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Cell-cell interactions and matrix deposition in tendon development *in vitro* and *in vivo*

A thesis submitted to the University of Wales
for the degree of Doctor of Philosophy.

by

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SUMMARY

Tendons transmit tensile load from skeletal muscles to bone. They fundamentally consist of densely packed longitudinal collagen fibres separated by rows of tendon fibroblasts. The cells have extensive cell-cell contacts, longitudinally within the rows and between rows via cell processes. This thesis explores how cell-cell interactions and the cytoskeleton relate to matrix secretion and organisation in developing chicken tendons and cell cultures.

In initial developmental stages, indicated by tenascin and type I collagen expression, cells were associated with cadherins, vinculin and connexin 32, and the presence of actin fibres. Subsequently, type III collagen delineated early fascicular structure, and later still connexin 43 appeared, relating to mechanical loading and formation of a cellular regulatory network. In culture, monolayers laid down minimal matrix, high density micromass cultures laid down more and pellet cultures produced an extensive matrix, although 75% of their collagen was lost to the medium. Micromasses and pellets expressed similar cytoskeletal and cell junctional markers to the *in vivo* tendons; monolayers differed with early expression of connexin 43. TGF β , bFGF and PDGF singly or in combination had minor effects on collagen production and deposition and had no effect on cell junctional or cytoskeletal components.

Finally, a novel suspension culture system was developed to allow large numbers of cells to interact and deposit matrix without interference from medium changes. After establishing that cells aggregated rapidly in suspension culture, suspensions were placed in dialysis tubes contained within 50ml sterile plastic tubes and cultured on rollers, with medium being changed only in the large tubes, leaving the cell suspensions undisturbed. This produced structures up to 3cm in length, with good cell and matrix organisation and seeming to incorporate more collagen into the extracellular matrix than pellet cultures. This could form the basis for a simple scaffold-free tissue engineering approach for tendons and ligaments.

DEDICATION

— For Robin —

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CONTENTS

	PAGE
TITLE	i
SUMMARY	ii
DECLARATION/STATEMENTS	iii
DEDICATION	iv
ACKNOWLEDGEMENTS	v
CONTENTS	vi
LIST OF FIGURES	x
LIST OF TABLES	xii
ABBREVIATIONS	xiv
 CHAPTER 1: General Introduction	
 1.1 TENDON structure and function	 1
1.2 TENDON CELLS	4
Tendon cell metabolism	5
Fibrocartilaginous cells	6
1.3 TENDON MACROMOLECULES	6
1.3.1 Collagens	7
Fibrillar collagens	7
Short chain collagens	9
FACIT collagens	10
Types VI and VII collagen	11
Membrane bound and MULTIPLEXIN collagens	11
1.3.2 Elastin	11
1.3.3 Non-collagenous components of tendon ECM	12
Glycoproteins	12
Glycosaminoglycans	14
Proteoglycans	14
1.3.4 Inorganic components	17
1.4 CYTOSKELETON	18
1.4.1 Actin subunits and assembly	18

Actin in connective tissues	20
1.4.2 Intermediate filaments	21
1.4.3 Microtubules	22
1.5 CELL JUNCTIONS	23
1.5.1 Anchoring junctions	23
1.5.2 Gap junctions	26
Physiological roles of gap junctions and connexins	27
1.5.3 Tight junctions	29
1.6 TENDON DEVELOPMENT	29
1.6.1 Tendon development and morphogenesis	29
1.6.2 Collagen fibrillogenesis in tendon development	31
1.6.3 Markers of tendon development	32
1.7 TENDON REPAIR	34
Inflammation phase	34
Proliferation/Granulation phase	34
Remodelling phase	36
1.7.1 Growth factors in connective tissue repair	37
Insulin-like growth factor	37
Transforming growth factor	37
Basic fibroblast growth factor	38
Platelet derived growth factor	38
1.8 CELL AND TISSUE CULTURE AND TISSUE ENGINEERING	39
1.8.1 Monolayers	40
1.8.2 3-D cultures	41
3-D cultures and tissue engineering	42
1.8.3 Bioreactors and 3-D culture	43
Application of 3-D culture and tissue engineering to tendon and ligament	43
1.10 AIMS AND OBJECTIVES	45
 CHAPTER 2: General Materials and Methods	 46
 2.1 Source of material	 46
 2.2 Cell isolation and Culture	 46

2.3 Fixation procedures	47
2.4 Immunohistochemistry	48
2.6 Biochemical Analysis	49
2.6.1 Hydroxyproline Assay	49
2.6.2 Hoechst Assay	49
CHAPTER 3:	51
Matrix deposition and cell-cell interactions in tendon development	
3.1 INTRODUCTION	51
3.2 MATERIALS AND METHODS	52
3.3 RESULTS	53
3.4 DISCUSSION	75
CHAPTER 4:	81
Matrix deposition by tendon cells in culture: A comparison of 2-D and 3-D culture methods	
4.1 INTRODUCTION	81
4.2 MATERIALS AND METHODS	85
4.3 RESULTS	87
4.4 DISCUSSION	131
CHAPTER 5:	135

Effects of growth factors on cell-cell interaction and matrix secretion in high density pellet cultures	
5.1 INTRODUCTION	135
5.2 MATERIALS AND METHODS	142
5.3 RESULTS	143
5.4 DISCUSSION	180
CHAPTER 6:	188
Cell-cell interaction and tissue differentiation in roller cultures: development of novel culture techniques for producing large scale tendon-like structure in vitro	
6.1 INTRODUCTION	188
6.2 MATERIALS AND METHODS	191
6.3 RESULTS	195
6.4 DISCUSSION	271
CHAPTER 7: General Discussion	283
REFERENCES	290
APPENDICIES	325
Publications	

LIST OF FIGURES

CHAPTER 1

Fig 1.1: An overview of tendon structure.

Fig 1.2: A histological section showing the structure of tendon.

Fig 1.3: A schematic image depicting the complexity of tendon cell shape.

CHAPTER 3:

Fig 3.1: Tendon developmental sequence: Type I collagen.

Fig 3.2: Tendon developmental sequence: Type III collagen.

Fig 3.3: Tendon developmental sequence: Tenascin.

Fig 3.4: Tendon developmental sequence: Actin.

Fig 3.5: Tendon developmental sequence: Vimentin.

Fig 3.6: Tendon developmental sequence: Pan-cadherin.

Fig 3.7: Tendon developmental sequence: N-cadherin.

Fig 3.8: Tendon developmental sequence: Vinculin.

Fig 3.9: Tendon developmental sequence: Connexin 32.

Fig 3.10: Tendon developmental sequence: Connexin 43.

CHAPTER 4

Fig 4.1: Tendon fibroblast monolayers: ECM and cytoskeletal components.

Fig 4.2: Tendon fibroblast monolayers: Cell junctional components.

Fig 4.3: Tendon fibroblast Micromasses: Collagens.

Fig 4.4: Tendon fibroblast Micromasses: Actin, pan-cadherin and n-cadherin.

Fig 4.5: Tendon fibroblast Micromasses: Connexins.

Fig 4.6: Tendon fibroblast Micromasses: Vinculin.

Fig 4.7: Tendon fibroblast pellet: Type I collagen.

Fig 4.8: Tendon fibroblast pellet: Type III collagen, type I pro-collagen, fibronectin.

Fig 4.9: Tendon fibroblast pellet: Cytoskeletal and Cell junctional components.

Fig 4.10: Tendon fibroblast pellet: Connexins.

Fig 4.11: Tendon fibroblast pellet: SEM, 1 day pellets.

Fig 4.12: Tendon fibroblast pellet: SEM, 7 day pellets.

Fig 4.13: Tendon fibroblast pellet: SEM, 14 day pellets.

Fig 4.14: Tendon fibroblast pellet: SEM, 7 day pellets, transverse section.

Fig 4.15: Tendon fibroblast pellet: SEM, 7 day pellets, collagenase digestion.

Fig 4.16: Tendon fibroblast pellet: TEM, 1 day pellets.

Fig 4.17: Tendon fibroblast pellet: TEM, 2 day pellets.

Fig 4.18: Tendon fibroblast pellet: TEM, 5 day pellets.

Fig 4.19: Tendon fibroblast pellet: TEM, 7 day pellets.

CHAPTER 5:

Fig 5.1: Growth factor treated pellet cultures: Type I collagen.

Fig 5.2: Growth factor treated pellet cultures: Type III collagen.

Fig 5.3: Growth factor treated pellet cultures: Type I pro-collagen.

Fig 5.4: Growth factor treated pellet cultures: Tenascin.

Fig 5.5: Growth factor treated pellet cultures: Fibronectin.

Fig 5.6: Growth factor treated pellet cultures: Actin.

Fig 5.7: Growth factor treated pellet cultures: Vimentin.

Fig 5.8: Growth factor treated pellet cultures: Pan-cadherin.

Fig 5.9: Growth factor treated pellet cultures: N-cadherin.

Fig 5.10: Growth factor treated pellet cultures: Vinculin.

Fig 5.11: Growth factor treated pellet cultures: Connexin 32.

Fig 5.12: Growth factor treated pellet cultures: Connexin 43.

Fig 5.13: DNA content of growth factor treated pellets.

Fig 5.14: DNA content of growth factor combination treated pellets.

Fig 5.15: Collagen content of growth factor treated pellets and media.

Fig 5.16: Collagen content of growth factor combination treated pellets and media.

CHAPTER 6

Fig: 6.1: Schematic diagram of DispoDialyzer tube within centrifuge tube

Fig 6.2: Phase contrast fibroblasts aggregates.

Fig 6.3: Phase contrast chondrocyte aggregates.

Fig 6.4: Phase contrast fibroblasts aggregates: cadherin blocking agents.

Fig 6.5: Fibroblast aggregates: Type I collagen.

Fig 6.6: Fibroblast aggregates: Type II collagen.

Fig 6.7: Fibroblast aggregates: Type III collagen.

Fig 6.8: Fibroblast aggregates: Actin.

Fig 6.9: Fibroblast aggregates: Vimentin.

Fig 6.10: Fibroblast aggregates: Pan-cadherin.

Fig 6.11: Fibroblast aggregates: N-cadherin.

Fig 6.12: Fibroblast aggregates: Vinculin.

Fig 6.13: Fibroblast aggregates: Connexin 32.

Fig 6.14: Fibroblast aggregates: Connexin 43.

Fig 6.15: Chondrocyte aggregates: Sample immunolabels.

Fig 6.16: Photographs of tendon cell aggregates: long term suspension cultures.

Fig 6.17: Photographs of chondrocyte aggregates: long term suspension cultures.

Fig 6.18: Long term aggregates: Propidium iodide labelling.

Fig 6.19: Long term aggregates: Type I collagen.

Fig 6.20: Long term aggregates: Type II collagen.

Fig 6.21: Long term aggregates: Type III collagen.

Fig 6.22: Long term aggregates: Actin.

Fig 6.23: Long term aggregates: Vinculin.

Fig 6.24: Long term aggregates: Connexin 32.

Fig 6.25: Long term aggregates: Connexin 43.

Fig 6.26: Long term embryonic fibroblast aggregates: ECM components.

Fig 6.27: Long term embryonic fibroblast aggregates: Cytoskeletal and cell junctional components.

Fig 6.28: Long term aggregates, 50+100 kDa MWCO: Collagens.

Fig 6.29: Long term aggregates, 50+100 kDa MWCO: Tenascin, actin, vimentin.

Fig 6.30: Long term aggregates, 50+100 kDa MWCO: Cell junctional components.

Fig 6.31: Long term aggregates, 50 and 100 kDa MWCO: Connexins.

Fig 6.32: Long term aggregates, growth factor treated: ECM.

Fig 6.33: Long term aggregates, growth factor treated: Cytoskeletal components.

Fig 6.34: Long term aggregates, growth factor treated: Cell junctional components.

Fig. 6.35: 50kDa and 100kDa Collagen quantification: with DNA normalisation.

Fig. 6.36: 50kDa and 100kDa Collagen quantification: without DNA normalisation.

Fig. 6.37: 50kDa and 100kDa: 7 and 14 days.

Fig. 6.38: Collagen quantification of 300kDa MWCO tubes.

Fig. 6.39: Collagen quantification of IGF-1 treated cultures.

Fig. 6.40: Collagen quantification of TGF-beta treated cultures.

LIST OF TABLES

CHAPTER 2:

Table 2.1: Summary of primary antibodies.

Table 2.2: Summary of secondary antibodies.

