. **Yiwei Qiu**, Xiao Wang, Raj Rout, Yaonan Zhang, Andrew J. Carr, Zhidao Xia Afsie Sabokbar. *In vitro tendon tissue engineering with degummed Bombix silk as scaffold under optimal culture condition.* (delayed submission due to patent application procedure)
4. **Yiwei Qiu**, Xiao Wang, Raj Rout, Yaonan Zhang, Andrew J. Carr, Zhidao Xia, Afsie Sabokbar. *Neotendon formation by in vivo transplantation of the tenocytes*

cultured under defined culture condition. (delayed submission due to patent application procedure)

5. Xiao Wang, **Yiwei Qiu**, James T. Triffit, Andrew J. Carr, Zhidao Xia, Afsie Sabokbar. *Responses of PRP Treated Tenocytes to Silks In vivo by means of Diffusion Chambers.* (In writing)
6. Xiao Wang, **Yiwei Qiu**, Afsie Sabokbar, James T. Triffit, Andrew J. Carr, Zhidao Xia. *Improved human tenocytes proliferation and differentiation in vitro by optimized silk degumming. (Submitted to European Cells & Materials Journal under review)*
7. Xiao Wang, **Yiwei Qiu**, James T. Triffitt, Andrew J. Carr, Zhidao Xia, Afsie Sabokbar. *Biological Responses to Silks In vitro and In vivo.* (In writing)
8. Xiao Wang, **Yiwei Qiu**, Andrew J. Carr, Zhidao Xia. *Effect of degummed silk on macrophage and human tenocytes attachment and proliferation.* (In writing)
9. Yaonan Zhang, Xiao Wang, **Yiwei Qiu**, Jill Cornish, Andrew Carr, Zhidao Xia. *Dose-dependent effect of Indomethacin and Lactoferrin on normal human tenocytes in vitro.* (Submitted to European Cells & Materials Journal under review)

Appendix I: **PATENT SUBMITTED**

Yiwei Qiu, Andrew J. Carr, Zhidao Xia. *Optimal culture condition for tenocytes differentiation*. (ISIS Project number: 4265)

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