CHAPTER 5:

Table 5.1: Summary of growth factors combination pellet immunological results

ABBREVIATIONS

bFGF- Basic fibroblast growth factor
BMP- Bone morphogenic protein
CS- Chondroitin sulphate
DAPI- 4',6-Diamidino-2-phenylindole
DMEM- Delbecco's modified eagle medium
DS- Dermatan sulphate
DSHB- Developmental studies hybridoma bank
ECM- Extracellular matrix
EGF- Epidermal growth factor
FACIT- Fibril associated collagens with interrupted triple helices
FBS- Foetal bovine serum
GAG- Glycoaminoglycan
GF- Growth factors
GFP- Green fluorescent protein
GP- Glycoprotein
GTPase- Rho-type guanosine triphosphatase
HAV- Histidine, Alanine, and Valine
HCL- hydrochloric acid
HGF- Hepatocyte growth factor
HH- Hamburger and Hamilton, chick developmental stages
IGF- Insulin-like growth factor
IL-1- Interleukin-1
IMS- Industrial methylated spirits
KDa- KiloDaltons
LAP- Latency associated protein
LTBP- Latent TGF β binding proteins
MMP- Matrix metalloprotease
MSC- Mesenchymal progenitor cells
MTOC- Microtubule organising centre
MWCO- Molecular weight cut off
NBFS- Neutral buffered formalin solution
PAI-1- Plasminogen activator inhibitor-1
PBS- Phosphate buffered solution
PECAM-1- Platelet endothelial cell adhesion protein-1
PDGF- Platelet derived growth factor
PDZ- Peptide binding domain

PG- Proteoglycan

SEM- Scanning electron microscopy

TEM- Transmission electron microscope

TGF β - Transforming growth factor beta

TIMP- Tissue inhibitor of metalloprotease

TNF- α - Tumour necrosis factor-alpha

VE-cadherin- Vascular endothelial cadherin

VEGF- Vascular endothelial growth factor

ZO-1- Zonula Occludens-1

CHAPTER ONE
General Introduction

INTRODUCTION

The musculoskeletal system functions to provide mechanical support to and permit movement of, the body. The connective tissues involved in this system, in particular tendon, ligament, cartilage and bone, all resist the associated mechanical loads by producing a specific extracellular matrix (ECM), varying according to tissue type and strain experienced. The focus of this thesis is tendon, and in particular how tendon cells interact with one another and deposit organised extracellular matrix, in development and in tissue culture.

1.1 TENDON STRUCTURE AND FUNCTION

Fundamentally, tendons connect muscle to bone and function to transfer tensile loads generated by the muscle to bone. Not all muscles have tendons however, where a muscle simply holds two pieces of bone together. Its origin and insertion can be directly into the bone, without intervening tendinous tissue, for example in the hip. Where tendons are present they have a variety of functions associated with the primary one of force transfer, which relate to more general anatomical or biological function, as reviewed by Benjamin and Ralphs (2000), and summarised below. Firstly, tendons allow muscles to be remote from their site of action. Particularly in the limbs, the major flexor and extensors are too bulky to fit within the hand or foot, and so are positioned proximally with long tendons connecting them to their site of action. Secondly, they allow muscles to act through a confined space, for example the carpal tunnel, and permit change in direction of pull, tendons may wrap around a bony pulley such as the malleolus in the foot. Thirdly, tendons form part of the muscle. The tendon unit and muscle part can be matched in length with the required amount of movement, so that excessive amounts of expensive muscle tissue do not have to be maintained unnecessarily. Also, the mechanical properties of tendon are matched to the muscle-tendon unit. Tendons have different stretching properties according to the function of their muscle-tendon unit. An important function is an extreme variation on this. Strained tendons act as elastic energy stores, for example the Achilles tendon in man can reduce muscle input during movement. It is also possible for tendons to focus the pull of several muscles onto a single site, alternatively, they can allow one muscle to act on several sites. Tendons can support or even form part of the joint capsule, this occurs in the knee joint and in the joints of the fingers. Tendons extend from their junctions with the muscle, the myotendinous junction, to their attachment to bone, the enthesis. In between, they have a variety of forms, ranging from round, through to oval and finally to the extremely flattened aponeurosis, according to the muscle

and bone shape and function. Loading generally occurs uniaxially along the long axis of the tendon, and the collagen rich ECM is aligned in this direction.

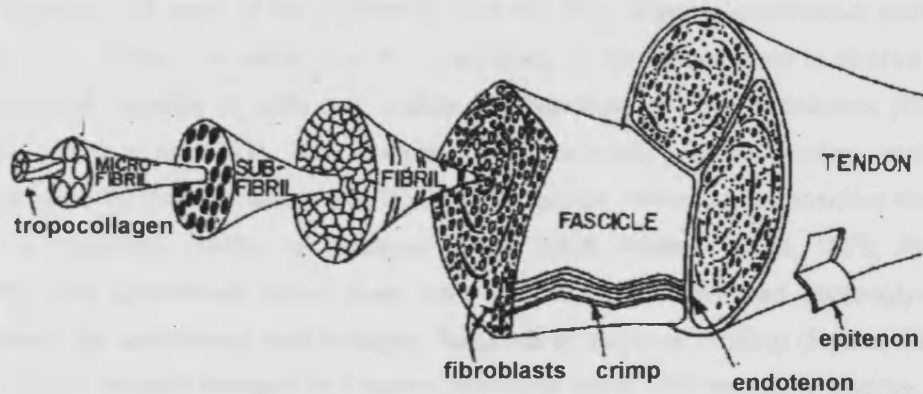


Figure 1.1. The hierarchical structure of tendon: organisation from whole tendon to collagen substructure. Modified from Kastelic, 1978.

Tendons run through tissues, and therefore have to be contained within specialised spaces within those tissues. At the simplest, there are spaces in the loose connective tissues, where the tissue simply forms a hollow tube for the tendon to run through; the connective tissue boundary is referred to as the paratenon. The main components of the paratenon include type I and type III collagen and elastic fibrils (Kvist et al, 1985) and the inner surface is lined with synovial cells (Jozsa and Balint, 1979). In a number of sites structures have to be more sophisticated, such as where tendon press on bony pulleys, run through confined spaces and/or undergo extensive movements. In these circumstances there may be a synovial bursa between tendon and bone, or a full scale synovial sheath. These consist of 2 thin layers, parietal and visceral, enclosed between these layers is a fluid for lubrication. The majority of tendons do not have a proper sheath and are surrounded by the paratenon, often confused with the tendon sheath and is essential for smooth tendon gliding and function.

In regions of extensive movement in the joints, tendons and their sheaths are retained by retinacula, ligaments that wrap around them and bond them to the bone, preventing bowstringing. Within their sheaths tendons generally have a

complex hierarchical structure. The whole tendon is enclosed by the epitenon, which consists of a dense connective tissue layer that contains a braided network of collagen fibres and protects the tendon, similar to a coaxial cable. (Reviewed by Benjamin and Ralphs, 2000). The outer surface of the epitenon is continuous with the synovial cell layer of the paratenon and the inner layer is continuous with the endotenon. Within the epitendon, the main body of the tendon itself is divided into longitudinal bundles of cells and matrix, the fascicles, by the endotenon (Elliott, 1965; Jozsa et al, 1991). The fascicles carry the loading of the tendon, and are surrounded by the endotenon which is a thin reticular network of connective tissue, with a crisscross pattern of collagen (Elliot, 1965, Kastelic et al, 1978; Rowe, 1985), this connective tissue layer has high levels of hydrated proteoglycans between the endotenon and collagen fascicles to improve binding (Rowe, 1985). The blood vessels present in tendon, although small and relatively sparse, are present in the endotenon layer together with tendon innervation (Elliot, 1965; Hess et al, 1989; Benjamin and Ralphs, 1997). This connective tissue part of the tendon often has a delicately layered collagen pattern, however, it can also become quite dense, forming layers upon layers of collagen wrapped around the fascicles.

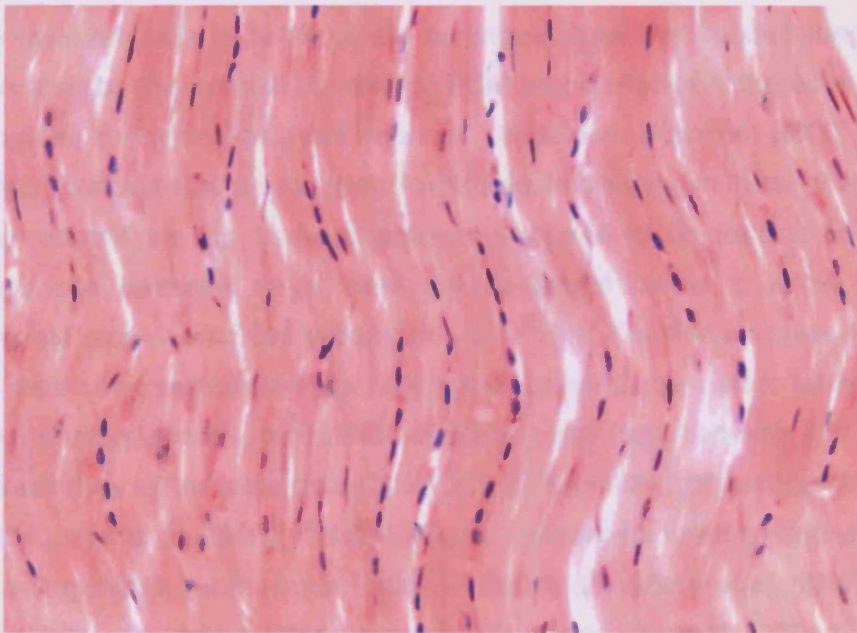


Figure 1.2 Histological section of tendon (LS). Haematoxylin / Eosin stain
(Courtesy of Dr J. Ralphs).

Collagen fibres have also been reported to lie transversely and horizontally, with longitudinal fibres crossing over one another forming plaits and spirals (Chansky and Iarrotti, 1991; Jozsa et al, 1991). This variation in collagen fibre orientation provides the tendon with an increase capacity for dealing with rotational, transverse and horizontal forces (Fan et al, 1997). The ratio of longitudinal versus horizontal fibres ranges from 10:1 to 26:1 dependant on tendon location (Jozsa et al, 1991). The majority of type I collagen fibres run parallel and longitudinally with rows of tendon fibroblasts positioned between them. These fibroblasts have a complex shape and relationship with the collagen fibre bundles (McNeilly et al, 1996).

Tendons consist of various specialised regions, namely the myotendinous junctions, connecting the muscle fibres to the tendon proper, the enthesis or osteotendinous junction, connecting tendon to bone and the wrap-around regions where tendons change the direction of their 'pull', due to a pulley system.

1.2 TENDON CELLS

The resident cells in tendons are arranged in evenly spaced longitudinal rows separated by the parallel bundles of ECM and have been characterised as either elongated tenocytes or more rounded tenoblasts (Kannus, 2000; Chuen et al, 2004). To date there are no specific markers to distinguish these cells and they are often treated as the same population, referred to as tendon fibroblasts. The differences between these cells are described below to allow the reader a complete overview of cell populations in tendon. For this purpose, the tenoblasts can read as young tendon fibroblasts and tenocytes as mature tendon fibroblasts. In developing tendons, tenoblasts synthesise and deposit matrix and as the tendon matures they either undergo apoptosis or differentiate into tenocytes (Ippolito et al, 1980; Jozsa et al, 1991). Tenoblasts are often considered as activated tenocytes regarding matrix synthesis or intrinsic healing (Davidson et al, 1997). The size of tenoblasts can vary in length from 20-70µm and between 8-20µm in width, with ovoid nuclei (Ippolito et al, 1980). The morphological features of young tenoblasts have features suggesting active matrix synthesis (Kannus, 2000), with well developed rough endoplasmic reticulum and Golgi, although there are few mitochondria in the cytoplasm (Moore and De Beaux, 1987). The mitochondria do however have well defined cristae (Ippolito et al, 1980), suggesting an efficient energy producing system. There are also fewer actin filaments detectable in tenocytes than in tenoblasts (Jozsa and Balint, 1979, Ippolito et al, 1980).

As development proceeds and the matrix to cell ratio increases, more tenoblasts adapt into tenocytes. They increase in length and decrease in width and each cell has less cytoplasm as the nucleus takes up almost the entire length of the cell (Ippolito et al, 1980). The ratio of inter-conversion between tendon fibroblast states has been suggested to govern the tendon response to exercise or trauma (Chuen et al, 2004). However, both tendon cell states are active in matrix production (Jozsa et al, 1991; O'Brien, 1992). Tenocytes have been shown to be located evenly throughout the tendon in longitudinal parallel rows (Chuen et al, 2004) whereas tenoblasts appear in clusters in the peripheral region of the tendon, devoid of collagen fibre anchorage (Chuen et al, 2004). This location together with the increased level of MMP-1 produced by tenoblasts suggests a role for this population in a migratory response to injury (Stamenkovic, 2003). It is possible these cells belong to the epitendon of peripheral endotenon, whose cells are also known to migrate to injury sites (Manske and Lesker, 1984). In the remainder of this thesis the cells of tendon will be referred to as tendon fibroblasts.

Tendon cell metabolism

Tendon fibroblasts have been shown to utilise all three of the metabolic pathways used by eukaryotic cells, namely the Krebs's cycle, glycolysis pathway and the pentose phosphate shunt (Tipton et al, 1975 and 1978; Jozsa et al, 1979; Kvist et al, 1987). During development all three pathways are active, reflecting the energy requirements of tissue morphogenesis and growth (Jozsa et al, 1979). During maturation, metabolic activity involving the aerobic Krebs's cycle and pentose shunt pathway decreases and the anaerobic glycolytic pathway predominates (Hess et al, 1989; Kannus and Jozsa, 1991). This increase in anaerobic activity correlates with an over all decrease in the metabolic activity of mature tendon (Kannus, 2000). Sustained periods of load and tension subject mature tendons to low oxygen levels, as blood vessels are squeezed preventing blood flow, and the use of the glycolytic pathway compensates for ischemia and prevents necrosis during these periods (Kannus, 2000). However, this low metabolic rate, results in a slow rate of recovery after activity or in the repair processes (Vailas et al, 1978; Williams, 1986).

In transverse section mature tendon fibroblasts are stellate and thin (see fig. 1.3). They have lateral, sheet-like cytoplasmic processes extending around collagen fibre bundles and are in contact with processes of cells in adjacent rows through gap junctions (Jozsa et al, 1991; McNeilly et al, 1996; Ralphs et al, 2002). This

multitude of intercellular contacts creates a communicating network that allows the tendon fibroblasts to respond to stimuli and coordinate these responses e.g. increased load (McNeilly et al, 1996; Benjamin and Ralphs, 2000).

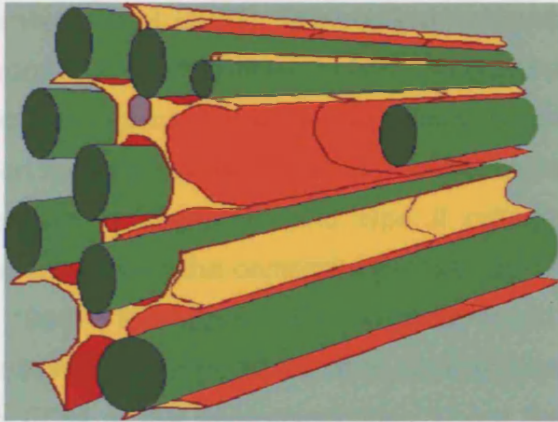


Figure 1.3 An interpretation of the three-dimensional structure of tendon, showing clearly the stellate shape of the cells at cross-section (yellow) and the longitudinal collagen (green) spanning multiple cell lengths. (Courtesy of Dr JR Ralphs)

Other tendon associated cells make up between 5-10% of cellular elements, these include synovial and vascular cells (Kannus, 2000). The epitenon and endotenon each contain a distinct cell population, as does the paratenon. Each cell population can be isolated and have been shown to react differently to various treatments or injury (Fujita et al, 1992; Wojciak and Crossan, 1993; Tsuaki et al, 2000; Klein et al, 2001, 2002).

Fibrocartilage cells

In regions where the tendon is wrapped around a bony or fibrous pulley fibrocartilaginous regions of tendon are present (Vogel and Koob, 1989; Benjamin and Ralphs, 2004). These regions protect the tendon from being damaged by compressive loading against the pulley (Ralphs et al, 1991), for example the peroneus longus grooving the cuboid bone. There are three types of fibrocartilage present in tendons these can affect cells of the epitenon, endotenon and of the tendon sheath showing the complexity of this tissue specialisation (Benjamin et al, 1995). The fibrocartilaginous cells lie in lacunae like chondrocytes in articular cartilage (Merrilees and Flint, 1980). Fibrocartilage also has a structural role in other areas including the menisci of the knee joint (Adams and Muir, 1981) and the intervertebral disc (Humzah and Soames, 1988).

1.3 TENDON STRUCTURAL MACROMOLECULES

Tendon matrix consists primarily of type I collagen. It constitutes over 95 % of the entire collagen content, with the rest being a mixture of types III, V, VI, IX (Gelberman et al, 1988; Vogel and Koob, 1989), XII and XIV (Walchli et al, 1994).

Matrix composition varies in specialised regions of tendons. The enthesis and other fibrocartilaginous regions may contain type II collagen and aggrecan (Waggett et al, 1998) and the myotendinous junctions are rich in tenascin (Jarvinen et al, 2003). Proteoglycans found in tendon include decorin, biglycan, fibromodulin and lumican. In fibrocartilaginous regions the composition of collagen and proteoglycans vary to the tendon proper. Also aggrecan and versican have been found in areas of tendon under compression (Vogel et al, 1994). There is an increase in aggrecan, and type II collagen mRNA is present, thought to be associated with the compressive loads that this region is subjected to (Waggett et al, 1998). Fibronectin and thrombospondin-1 are ubiquitous in tendon (Jozsa et al, 1989; Kannus et al, 1998). Immunohistochemistry showed both to be prominent in the ECM of the anatomical sites where firm bindings are needed, for example between muscle fibres and fibre bundles, between the collagen fibres of a tendon and at myotendinous junctions, osteotendinous junctions and articular cartilage (Kannus et al, 1998).

1.3.1 Collagens

Collagens are the main structural components of all ECMs. There are at present 27 collagens identified (Mayne and Brewton, 1993; Myllyharju and Kivirikko, 2001; Sato et al, 2002; Pace et al, 2003). Collagen is characterised by its triple helical structure. The triple helical domain comprises of three polypeptide chains, each with a left-handed helical configuration wound around each other to form a right-handed super helix. There is a glycine amino acid at each third residue and this, the smallest amino acid, is vital in the establishment of a stable helix as it is the only amino acid with a side chain small enough to fit in the core of the helix without distorting it. In the repeating sequence Gly-X-Y, the X is nearly always proline residue and the Y hydroxyproline. It is the hydroxy group from this amino acid that is essential in the formation of hydrogen bonds that stabilise the triple helix.

Fibrillar Collagens

Type I, II and III collagen are the major fibrillar collagens and provide structural integrity and mechanical strength for all connective tissues. Together these collagens account for 80% of total collagens in the body (Jozsa et al, 1989). All these collagen types assemble intracellularly into a procollagen triple helix formation (Birk and Zycband, 1994). The procollagen is synthesised as 300nm long molecules in the rough endoplasmic reticulum (McLaughlin and Bullied, 1998; Lamande and Bateman, 1999; Canty and Kadler, 2002). Procollagen is then

strengthened by covalent lysine-derived crosslinks between the monomers as a result of the enzyme lysyl oxidase (Eyre et al, 1984). Procollagen molecules are then secreted from the cell. The propeptides at the N- and C- termini that prevent self-assembly intracellularly, are cleaved by specific procollagen N-proteinases (or ADAMTS-2) and a procollagen C-proteinase, which is identical to BMP-1 (Kessler et al, 1996; Li et al, 1996; Leung et al, 1979; Prockop and Fertala, 1998). When the C- and N termini have been cleaved the secreted collagen molecules from the cell self-assemble into triple helical fibrils extracellularly (Fessler and Fessler, 1978), with the characteristic repeating banded sequence at 67nm (Kadler et al, 1996). Cleavage of the C-terminus alone is enough to induce collagen self-assembly (review Kadler, 1996). Collagens with incomplete cleavage are present in the developing chick tendon (Fleischmajer et al, 1983 and 1987), and are thought to be involved in fibril diameter regulation (Fleischmajer et al, 1983 and 1987, 1988).

Type I collagen is the most common fibril-forming collagen of the body. It is found mainly in fibrous tissue and has varying organisations to meet the differing functions of these tissues. In skin it forms a sheet-like structure, in bone it is reinforced by calcium hydroxyapatite, but in tendon it has a rope-like structure (Birk and Zycband, 1993). Type I collagen interacts with the protein cores of the proteoglycans decorin and fibromodulin, which are involved in regulating fibrillar diameter (Schonherr et al, 1995a, b) and can also form heteropolymers with type III collagen (Fleischmajer et al, 1990). The production of type III collagen is essential for normal type I collagen fibrillogenesis (Lui et al, 1997). Type III collagen is found throughout tendon proper, in the endotenon, in unmineralised fibrocartilage regions (Kumagai et al, 1994) and in the epitenon (Murphy et al, 1997). Type III collagen fibres are thinner than type I fibres, resulting in a more compliant tissue (Borel and Monboisse, 1993). Type II collagen, the major collagen in cartilage (90% of total collagen), provides resistance to compressive force, supports chondrocyte adhesion and possibly influences the differentiated phenotype of chondrocytes (Lemare et al, 1998). It forms a network of fibrils that contain proteoglycans, due to a higher content of hydroxylysine and glycosyl and galactosyl residues compared to type I collagen (Mayne et al, 1989). It is not generally distributed in tendon (Waggett et al, 1998), but can be observed in regions of fibrocartilage (Ralphs et al, 1991). There are two splice variants, type IIA and type IIB (Ryan and Sandell, 1990). Type IIA is expressed in pre-

chondrogenic tissue, whereas type IIB is expressed only in chondrogenic tissues (Sandell et al, 1990; Nah et al, 2001; McAlinden et al, 2002).

The minor fibrillar collagens are type V and type XI collagens. Type V collagen is associated with types I, II and III collagen, forming heterotypic fibrils with each (Keene et al, 1987; Fleischmajer et al, 1990). Type V collagen is involved in regulating type I fibril diameter by forming the core of type I fibrils (Lindsenmayer et al, 1990). It is widespread in virtually all connective tissues and occurs as non-banded fibrils with small diameters. Type XI collagen is found mainly in cartilaginous tissue (von der Mark, 1999), but is also seen in developing tissues where it forms cross-type heterotrimers with type V (Balmain et al, 1995; Nalin et al, 1995; Yoshioka et al, 1995; Fichard et al, 1995). It is found in the same fibrils as type II (Vaughan, 1998) forming the core of type II collagen heterofibrils (von der Mark, 1999), and thought to influence the fibril diameter. The section of the type II fibril combined with type XI collagen is possibly where the fibril interacts with matrix proteoglycans (Smith et al, 1985). Both types V and XI collagen have large N-terminal non-collagenous domains. The incorporation of these non-collagenous domains into heterotypic fibrils is suggestive of involvement in collagen assembly, growth and diameter regulation (Fessler et al, 1985).

Short Chain Collagens

Types VIII and X collagen are distinguished by shorter helical domains than the fibril-forming collagens and have globular extensions at one or both ends (Sutmuller et al, 1997). They both form hexagonal lattice structures and have distinct distributions. Type VIII is found primarily in a specialised membrane produced by endothelial cells in the cornea, called the Descemet's membrane (Sawada et al, 1990), although it is also associated with type IV collagen. Type X collagen is found in hypertrophic cartilage (Schmid and Lisenmayer, 1985), and is thought to be involved in growth plate development and calcified cartilage formation (Kwan et al, 1989; Kirsch and von der Mark, 1992; Alini et al, 1994). However, it has also been suggested that type X collagen interacts with type II collagen to prevent calcification (Poole et al, 1989). It has been described in the tendon enthesis or ligament bone-interface, where fibrocartilage cells undergo hypertrophic changes as bone grows into the enthesis (Niyibizi et al, 1996; Ralphs and Benjamin, 2000).

FACIT collagens

Fibril Associated Collagens with an Interrupted Triple helix (FACIT) collagens act mainly as molecular linkers between fibril forming collagens and other ECM molecules (Shaw and Olsen, 1991). FACIT collagens include type IX, XII, XIV, XVI and XIX collagen. Type IX collagen has been shown to co-localise with type II collagen (von der Mark, 1999) and is involved in type II fibrillogenesis (Eyre et al, 1987). It binds covalently to type II collagen and acts to stabilise the network. It can also form heterotypic fibrils with type XI collagen. Type IX collagen is found in cartilage and fibrocartilage, such as the intervertebral disc, and is thus a possible component of tendon fibrocartilage. Both IX and XII can be glycanated with chondroitin/dermatan sulphate GAG side-chains and classified as proteoglycans (Koch et al, 1992).

Type XII collagen is associated with type I collagen and located in the skin, perichondrium, periosteum, and tendon, among others (von der Mark, 1999). Type XII collagen is also found in the developing chick, prior to hatching in tissues exposed to high mechanical stresses, ie. tendon and ligament (Dublet and Van der Rest, 1991; Walchli et al, 1994). It is localised at the surface of tendon collagen fibrils (Khang et al, 1993).

Type XIV collagen is mainly found in tissues rich in type I collagen, also where high mechanical stresses are involved. Type XIV collagen has been shown to take over the role from type XII collagen (Walchli et al, 1994) and is the predominant FACIT protein in virtually all tissues. Type XIV collagen has been found in developing tendon with type XII collagen (Dublet and Van der Rest, 1991), and is thought to be involved with regulating fibril formation much the same as type V collagen (Linsenmayer et al, 1990). It does this by stabilising the immature collagen fibrils during early fibril growth phase (Young et al, 2000). Type XIV collagen is also known to bind to decorin (Font et al, 1993) and is present in its proteoglycan form in cartilaginous tissues (Watt et al, 1992). Type XVI collagen is found in a variety of tissues, including the heart, kidneys, intestine, ovaries, testes, eyes, arterial walls and smooth muscles (Lai and Chu, 1996). Type XIX collagen has only been characterised at cDNA and genomic levels, but is expressed in fibroblastic cell lines (Myers et al, 1994). The roles of type XVI (Lai and Chu, 1996), type XIX (Myer et al, 1997) and type XX collagen (Koch et al, 2001) have not been established.

Type VI and VII Collagen

Type VI collagen is essentially a glycoprotein with a collagenous central domain, it is often referred to as a microfibrillar collagen. Its functions involve binding cells to fibrillar collagens, decorin and hyaluronan (Bindanst et al, 1992; Kielty et al, 1992; Pfaff et al, 1993). In tendon type VI collagen has a wide distribution in the ECM, whereas in articular and fibrocartilage it appears in the pericellular region of the matrix, close to the cell and aids in the chondrocyte attachment to the matrix (Poole et al, 1992). Type VII is a major component of anchoring fibrils attaching basement membranes of multilayered epithelia to the underlying connective tissues (Sakai et al, 1986).

Membrane bound and MULTIPLEXIN collagens

Membrane bound, or transmembrane collagens include types XVII and XIII. They each have intracellular domains and are involved in cell adhesion and possibly signal transduction (Kadler, 2002). Type XVII collagen is located in hemidesmosomes and its mutations have been linked to the cause of non-lethal blistering of the skin where tissue separates from the cutaneous basement membrane zone (Gatalica et al, 1997). Type XIII collagen has been found in the skin epidermis, placenta, intestine, bone, cartilage and striated muscle and others, although only in cDNA and genomic levels (Peltonen et al, 1997). Research using mouse models indicate that type XIII is a transmembrane component of focal adhesion sites (Peltonen et al, 1999).

Types XV, XVI and XVIII collagens are MULTIPLEXIN (multiple triple helix domains and interruptions) collagens. These are non-fibrillar collagens, type XV collagen has only been identified at cDNA and genomic levels, whereas type XVIII collagen has been isolated in a Western blot from extracts of mouse embryonic stem cells (Muragaki et al, 1995). Type XV collagen is widespread and has been found in the basement membrane zones of the placenta, adrenal glands, kidneys, heart, skeletal muscle and gonads (Myers et al, 1996). The distribution of type XVIII collagen is more specific and its three variants are expressed in a tissue specific manner, including the liver, lungs kidneys, heart, brain and skeletal muscle (Muragaki et al, 1995; Myers et al, 1996).

1.3.2 Elastin

Elastin is a hydrophobic protein of the ECM known to give tissues their elastic qualities. It is widely distributed, but most abundant in artery walls, lungs,

intestines and skin. Elastin has many external linkages making it a stable protein. As with most structural proteins other roles have been found for elastin, an elastin receptor has been identified and there are now known to be various biological properties of elastin and elastin-derived peptides (Debelle and Tamburro, 1999; Gosline et al, 2002). Elastin generally exists as an amorphous element surrounded by a rim of microfibrils (Karrer and Cox, 1960; Haust, 1965). Elastin fibrillogenesis occurs close to the cell surface and it is thought that the microfibrils act as a scaffold for the elastin to be deposited (Robert et al, 1971; Cleary et al, 1981; Cleary and Gibson, 1983).

In tendon development elastin appears as fibrillar tracts at the periphery of the tendon, and fibrillin appears a little later (Ros et al, 1995). Elastin becomes more widespread with the onset of muscle contraction and has been suggested that it helps to maintain the structural integrity of developing tendons (Benjamin and Ralphs, 2000). In adult tendon the elastin content is approximately 1-2% of tendon dry mass (Kannus, 2000) and in healing there is an increase in elastin deposition (Gigante et al, 2003).

1.3.3 Non-collagenous components of the ECM

Extracellular matrix molecules that surround collagens are soluble polymers and these include: glycoproteins, glycosaminoglycans (GAGs), proteoglycans.

Glycoproteins

Glycoproteins are multifunctional proteins with carbohydrates attached covalently to the peptide portion of the molecule. They interact with cells and the surrounding matrix molecules. A subgroup of matrix glycoproteins is involved in cell adhesive. In tendon these include; fibronectin, vitronectin, laminin, tenascins, thrombospondins (cell-matrix interactions modulators), von Willebrand factor (collagen associated), undulin (type XIV collagen $\alpha 1$ chain fragment), osteonectin and osteopontin (each found in calcifying regions of tendon) (Miller and McDevitt, 1988; Jozsa et al, 1991; Maillard et al, 1992; Usui et al, 1992; Ehnis et al, 1997; Kannus et al, 1998; Halverson et al, 2003).

Fibronectin

Fibronectin is a widely distributed adhesive glycoprotein. Its general functions include controlling cellular migration throughout development and wound healing, and in regulation of cell growth and differentiation (Burton-Wurster et al, 1997). It is

composed of 2 large subunits joined by disulphide bonds near the C-terminus. Along the 2 subunits are multiple binding sites for GAGs, collagens and cells (Akiyama et al, 1981). Levels of fibronectin synthesis increase in healing tendon (Williams et al, 1984). Fibronectin and its isomers have the potential to participate in cell signalling through the integrin binding RGD sequence (arginine, glycine, aspartate; Pierschbacher and Ruoslahti, 1984), and whilst they are thought to be critical in chondrocyte biology and matrix organisation (Burton-Wurster et al, 1997), little is known about its role in tendon. However, vitronectin is seen in the ECM of the myotendinous and osteotendinous junctions (Kannus et al, 1998).

Tenascins

The tenascin family have 5 known members, tenascin C, R, X, Y and W (Erickson, 1993; Weber et al, 1998; Chiquet-Ehrismann, 2004). This glycoprotein family is thought to be involved in the migration of cells, and can act as an anti-adhesive agent (Chiquet-Ehrismann et al, 1988). However, tenascin has been shown to encourage adhesion with certain cell types, e.g. endothelial cells (Sriramarao et al, 1993). The N-terminus is responsible for the anti-adhesion properties, while the C-terminus is involved in its adhesive qualities (Spring et al, 1989). Tenascin-C is the most studied of the family and is upregulated in tendon during embryonic development, but present throughout and into mature tissue (Hurle et al, 1990; Ros et al, 1995; Kardon, 1998). Tenascin-C is the first identifiable ECM component of developing tendons (Hurle et al, 1990). Experiments performed with tenascin transgenic knockout models have shown that in the absence of tenascin-C tendons will still form with accurate structure and morphology (Kardon, 1998). It is also interesting to note that it is found in ECM of connective tissue regions where especially high forces are transmitted from one tissue type to another, for example at tissue-tissue boundaries, such as the myotendinous junction (Erickson and Bourdon, 1989; Kannus et al, 1998).

Laminins occur mainly in the basement membranes (Beck et al, 1990), and are the first glycoproteins to appear during development (Leivo et al, 1980). They have an important role in the development of the myotendinous junction (Pedrosa et al, 2000). Laminins are involved in cell adhesion, migration and differentiation (Timpl et al, 1983; Dziadek et al, 1995; Ryan and Christiano, 1996). There are 6 isoforms of laminin, each with distinct biological functions.

The thrombospondin family of glycoproteins are involved in cell adhesion interactions. Thrombospondin 1 and 2 are closely related and form subgroup A, whereas thrombospondin 3, 4 and 5 (more commonly known as cartilage oligomeric matrix protein) form subgroup B (Adams, 2001). Cartilage oligomeric matrix proteins or COMP consists of 5 identical coiled glycoprotein subunits joined by disulphide bonds (Efimov et al, 1996). Initially discovered in cartilage its distribution is now known to be more widespread, including expression in tendon, ligament, meniscus, vasculature, skeletal muscle, bone, eye, heart and placenta (Fang et al, 2000; Muller et al, 1998; Vogel and Meyers, 1999; Reissen et al, 2001). Experiments on COMP in tendon have identified a possible function as a response and resistance to load (Smith et al, 1997). COMP expression in tendon is affected by age, disease and exercise (DiCesare et al, 1994; Smith et al, 1997; Delot et al, 1998; Smith et al, 1999; Cherdchutham et al, 1999; Smith et al, 2002).

Glycosaminoglycans

GAGs consist of repeating disaccharide units. GAGs with specific physiological significance include; hyaluronan, dermatan sulphate, chondroitin sulphate, heparan, heparan sulphate and keratan sulphate. Each has a wide range of functions including providing structural integrity for cells and creating spaces for migratory cells. In tendon the concentration of GAG is lower than in any other connective tissue (Kannus, 2000), being approximately 0.2% of the total dry mass, and being elevated to 3.5-5% in fibrocartilaginous regions (Merrilees and Flint, 1980). In tendon the majority of GAG is dermatan sulphate (60%), whereas in the fibrocartilaginous regions it is chondroitin sulphate (Merrilees and Flint, 1980). Hyaluronic acid (HA) comprises 6% of the total GAG in tendon. Heparan sulphate GAGs have been located at the myotendinous junction of tendon and are implicated in biological processes such as growth factor signalling, cell adhesion and wound healing (Jarvinen et al, 1991; Nakato and Kimmata, 2002).

Proteoglycans

Proteoglycans are a large and diverse family of molecules. They are a subclass of glycoproteins and each has one or more GAG side-chains attached to their core proteins. They are involved in multiple biological processes and their distribution is ubiquitous. In tendons, an increase in the levels of proteoglycans is often related to tendon pathology (Benazzo et al, 1996). In the ECM, they can be divided into two groups; large aggregating proteoglycans and small leucine-rich proteoglycans (SLRPs; Kolset et al, 2004).

Large proteoglycans

The large aggregating proteoglycans are part of the hyaluronan binding family (Roughley and Lee, 1994), this is due to their ability to bind to hyaluronan non-covalently and form supramolecular aggregates (Margolis and Margolis, 1994). This group comprises of aggrecan, versican, neurocan and brevican. Neurocan and brevican are unique to nervous tissue (Milev et al, 1998).

Aggrecan is a large aggregating proteoglycan with a protein core of 250-300 kDa, with around 100 chondroitin sulphate chains and 50 keratan sulphate chains. It aggregates with HA and binds water so the tissue becomes hydrated and fills interstitial spaces to resist compression (Watanabe and Yamada, 2002). Some proteoglycans, including aggrecan, can trap up to 50 times their own weight in water, this can also aid the diffusion of water soluble molecules and the migration of cells (Karpakka et al, 1991). Aggrecan accounts for approximately 10% of the dry weight of cartilage and is found in small amounts in the tendon proper, but in larger amounts in fibrocartilaginous regions of tendon (Waggett et al, 1998). It is thought that its roles overlap somewhat with versican.

Like aggrecan, versican can bind to HA forming supramolecular aggregates that bind to link protein, an ECM molecule of cartilage (Roughley and Lee, 1994). Certain domains of versican promote chondrocyte proliferation by destabilizing cell adhesion (Zhang et al, 1999) and it can inhibit mesenchymal chondrogenesis, though not condensation (Zhang et al, 1998). The roles of versican include regulation of cell motility, growth and differentiation (Perides et al, 1992). Versican is found in mid-region of tendon and ligament, but also in fibrocartilaginous regions (Cambell et al, 1996; Waggett et al, 1998; Moriggi et al, 2001).

Small leucine-rich proteoglycans

The second group of proteoglycans are the small leucine-rich proteoglycans (SLRP's), each playing an important role in matrix metabolism and collagen fibrillogenesis (Hocking et al, 1998). Included in this group are decorin, biglycan, fibromodulin and lumican, each characterised by a leucine-rich core protein (Iozzo et al, 1997) and differentiated from each other due to the number and type of GAG chains each has attached.

Decorin is so named because it decorates collagen fibrils (Krusius and Ruoslahti, 1986). It has a short core protein with a molecular weight of 40kDa with one of

either chondroitin sulphate or dermatan sulphate as its GAG chain attached to its N-terminus (Weber et al, 1996; Iozzo, 1998). In bone the GAG is predominantly chondroitin 4-sulphate, whereas in tendon and cartilage a dermatan sulphate chain is most common (Cheng et al, 1994). Its distribution is wide in connective tissues and it binds to types I, II, III, V, VI and type XIV collagen (Vogel et al, 1984; Bidanset et al, 1992; Whinna et al, 1993; Hedbom and Heinegard, 1993; Font et al, 1993). The core protein of decorin binds to type I collagen near the C-terminus (Keene et al, 2000), near to major intermolecular cross-linking sites of collagen heterotrimers. Decorin binding therefore, has the potential to affect the cross-linking of collagen (Svensson et al, 1995). The characteristic horseshoe shape that decorin forms creates a large concave surface that is the correct diameter to accommodate one triple helix collagen fibril (Schonherr et al, 1995; Weber et al, 1996). Decorin prevents collagen fibrils from aggregating laterally (Birk et al, 1995) through binding to types I and VI collagen (Weber et al, 1996; Wiberg et al, 2001), and can inhibit types I and type II collagen fibrillogenesis *in vitro* (Vogel et al, 1984; Bidanset et al, 1992). In decorin null mice, collagen fibrils have irregular contours and thin fibrils abnormally fuse with thicker fibrils causing the skin to become fragile with disorganised collagen-fibril morphology (Danielson et al, 1997). Decorin also binds to the growth factor TGF β resulting in its selective inactivation (Yamaguchi et al, 1990). As TGF β functions include the regulation of cell differentiation, growth and matrix synthesis (Iozzo et al, 1998), the binding of decorin to TGF β has a substantial regulatory role, acting to sequester the growth factor extracellularly (Hildebrand et al, 1994).

Biglycan has two chondroitin sulphate or dermatan sulphate chains GAG chains and similar leucine-rich repeats to decorin (Hocking et al, 1998; Iozzo, 1998). Its role still remains unclear, although it is known to bind TGF β (Hildebrand et al, 1994) and type I and VI collagen *in vitro* (Schonherr et al, 1995; Wiberg et al, 2001). Biglycan knockout mice have defective bone formation with a diminished capacity to produce marrow stromal cells (Young et al, 2003). Biglycan is found in greater quantities in fibrocartilaginous regions of tendon (Robbins and Vogel, 1994) and tends to be found in the pericellular matrix of dividing cells, in cartilage (Knudson and Knudson, 2001). However, chickens and other avian species lack biglycan and use a differentially expressed glycosylated form of decorin (Johnstone et al, 1993).

Fibromodulin and Lumican are structurally similar to decorin and biglycan although they contain keratan-sulphate GAG chains instead of chondroitin and dermatan sulphate chains (Knudson and Knudson, 2001). Fibromodulin and lumican bind to the same binding site on a type I collagen fibril, distinct from that of decorin (Yoon and Halper, 2004). Lumican has been found in tendon and in tendon fibrocartilage (Ezura et al, 2000). It affects the spacing and diameter of collagen fibrils and is important in corneal transparency (Rada et al, 1993) - lumican null mice have opaque corneas with abnormally thick collagen fibres (Chakravarti et al, 1998). Fibromodulin also has the ability to regulate collagen fibril formation (Ezura et al, 2000) and like decorin inhibits assembly of type I and type III collagen fibrils (Hedbom and Heinegard, 1989). It is found in many tissues including tendon, but is more abundant in cartilage (Hedlund et al, 1994). Fibromodulin knockouts alone show a reduction in tendon stiffness, irregularly contoured collagen and more fibrils with a smaller diameter (Svensson et al, 1999). Lumican knockouts show irregular collagen fibril contours not only in tendon but also in the cornea and skin, forming collagen fibrils with an increased diameter (Chakravarti et al, 2000; Chakravarti, 2002). Double fibromodulin and lumican knock-outs are smaller than the wild-type mice and show extreme tendon weakness (Chakravarti et al, 2003). The tendons have unusually high frequencies of collagen fibrils with too large or too small a diameter. It has been suggested that fibromodulin is required in early fibrillogenesis to stabilise collagen fibril intermediates with small diameters, whereas lumican is needed at a later stage to prevent the lateral growth of the fibrils (Chakravarti, 2002).

1.3.4 Inorganic Components

Certain elements of the ECM that are often overlooked are the inorganic components, which make up 0.2% of tendon dry mass (Kannus, 2000). Most inorganic components are present in trace levels, between 0.02 and 120ppm (Spadaro et al, 1970). Calcium has the highest concentration, with an increase at the insertion, and in calcifying tendons - levels increase 10-20 fold (Jozsa et al, 1989). Other inorganic ions found in tendon include magnesium, manganese, cadmium, cobalt, copper, zinc, nickel, lithium, lead, fluoride, phosphorus and silicon. These are all known to be important in growth, development and normal tendon metabolism, as in other tissues (Schor et al, 1973). The precise functions of these trace elements are mostly unknown, but copper is important for collagen crosslinking (Farquharson et al, 1989), manganese has a role in enzymatic reactions and calcium is important in the development of the osteotendinous

junction (Minor, 1980). Zinc and nickel have roles in the interaction of COMP with types I and II collagen (Rosenberg et al, 1998).

1.4 CYTOSKELETON

The cytoskeleton of eukaryotic cells is a dynamic intracellular system of protein polymers constructed throughout the cytoplasm, controlling cell shape, internal spatial organisation and motility. The three prominent components of this cellular arrangement are actin microfilaments, intermediate filaments and microtubules. These systems, although composed of different proteins, are in constant interaction with each other.

1.4.1 Actin subunits and assembly

Actin is one of the most highly conserved eukaryotic proteins (Egelman and Orvola, 1995) and makes up 5% of all intracellular protein. It plays an active role in many cellular functions, including cell movement, change of cell shape and particle movement (transport of particles and receptors over the cell surface, vesicle transport and intracellular pathogens), cell division and muscle contraction (Kabsch and Vanderkerckhove, 1992; Cramer et al, 1994; Furukawa and Fechheimer, 1997). There are three actin isoforms, α -, β - and γ - actin (Kabsch et al, 1990), which express 6 actin isoforms in mammals (Vandekerckhove and Weber, 1978; Herman, 1993). Alpha actins are found in muscle and in myofibroblasts, as α -smooth muscle actin (Premdas et al, 2001), whereas β -actins and most γ -actins are found in non-muscle cells (Vandekerckhove and Weber, 1978; Herman et al, 1993). Alpha-smooth muscle actin is expressed by many, possibly all, connective tissue cells (Kinner et al, 2001; Kinner and Spector, 2002; Kinner et al, 2002).

In non-muscle cells, actin occurs as globular actin monomer (G-actin), or as polymerised filaments (F-actin), particularly concentrated just beneath the plasma membrane. Actin filaments are dynamic, polymerising and depolymerising in response to the cellular environment and the presence of other controlling proteins (Bonder et al, 1983; Egleman and Orvola, 1995). Actin structure, assembly and function are based on the assembly dynamics of the actin filament. Filaments initially assemble at a nucleation protein and then assemble directionally (Welch, 1999; Mullins and Pollard, 1999). Structure and intracellular environment determine that they are assembled at one end, the positive end, and disassemble

at the other, the negative end, converting ATP to ADP + P_i and instigating a 'treadmilling' effect (Wegner, 1976; Neuhaus et al, 1983; Cleveland et al, 1982; Carlier et al, 1989; Carlier et al, 1992; Pollard et al, 2000).

Actin organisation and function in non-muscle cells

The cellular organisation of actin is controlled by its interactions with other proteins, for example profilin, thymosin, and actobindin. Each sequester G-actin or actin dimers, creating a cytoplasmic actin pool (Carlsson et al, 1977; Lambooy and Korn, 1988; Safer et al, 1991; Vancompernelle et al, 1992; Goldschmidt-Clermont et al, 1992; Bubb et al, 1998). Actin can be released from these proteins by others, for example profilin, which releases actin from thymosin, allowing its participation in filament assembly (Pantaloni and Carlier, 1993).

There are essentially three possible 3-D arrays of filamentous actin: gel-like network, tight parallel bundles and contractile bundles, the structures being formed by association between actin and different binding proteins. Gel-like networks support the plasma membrane and potentially determine cell shape. They are formed by interactions of actin filaments with the actin binding protein filamin, which is a flexible molecule that links filaments where they cross one another (Tu et al, 2003). These flexible links between crossing fibres result in the formation of the gel. Gel characteristics can be changed by ion concentration (Ishiura and Okada, 1979). At low concentration the gel is quite stable, whereas at higher concentration it is more likely to flow. The changes between forms is referred to as 'gel to sol' or 'sol to gel' transitions and is associated with movements at the cell surface. These may also be affected by enzymatic cleavage of actin filaments by enzymes such as gelsolin, enhancing sol formation (Yin and Stossel, 1979; Yin et al, 1981a and b; McGough et al, 2003). Fibrous arrays of actin filaments take two forms. Firstly, tightly packed bundles with parallel, similarly orientated filaments are formed by binding proteins such as fascin, fimbrin and villin. These support membrane structures such as microvilli (Bartles, 2000; Kureishi et al, 2002; Passey et al, 2004). Secondly, actin filaments can form contractile filaments, or actin stress fibres. These are arranged in an anti-parallel fashion, but are more widely spaced than actin bundles (Furukawa and Fechheimer, 1997). The actin related proteins associated with stress fibres are predominantly α -actinin and tropomyosin, which allow space for myosin molecules to bind between filaments (Langanger et al, 1986). The initiation of actin stress fibre formation begins with the binding of tropomyosin to stabilise the filaments. Tropomyosin excludes filamin

binding and allows α -actinin to bind to its barbed end (Weihing, 1985), and promotes myosin II binding. There are two types of myosin molecule binding to actin in non-muscle cells, myosin I and II. In essence, myosin I moves vesicles along normal actin filaments and it is myosin II that slides the filaments across each other creating the stress fibre (Cramer, 1999). The actin stress fibre contraction mechanism is based on the hydrolysis of ATP and they form as a response to tension generated across of the cell (Cheney et al, 1993).

Actin in connective tissues

Actin, in particular α -smooth muscle actin, is associated with a wide range of connective tissue cells (Menard et al, 2000; Premdas et al, 2000; Kinner and Spector, 2001). Tendon cells *in vivo* contain orientated stress fibres linked cell to cell along the cell row by adherens junctions (Ralphs et al, 2002). These hold cells together along the line of principal strain, and may also be involved in mechanotransduction. Mechanical strain applied to tendon cells *in vitro* produces an increase in n-cadherin, vinculin and tropomyosin, indicating the cells increase the strength of their attachments (Ralphs et al, 2002).

In vivo, actin stress fibres direct the initial elongation of the cells and control the subsequent deposition of an orientated matrix in the developing annulus fibrosus of the intervertebral disc (Hayes et al, 1999). Thus, the actin cytoskeleton may have this role in the development of tendon. Fibroblasts cultured in monolayer, have fibronectin and tenascin orientated at the cell surface. If the cytoskeleton is disrupted this is lost (Domnina et al, 1996). Tendon cells embedded into a collagen matrix initially orientate to the collagen, but newly synthesised collagen appears to be orientated by the cells and associated with the cytoskeletal orientation (Matsumoto et al, 1998). Chondrocytes show a fine network of actin fibres (Benjamin et al, 1994), rather than the large filaments seen in fibroblasts. If chondrocytes express actin stress fibres *in vitro*, they lose their phenotype and become more fibroblastic (Benya et al, 1982; Mallein-Gerin et al, 1991).

Mechanotransduction is the process by which cells convert mechanical stimuli into biochemical signals, and often involves the actin cytoskeleton. The role of actin in mechanotransduction has been investigated in the microvilli of the proximal tubule and small intestine, the hair cells of the inner ear and the cellular processes of osteocytes (Weinbaum, 2001, Yuefeng et al, 2004). Microvilli and osteocyte cell processes have the same actin related proteins, fibrin and α -actinin (Tanaka-

Kamoka et al, 1998). 'Bending' of microvilli is thought to be sufficient to activate ion channels at the tip or amplified stress at the cytoplasmic terminus of the actin bundle, where it is anchored into intermediate filaments (Weinbaum, 2001). Osteocyte cell processes are housed in mineralised canaliculi surrounded by a fluid annulus, containing pericellular matrix. The fluid drag from the annulus produces a tension on the transverse fibres in the pericellular matrix, which is then transmitted across the membrane of the cell process to the axial actin filaments in the cytoskeleton of the cell process (Weinbaum, 2001, Yuefeng et al, 2004), enabling cells to respond to external loading. Actin is also associated with integrin binding-based outside-in mechanical signalling (Juliano et al, 2004) via tyrosine kinase and phosphorylation of junctional intermediates such as FAK and paxillin (Turner, 2000; Wozniak et al, 2004).

1.4.2 Intermediate filaments

Intermediate filaments are structurally distinct from actin and microtubules, and their diameter falls between these two, hence their name. In vertebrates, cytoplasmic intermediate filaments can be grouped into 3 classes, 1) keratin filaments (McLean and Lane, 1995), 2) vimentin and vimentin-related filaments (Vikstrom et al, 1992) and 3) neurofilaments (Geisler et al, 1982). Actin and microtubules are made of globular subunits, whereas intermediate filaments are made of long fibrous subunits. They have repeated sequences of amino acids called heptad repeats, and form an extended α helix, although the amino-head and carboxy-tail terminals are non- α -helical (Stewart, 1993). To form intermediate filaments two monomers become 'coiled-coil' dimeric structures, due to the heptad repeats (which contain a greater than average number of hydrophobic residues) in the helical rod. This dimer associates with another, in an anti-parallel manner, creating a non-polarised tetramer, unlike actin and microtubules, whose functions depend on polarity (Steiner and Parry, 1985). Final stages of intermediate filament aggregation show the tetramers binding to an intermediate filament and aligning themselves along the axis of the filament, then packing together in a helical formation that is 10nm in diameter (Fuchs and Weber, 1994). The terminals at either end may function to let the filaments interact with other components of the cell, therefore allowing the filaments orientation within the cell (Fuchs and Weber, 1994).

Intermediate filaments provide the cell with mechanical stability, and can withstand greater forces than actin filaments or microtubules due to the overlapping arrays of subunits (Janmey, 1991; Stewart, 1993). Vimentin null mice show no obvious defects and can reproduce successfully (Colucci-Guyon et al, 1994), indicating possible compensatory roles between intermediate filaments. Intermediate filaments are also responsible for connections between actin and microtubules (Chang and Goldman, 2004). In tendons, vimentin-based intermediate filaments are especially prominent in pressure resisting tendon fibrocartilage (Ralphs et al, 1991) and are a dynamic feature of chondrocytes, changing their expression rapidly as pressure conditions vary on the tissue (Durrant et al, 1999).

1.4.3 Microtubules

Microtubules are the largest structures of the cytoskeleton and consist of linear, tubular polymers of tubulin, a globular protein. There are three tubulin isomers, α , β and γ (Burns et al, 1991). Microtubules consist of α and β polypeptides, during nucleation an α and β tubulin molecule join to form a heterodimer (Amos and Baker, 1979). These attach to the other dimers and form oligomers, which then elongate and produce the protofilaments. Similarly to actin filaments, microtubules have positive and negative ends (Bergen and Borisy, 1980) and therefore show characteristic 'treadmilling' dynamics (Cleveland et al, 1982). The negative ends are anchored to a microtubule organising centre, which is usually a centrosome (Brinkley et al, 1985) of which γ -tubulin is a part (Job et al, 2003). Microtubules function to provide intracellular tracks for the cell and transport vesicles, granules and organelles around the cytoplasm (Kreis et al, 1990). An important function of microtubules within cells is their role in the mitotic spindle in the separation of chromosomes during anaphase in mitosis (Lambert and Schmitbenner, 1982; Gorbsky et al, 1988). They are also the predominant components of cilia and flagella vital for the propulsion of debris or movement of small protzoa and of course sperm (Gibbons, 1981; Burgess et al, 1991). Chondrocytes often have a non-motile stereocilia extending into their ECM, possibly involved in a homeostatic feedback loop from the external environment to the cytoskeleton (Poole et al, 1985).

1.5 CELL JUNCTIONS

Direct cell-cell junctions, or cell-matrix junctions are fundamental to the construction and maintenance of tissue and organs. There are three categories of

such junctions: anchoring junctions, linking cells to one another and to the ECM; gap junctions, allowing cells to directly pass signals from one another, and sealing or tight junctions that seal spaces between cells.

1.5.1 Anchoring Junctions

In addition to being components of the anchoring junctions outlined above, various membrane proteins are associated with general cell adhesive behaviour, to other cells or to the matrix. These may be a short-lived interaction or become stabilised into adherens junctions. These first interactions are vital in cell recognition and attachment. Cell-cell adhesion does not just stick cells together but organizes cells into 3-D patterns (Gumbiner et al, 2000), and prior to any communication between cells they must first form an adhesive bond. Here the super-family of cell adhesion molecules namely cadherins is focused on, with a comment on integrins.

Cadherins are transmembrane glycoproteins with an extracellular N-terminal and an intracellular C-terminal, generally passing through the cell membrane once. The N-terminal consists of cadherin domains, these vary in number each homologous to one another. The C-terminal binds through a protein complex to cytoskeletal actin (Pavalko and Otey, 1994; Steinberg and McNutt, 1999). There are five different classes of cadherin; type I or classical, to which e, p and n-cadherin belong, type II, desmosomal, protocadherins seven-pass transmembrane and flamingo cadherins (Angst et al, 2001). There are other members of the cadherin family that do not fit neatly into these groups e.g. T-, KSP- and LI-cadherin (Wheelock and Johnson, 2003).

The key feature of the extracellular domain is that they bind to the same cadherin type as the adjacent cells, via an extracellular recognition site, the HAV tripeptide sequence histidine, alanine and valine or HAV, at the n-terminal (Blaschuk et al, 1990). Either side of this sequence are Ca^{2+} binding regions (cadherin binding is Ca^{2+} dependant) which control the specificity of the cadherin binding. Synthetic peptides containing this sequence can interrupt normal cell-cell adhesion by competing with endogenous cadherins for binding (Blaschuk et al, 1990, 1994; Johnson et al, 1999; Knudsen et al, 1998, Takechi, 1990). The intracellular C-terminal is highly conserved and is the site of interaction between the cadherin and the actin cytoskeleton. A number of proteins including catenins, vinculin and focal adhesion kinases mediate this attachment, which is essential for cadherin

clustering and the construction of an adherens junction if the interaction persists (Wheelock and Johnson, 2003).

There are many members of the cadherin super-family with different members having a degree of tissue specific expression. In connective tissues the predominant cadherin is n-cadherin, initially described in neural cells (Brakenbury, 1988; Rutishauser, 1989; Takeichi, 1990). It is expressed by tendon fibroblasts (Ralphs et al, 2002), where it is upregulated in response to mechanical loading, and pre-chondrocytes (DeLise and Tuan, 2002), where it is involved in cell condensation and chondrogenesis. Generally, in development cadherins are important in cell sorting, concentrating cells of particular types to allow tissue formation (Wheelock and Johnson, 2003).

To link the transmembrane cadherin molecules to the actin cytoskeleton a complex intracellular junction is formed, included at this complex are catenins, β -catenin, γ -catenin (also known as plakoglobin) α -catenin and p120 catenin (Stappert and Kemler, 1994; Aberie et al, 1994; Hulsken et al, 1994; Jou et al, 1995; Pokutta and Weis, 2000; Rubinfeld et al, 1995; Yap et al, 1995; Anastasiadis and Reynolds, 2000). The proteins α -actinin, vinculin, nectin, afadin and ZO-1 (PDZ protein) all play key roles in forming adherens junctions (Knudsen et al, 1995; Rimm et al, 1995; Watabe-Uchida et al, 1998; Weiss et al, 1998; Shimizu and Takai, 2003; Okabe et al, 2004).

Cadherin activity is mainly regulated by protein phosphorylation, which is a response to a variety of signals including growth factors (Shibamoto et al, 1994; Dumstrei et al, 2002), hormones (Blaschuk et al, 1994) and signals from gap junctions (Maschhoff and Baldwin 2000). Cell-cell interactions can be affected in terms of their strength by modulating the expression level or altering the activity. If you increase the cadherin content the adhesion ability of the cells will increase (Wheelock and Johnson, 2003). Cadherins are not solely involved in cell adhesion they can indirectly cause a change in cell shape and polarity (Reinsch and Karsenti, 1994; Harris and Peifer, 2004; Johnson and McConnell, 2004). The signalling pathways activated by the formation of adherens junctions include those regulated by GTPases, receptor tyrosine kinases and the Wnt pathway (Wheelock and Johnson, 2003). Cell interactions mediated by cadherins are also a pre-requisite for many other membrane receptor interactions, including gap junctional

formation (Frenzel and Johnson, 1996) and tight junction formation (Tsukita et al, 2001), discussed later.

Junctions between cells and matrix

Integrins are cell adhesion molecules, however these cell surface molecules are involved in cell-matrix interactions. Integrins are produced by a wide variety of cells, and most cell types express several different types of integrin. They exist as non-covalently bound α -integrin and β -integrin heterodimers. The combination of α and β integrins determines the binding capabilities of the dimer (Ruoslathi and Pierschbacher, 1987). Integrins vary from other cell surface receptors in that they bind to their ligands with a low affinity, and that they are usually present at a large concentration at the cell surface. However, they only bind to their ligands when they gather at a region of the cell surface above a threshold concentration (Berman et al, 2003).

In addition to roles in matrix adhesion, integrins can be involved in cell-cell binding (Martin-Bermudo and Brown, 2000), integrins can bind ECM to the cell surface, notably fibronectin (Hemler, 1990), but also other components, such as laminin, collagens, entactin, tenascin, thrombospondin, von Willebrand and vitronectin (Hemler, 1990; Rouslahti and Pierschbacher, 1987). Fibronectin fibril formation is prevented at cell surfaces if integrin binding is blocked (Darribere et al, 1990). This can have roles in general ECM organisation as actin-associated integrin orientation of fibronectin at the cell surface, which can also interact with collagen (Hayes et al, 1999). The adhesive roles of integrins are also involved in mechanotransduction. Application of load to integrins at the cell surface causes changes at the integrin/actin interface, resulting in signal transduction processes, such as the phosphorylation of FAK and other integrin/actin associated proteins (Cary and Guan, 1999; Wozniak et al, 2004).

Growth factors regulate integrin expression these can include TGF β , IGF-1, bFGF and PDGF (Loeser, 1994 and 1997; Sepp et al, 1994; Brooks et al, 1994; Kirchberg et al, 1995). Tendon cells treated with bFGF and PDGF show an increase in the regulation of integrins *in vitro* (Harwood et al, 1999), and at the myotendinous junction integrins have been associated as having vital roles in development (Swadison and Mayne, 1989; Miosge et al, 1999; Ferguson et al, 2003; Nawrothki et al, 2003).

Focal Contacts are junctions allowing the actin cytoskeleton of the cell to interact with the ECM, arranged in a similar fashion to adherens junctions but with cadherins substituted for integrins within the junctional complex. They act as anchors for the cell and relay signals involved in cell motility and survival (Schaller, 2001). They consist of a complex of integrins, and multiple intracellular attachment proteins, including talin, paxillin, vinculin and α -actinin, it is α -actinin that binds directly to the actin filaments (Critchley et al, 1999; Critchley, 2004; Nayal et al, 2004).

Desmosomal junctions use cadherins, namely desmoglein and desmocollin, to form plaques connecting the intermediate filaments of adjacent cells, rather than the actin cytoskeleton of adherens junctions (Garrod et al, 1996). Desmosomes are prominent cellular structures especially in tissue that experience mechanical stress (Wheelock and Johnson, 2003). These junctions function to resist stretch and play a structural and tension-bearing role in the cell. Hemidesmosomes use integrins to attach the intermediate filaments to ECM molecules (Jones et al, 1994; Green and Jones, 1996). Their best known function is to mediate the adhesion of epithelial cells to the basal lamina (Briggaman and Wheeler, 1975; Gipson et al, 1983). No desmosomal junctions or their associated proteins have been identified within tendon (Ralphs, personnel communication).

1.5.2 Gap junctions

Gap junctions provide intercellular communication between cells within tissues and organs. Comprised of 6 transmembrane proteins called connexins, forming a hemi-channel called a connexon. A gap junction forms when two connexons on adjacent cell membranes become aligned creating a continuous aqueous pore (Unwin, 1986). The connexins rise above the surface of the cell, and create a 2-4 nm gap between the cell membranes, hence the name 'gap' (Goodenough et al, 1996). Connexins have been classified into two main subgroups, α and β dependant on similarities in the amino acid sequence (Willecke et al, 2002). However, the numerical names of the connexins are more widely used referring to the molecular weight of the sequence in kilodaltons (Goodenough et al, 1996).

Gap junctions allow inorganic ions and other small water-soluble molecules, for example Ca^{2+} , IP^3 or cAMP, sugars, amino acids and nucleotides (anything up to 1000Da), to passively diffuse between the cytoplasm of adjacent cells (Warner et

al, 1995). The diffusion of these metabolites and nutrients depends on channel type, connexin 32 channels are more permeable to adenosine than connexin 43 channels, but ATP passes more readily through connexin 43 channels (Goldberg et al, 2002). This means the cells are communicating electrically and metabolically with each other (Kumar et al, 1996). However, it is important to note that these junctions are relaying signals, not originating them.

Connexins are generally regulated by phosphorylation. The primary region to undergo phosphorylation is the C-terminal of the protein (Lampe and Lau, 2004). The permeability of gap junctions is governed by cytosolic pH and calcium levels, if the pH level increases or the Ca^{2+} level decreases, gap junctional permeability will increase (Bennett and Verelis, 1992). Calcium dependant cell adhesion molecules, (CAMs) namely cadherins are found localised with gap junctions, and the formation of adherens junctions are known to be a prerequisite for gap junctional formation (Musil et al, 1990; Jongen et al, 1991; Meyer et al, 1992; Fujimoto et al, 1997; Segretain and Falk, 2004). There are in fact several proteins present at both adherens and gap junctions, indicating their subcellular domains are tightly regulated and that they both may play a role in cytoskeletal anchorage (Giepmans, 2004), discussed later.

Physiological roles of gap junctions and connexins

Connexin knock-out mice have outlined many aspects of connexin and gap junction function. The cellular functions of gap junctions include the propagation of actions potentials in electrically excitable tissues, including the myocardium (Gourdie et al, 1992; Reaume et al, 1995), communicating between smooth muscle (Brink et al, 1998) and relaying information across synapses (Bruzzone et al, 1997; Houlden et al, 2004). Connexin 43 knockout mice die, as connexin 43 is required for normal heart development (Reaume et al, 1995; reviewed in Barker and Gourdie, 2002). Knock-in mouse lines, where connexin 43 gene is replaced with connexin 32 or 40 rescued the lethality of connexin 43 deficient mice, although they retained distinct morphological and functional defects (Plum et al, 2000). Connexin 50 can compensate for connexin 46 in the prevention of cataracts, but is required for normal ocular growth (White, 2002). Connexin 32 mutations are associated with x-linked Charcot-Marie tooth disease, a mild peripheral neuropathy associated with myelin dysfunction (Houlden et al, 2004), but connexin 32 knock-out mice show a decrease in connexin 26, suggesting connexin 32 has a stabilising effect on connexin 26.

Extensively studied in bone, connexin 43 and 45 are expressed by osteoblasts and osteocytes, where they are involved in bone cell differentiation and remodelling (Lecanda et al, 1998; Upham et al, 2003; Thi et al, 2003; Borke et al, 2003). Although no specific roles for the different connexin networks have been determined, one type can compensate for the other (Minkoff et al, 1999). In cartilage, the cells are normally isolated from one another by an extensive matrix, however, chondrocytes in precartilaginous mesenchymal condensations express connexin 43 (Schwab et al, 1998; Zhang et al, 2002). They also re-express connexin 43 when they come into contact with one another, *in vivo*, in cell clusters that are characteristic of osteoarthritis or *in vitro* (Donahue et al, 1995; Loty et al, 2000; Hellio Le Graverand et al, 2001).

Connexin expression in fibroblasts has shown connexin 43 and 45 are present in the outer annulus of the intervertebral disc (Bruehlmann et al, 2002), connexin 43 is expressed by corneal stromal cells (Clover et al, 1996; Laux-Fenton et al, 2003) and by synovial fibroblasts (Kolomytkin et al, 1997 and 2002). Connexin 32 and 43 are found in tendon and the periodontal ligament (McNeilly et al, 1996; Yamaoka et al, 2000). In tendon connexin 32 and 43 coordinate cellular response to mechanical load and have very specific distributions (McNeilly et al, 1996; Ralphs et al, 1998; Waggett et al, 1999). The lateral cell processes radiating from the stellate shaped tendon fibroblasts communicate with the adjacent cell rows using gap junctions comprising of connexin 43, known to be regulated by mechanical strain (Banes et al, 1995a). Conversely, the gap junctions located within the longitudinal cell rows themselves consist of connexin 32 and 43 (McNeilly et al, 1996). Both connexins 32 and 43 can only form homomeric connexons (Elfgang et al, 1995), implying these connexins have different functions. The functions appear to be associated with sensing load and modulating collagen synthesis, collagen synthesis is stimulated by communication via connexin 32, but inhibited by communication via connexin 43 (Waggett et al, submitted; Waggett et al, 2001). Connexin 26 mRNA has been identified within tendon (Banes et al, 1995a), although none has been found at the protein level (Ralphs et al, 2002), suggesting either protein levels are too low, or connexin 26 is not translated.

Connexins are associated with other cell-cell junctions and electron micrographs show distinct tight junctional strands found surrounding gap junctional plaques (Kojima et al, 1999). Precipitation experiments show connexin 32 is associated with occludin, ZO-1 and claudins, proteins that form the core of tight junctions, see

below (Kojima et al, 2001). ZO-1 is associated with other connexins; 43, 45, 50, 39.1 and 36 (Giepmans and Moolenaar, 1998; Giepmans et al, 2001a, b; Barker et al, 2002; Kausalya et al, 2001; Laing et al, 2001; Neilsen et al, 2002 and 2003; Li et al, 2004).

1.5.3 Tight Junctions

Tight junctions fuse epithelial or endothelial cells forming continuous selective barriers, although more recently they have been found in myelinated cells (Gonzalez-Mariscal et al, 2003). The three integral proteins of tight junctions are occludin (ZO-1), junction adhesion molecules (JAM) and the claudin family of proteins (Schneeberger and Lynch, 2004). Tight junctions play a role in maintaining the concentration differences of ions, water, and small hydrophilic molecules between cells, and are involved in organising morphogenesis, cell polarity, cell proliferation and differentiation (Schneeberger and Lynch, 2004). Tight junctions are often seen in conjuncture with adherens, desmosomes and gap junctions (Gumbiner, 1987), as discussed above.

1.6 TENDON DEVELOPMENT

There are relatively few studies on the development of tendon, particularly in comparison with studies on cartilage. There are however, similarities between the two. From a mesenchymal origin the cells condense, increasing cell-cell contacts and inter-cellular communication (Hall and Miyake, 2000). This process has been extensively studied in cartilage, demonstrating important roles for gap junctions and cadherin based adhesion (Coelho and Kosher, 1991; Wolpert, 1992; Zimmermann, 1984; Oberlander and Tuan, 1994; Tavella et al, 1994; Hall and Miyake, 2000; DeLise et al, 2000; DeLise and Tuan, 2002a, b). There is very little information however, about cellular communication between tendon progenitors during condensation (Hall and Miyake, 2000). In contrast to cartilage, cells in tendon primordia retain contact with adjacent cells through ever lengthening cell processes, whereas in the cartilage primordia cells are completely pushed apart, breaking the cell-cell contacts.

1.6.1 Tendon development and morphogenesis

The development of tendon is best known in the limbs. The most commonly used species is chick, although extensive work has also been carried out on mouse embryos with the advent of gene knock-out experiments. In early development, tendons develop from the mesoderm, formed at the trilaminar germ disc stage.

The mesoderm forms the paraxial lateral plate and intermediate mesoderm. Tendon development is dependant on the paraxial mesoderm that accumulates bilaterally to the neural tube and notochord (Pourquie, 2001). It is the segmentation of the paraxial mesoderm, from rostral to caudal in direction that produces the somites (Pourquie, 2001). Until recently the somite was thought to differentiate further into sclerotome, myotome and dermomyotome (Christ and Ordahl, 1995). Recent experiments have brought to light another segment of the somite that lies dorsally to the sclerotome. (Brent et al, 2003). It has been named the syndetome and it is thought to give rise to future tendon cells of the axial skeleton. Tendon progenitors become incorporated into the limb mesenchyme as development proceeds (Oldfield and Evans, 2003). In the developing chick limb-bud, a tenascin-rich sheet of ECM accumulates beneath the ectodermal basal lamina (Hurle et al, 1990). This is called the mesenchymal lamina, and provides a scaffold for the assembly of pre-tendinous mesenchymal cells. This is not present in mammalian development, but the specialised nature of this region is shown by the expression of *eya* and *six* genes, marking it out in the same place (Riou et al, 2002). These cells will eventually segregate and align to form individually and anatomically distinct tendons (Ros et al, 1995, Kardon et al, 1998; reviewed by Benjamin and Ralphs, 2000).

In the developing limb, tendon progenitors lie in the dorsal and ventral regions, which become extensor and flexor tendons respectively, with cartilage progenitors positioned centrally (Schramm and Solursh, 1990; Schweitzer et al, 2001). These blastemas can be subdivided into proximal, intermediate and distal parts (Kardon, 1998). These differ in their initial structure and association with the early musculature. The distal primordia differentiate independently of the musculature, but require interactions with it later, or these regions of tendon degenerate (Kieny and Chevalier, 1979; Kardon, 1998). The proximal and intermediate primordia require closer interactions with muscle, possibly involving FGF4 (Edom-Vovard et al, 2001). Tendon cells become orientated on an organised tenascin scaffold in the intermediate and distal primordia, and tenascin expression is upregulated in proximal primordia (Kardon, 1998). As cell rows form, orientated matrix is deposited. This may be based around the tenascin pattern as above, but also, in other highly organised fibrous structures cytoskeletal architecture, cell interactions and cell orientation are also important (Hayes et al, 1999).

1.6.2 Collagen fibrillogenesis in tendon development

Collagen fibril formation in developing tendon involves a hierarchy of extracellular compartments, created by fibroblasts (Trelstad, 1982; Trelstad and Birk, 1984; Birk and Zycband, 1993). Collagen segments are intermediates in collagen fibril assembly in soft connective tissue. There are indications that the ends of developing collagen fibrils show distinct differences involving long and short tapered ends throughout stages of development (Birk et al, 1989, 1990). This pattern was found in numerous tissues including the skin and cornea, demonstrating it as a general mechanism in the growth and morphogenesis of connective tissues (Birk and Trelstad, 1984; Birk et al, 1991; Ploetz et al, 1991).

The recent studies of Canty et al, (2004), into collagen deposition in developing tendon has provided evidence of vesicles carrying procollagen fibrils, with 28nm in diameter, being targeted to specific areas of the plasma membrane. These vesicles are well known (Hirschberg et al, 1998; Toome et al, 1999; Polishchuk et al, 2000), but the areas of plasma membrane are a relatively new observation. Termed 'fibripositors', these plasma membrane protrusions have a significant role in collagen deposition, essentially their ability to allow collagen nucleation within the lumen at the base of the fibripositor, well within the cell, and collagen deposition at the tip into intercellular spaces formed between tendon cells. They are present in early tendon development but only for a short period (Canty et al, 2004).

When collagen fibrils are secreted from the fibroblast they remain in close proximity with the cell membrane, and self-aggregate into collagen fibres. The first extracellular compartment consists of 2-3 fibrils, in a series of narrow channels created by the membrane of the fibroblasts, running from deep within the cytoplasm, often associated with the Golgi region, out to the ECM. These channels fuse laterally and the fibril segments aggregate and form longer fibrils. The second ECM compartment defined is also close to the cell surface. The region is determined by one or more fibroblasts, where collagen fibres coalesce to form fibril bundles. Between 12 and 15 days of development, collagen assembly is predominantly tapered end-end attachments (Birk et al, 1995). This yields longer collagen fibrils without increasing the diameter. The third compartment for collagen fibrillogenesis has boundaries formed from 2-3 adjacent fibroblast membranes. This enables the cells to physically position and organise the collagen macroaggregates within the matrix (Birk and Zycband, 1993).

There is a dramatic increase in collagen fibril diameter at 17 days of development (Brodsky et al, 1982; Eikenberry et al, 1984; Birk et al, 1990) that is consistent with the increase of mechanical strength of developing tendon (Ros et al, 1995). At this stage there is no cell proliferation to facilitate an increase in the matrix:cell ratio (Nurminskaya and Birk, 1998). This also correlates with a remarkable decrease in fibril-associated decorin, which occurs between 16-19 days of development in the chick (Birk et al, 1995). This lack of fibril-associated decorin allows lateral assembly of the collagen fibrils. Coinciding with this point of development is a reduction in the expression of the FACIT collagen type XIV, (Young et al, 2000) together with a decrease in type XII collagen (Zhang et al, 2003). Type XIV collagen is restricted to immature collagen fibrils suggestive of stabilisation of early collagen fibril segments allowing only end-to-end growth (Young et al, 2000). The decrease and the spatial shift of type XII collagen expression, shows that it becomes associated with endotenon of developing fascicles (Zhang et al, 2003) and corresponds with type III collagen shift of expression to the endotenon (Birk and Mayne, 1997). Both decorin and type XIV collagen play a regulatory role in collagen fibrillogenesis, but type XII collagen has a functional role in integrating fascicles into functional units with type III collagen (Keene et al, 1991; Young et al, 2000; Schuppan et al, 2001). Gene expression of TGF β and IGF-1 are increased at this stage, possibly inducing the increased expression of type I, VI and XI collagen observed (Nurminskaya and Birk, 1998). Also fibromodulin expression is increased, possibly to replace decorin and to help stabilise the maturing fibrils (Nurminskaya and Birk, 1998; Ezura et al 2000). Lateral fusion predominates as development proceeds (Fleischmajer et al, 1998), with collagen diameter regulation from surface decorin and type V collagen (Birk et al, 1990; Weber et al, 1996).

1.6.3 Markers of tendon differentiation

A problem in studies of tendon development and differentiation is that there is no clear marker for tendon. In cartilage, there are matrix markers such as type II collagen, aggrecan, and the transcription factor sox9 (Benya et al, 1982; Asou et al, 2002), in tendon matrix components are expressed by numerous other connective tissues and there does not seem to be an 'exclusive' transcription factor marker either. An important indicator is therefore structure, with cell rows and parallel matrix, in the correct anatomical position. However, some matrix and molecular markers are useful up to a point, and these are outlined below.

The transcription factor scleraxis can be used as an early stages marker before tendon primordia become recognisable by other means. It is a marker of ligament as well, and is expressed in other connective tissues (Cserjesi et al, 1995; Schweitzer et al, 2001), but can serve as an early marker, if not an exclusive one. In tendon development there are 4 phases of scleraxis expression (summarised in Oldfield and Evans, 2003). The first is at Hamburger and Hamilton (HH) stage 21 (day 3.5), cells in the proximo-medial region of the limb bud begin to express scleraxis. The expression is superficial in the dorsal and ventral domains of the limb, and it is too early to identify tendon cells using tenascin as a marker (Kardon, 1998). The second stage occurs between HH stages 25-27 (4.5-5 days), here the scleraxis-expressing cells join together in a proximal-dorsal fashion and begin to form the first fibrous elements of tendons. The third stage, at HH28 (5.5 days), and true tendon fibres can be seen, with fibres becoming more elongate, nearer to mature tendon structure. The final stage occurs at HH31 (7 days) where scleraxis expression is present throughout the tendon (Schweitzer et al, 2001).

Other transcription factors may be expressed in tendon related regions include *pea 3* and *Erm*, regulating scleraxis expression in the syndetome (Brent and Tabin, 2004), *six1*, *six2* (Oliver et al, 1995), *Eya1* and *Eya2* (Xu et al, 1997). *Six1* and *Eya2* expression is restricted to the dorsal extensor tendons of the digits, whereas *six2* and *Eya1* are seen ventrally, and precede the expression of *Eya 2* (Oliver et al, 1995). Another factor involved in tendon development is the LIM homeodomain transcription factor (*Lmx1b*). In *Lmx1b* knockout mice the ventral-dorsal asymmetry caused by the six genes is lost (Dreyer et al, 2004). They are useful markers of regions of early tendon, therefore, if not of tendon tissue itself.

Extracellular matrix markers are tenascin, and a range of collagens including types I, III and VI, and various small proteoglycans. Tenascin is the earliest marker of these, although later than scleraxis (Kardon, 1998). The others become clearer later, although it is only because of their relationship to tendon structure that they can be used, as types I and III collagen, for example, as expressed prominently in the mesenchyme from which they are derived (Hurle et al, 1990; Ros et al, 1995). Growth factors and other signal related molecules are also specifically associated with tendon at some stage. *Eph-A4*, a tyrosine-kinase receptor is expressed in the proximal portion of the tendon, and in the body of the tendon in later development (Patel et al, 1996; D'Souza and Patel, 1999). The *follistatin* gene is expressed in a ventral-distal fashion and restricted to the tendon periphery (D'Souza and Patel,

1999). Follistatin encodes a protein that acts as an antagonist to some TGF β superfamily members, including BMP-2, BMP-4 and Activin (Reviewed in Patel, 1990). This is important as BMP-2 and BMP-4 induce chondrogenesis (Merino et al, 1998). Conversely, other members of the TGF β superfamily, namely growth and differentiation factors (GDF) 5, 6 and 7 have been shown to induce tendon development (Wolfman et al, 1997). Consequently, follistatin could have a regulatory role in GDF's during tendon development.

1.7 TENDON REPAIR

Tendons may be damaged in a number of ways. They may be crushed or fully or partially severed; they may be surgically cut, torn by excessive loading or damaged in disease, for example tendonitis. Tendon repair is promoted by intrinsic factors (endotenon and epitenon cells) and extrinsic factors (e.g. neutrophils and macrophages; Potenza, 1962; Gelberman et al, 1984; Manske et al, 1984; and Jaibaji et al, 2000). There are three recognised stages in the process: inflammation, proliferation and remodelling (Frank et al, 1999). Overuse injuries, or tendinosis, caused by repeated low-level traumas, tend not to initiate an inflammatory response (Frank et al, 1999).

Inflammation phase

Tendon injury often results in the rupture of a blood vessel, causing inflammation that can last for up to 4 days post-injury (Wojciak and Crossan, 1993). Blood vessels, blood plasma and tissue fluids accumulate at the injury site. Platelets enter and bind to exposed collagen fibres stimulating clotting (Rao, 1993), filling the wound. Fibrin and fibronectin are present from one hour after injury and form cross-links with collagen (Lehto et al, 1990), producing a glue-like plug that prevents haemorrhage and provides some degree of continuity between the damaged tendon ends. Fibronectin enhances the migration and adhesion of cells (Wojciak-Stothard et al, 1997). The deposition of platelets also brings a supply of growth factors to the site, e.g. PDGF and TGF β (Mast, 1997; Anitua et al, 2004). Within hours, white blood cells migrate to the injured area and macrophagocytic inflammatory cells clear the area of necrotic tissue and debris (Wojciak and Crossan, 1993). When the wound area is clear of debris, the proliferation phase begins.

Proliferation/Granulation phase

During the proliferation/granulation stage there is an accumulation of fibroblasts, myofibroblasts and epithelia at the wound site (Gelberman et al, 1985). Angiogenesis commences and the newly formed capillaries communicate with the existing capillary network (Fenwick et al, 2002). Nascent tendon fibroblasts at the damaged end of the tendon begin to both proliferate and synthesise new matrix allowing for granular tissue formation (Williams, 1985). Tendon cells from the epitenon, endotenon or tendon proper have varying capacities for proliferation, matrix synthesis and migration and possibly different roles in healing. Investigations into these differences found epitenon cells are bigger and synthesise increased amounts of α -smooth muscle actin and vinculin (Ragoowansi et al, 2003). They also produced more MMP-2 and MMP-9 compared to endotenon cells, it was recommended that the epitenon cells should be specifically targeted to inhibit or treat tendon adhesions (Ragoowansi et al, 2003). Fibroblasts from the synovial sheath were taken and compared with fibroblasts from the endotenon. It became apparent that synovial fibroblasts are more active and have a greater capacity to produce matrix metalloproteases, implying a better ability to degrade matrix and consequently have a greater ability to migrate (Khan et al, 1998). Experimentation on sheath, epitenon and endotenon fibroblasts *in vitro* investigated their reactions to the TGF β isoforms 1, 2 and 3. There were small differences between cell type and growth factor treatments, but a general increase in types I and III collagen and a decrease in cellular proliferation was observed (Klein et al, 2002). Myofibroblasts present at the wound site, function to produce granulation tissue, but also help to contract the granular tissue aiding wound closure (Hinz and Giabbiani, 2003).

In the early stages of this phase tendon fibroblasts increase the production of GAGs, proteoglycans, vitronectin, fibronectin and types III, IV, V and VI collagen (Clark, 1996; Birk et al, 1997). The production of type III collagen at this time has been shown to stabilise the repair process due to its ability to form rapid crosslinks (Liu et al, 1995). Granulation tissue is well vascularised, highly cellular and poorly organised relative to the ordered ECM of normal tendon (Williams, 1985). Approximately 2 weeks after injury, the granulation tissue has matured, the scar is stronger and the collagen fibres begin to become aligned with those of the uninjured tendon (Clark, 1996). It is in this later stage of healing that type I collagen is synthesised (Leadbetter et al, 1992). The proliferative stage overlaps considerably with the final stage in tendon repair, the maturation and remodelling stage.

Remodelling phase

The remodelling phase is the longest and can last for a year or more. The number of macrophages, fibroblasts, myofibroblasts and capillaries decrease slowly, as does their synthetic activity (Mast, 1997; Molloy et al, 2003). The scar becomes denser and vascularity decreases with the concentration of GAGs (Lin et al, 2004). Type I collagen predominates and is densely packed to increase tensile strength (Jaibaji, 2000; Clark, 1996; Eckes, 2000). The type I collagen fibres remodel themselves until the tendon forms a strong permanent structure (Leadbetter et al, 1992). After 5-6 months the tendon fibroblasts or tenoblasts de-differentiate to less active tenocytes. Other experiments have shown that it is not until 12 months after tendon rupture that the tendon begins to recover some degree of organisation in its collagen fibrils (Bruns et al, 2000).

Although this repair process seems efficient, there are still biochemical and mechanical deficiencies that will persist indefinitely (Leadbetter et al, 1992) and the tendon will always be more susceptible to further damage (Mast, 1997). The ultimate tensile strength of the repaired tendon can be decreased by as much as 30% of its original value. This decrease is due to alterations in collagen type and distribution, PG content and cellularity (Gelberman et al, 1988; Hyman and Rodeo, 2000). This may sound less than ideal, however, in most cases it represents a perfectly adequate repair. Experiments have shown *in vivo* the natural reparative state of the tendon to be 48% tensile strength after 15 days (Reddy et al, 2001). Normal activity studies have shown that rabbits use up to 40% of the tendon tensile strength. So it is fair to suggest 100% recovery may not be necessary.

The natural healing process, even though it may take over a year, if accompanied with the appropriate exercise program (Stanish, 1984; Alfredson et al, 1998), will see most injuries fully recover (Paavola et al, 2000). However, where a tendon injury has complications (burns, crush injuries or sections of the tendon missing), the natural healing process is inadequate for a satisfactory recovery. Surgical procedures for ligament/tendon replacement have been developed over decades. Reviews presenting a concise evaluation of these methods for anterior cruciate ligament or flexor tendon repair, autograft, allograft or synthetic graft provide ample information on this subject (Wilson and Sammut, 2003; Sherman and Banffy, 2004). Together with an explanation of the role of the vasculature in these systems (Fenwick et al, 2002), it is possible to assess the current clinical standing

on this speciality. However, the response of tendon to these techniques is another large area of research.

1.7.1 Growth factors in connective tissue repair

The release of growth factors (i.e. TGF β , bFGF, PDGF, VEGF and IGF-1) and other chemotactic factors is highly influential in tendon repair. Each growth factor is required at specific times during the repair process (Molloy et al, 2000; Hsu and Chang, 2004). A short summary is presented here, with more details being given in chapters 5 and 6.

Insulin-like growth factor (IGF)

The growth factor IGF-1 has important actions during the inflammation and proliferative stages of tendon repair (Molloy et al, 2003). IGF-1 promotes cell proliferation and cell migration, in addition to increased matrix synthesis (Grazul-Bilska et al, 2003). It follows that with the elevated IGF-1 concentrations apparent during inflammation there is a corresponding increase in IGF receptor expression (Tsuzaki et al, 2000). IGF-1 has also been shown to be important in conjunction with other growth factors (Lynch et al, 1989). PDGF acts synergistically with IGF resulting in an increase in cellular proliferation (Tsuzaki et al, 2000). *In vivo* use of IGF-1 can increase the rate of Achilles tendon healing and reduce inflammation (Kurtz et al, 1999). Additionally, application of IGF and bFGF to healing tendons showed a statistically significant increase in the tensile strength of tendons (Letson and Dahners, 1994).

Transforming growth factor

The superfamily of TGF is large and includes BMPs and GDFs. The prototypical member, TGF β -1, is important at all stages of tendon repair (Tsubone et al, 2004). TGF β -1 is involved in the regulation of cell migration, proteinase expression, fibronectin binding, termination of cell proliferation and the stimulation of collagen synthesis (Chang et al, 2000; Bennet and Schultz, 1993; Natsu-ume et al, 1997 and Wojciak and Crossen, 1994). As with IGF receptors, TGF β receptors are also up-regulated at the cell surface during tendon healing (Ngo et al, 2001). Coincidentally, the up-regulation of TGF β receptors corresponds with the peak of types I and III collagen synthesis within scar tissue (Eckes et al, 2000), reiterating its role in collagen synthesis. Increased levels of TGF β are associated with adhesion formation in healing tendons *in vivo* (Chan et al, 1997) and neutralisation of TGF β activity using antibodies can improve post-operation range of motion in

tendon (Chang et al, 2000). A study using cartilage derived morphogenic protein 2 (CDMP-2/GDF-6) showed stimulation of matrix synthesis in healthy and osteoarthritic chondrocytes (Bobacz et al, 2002). Injections of CDMP-2, or the use of GDF-5/CDMP-1 coated sutures applied to damaged Achilles tendons resulted in an increase in the tensile strength, thickness and density (Forslund and Aspenberg, 2001; Rickert et al, 2001). Subjects treated with GDF-5/CDMP-1 saw chondrogenic material within the tendon a month after repair (Rickert et al, 2001).

Basic fibroblast growth factor

BasicFGF/FGF-2 is a stimulator of angiogenesis (Gabra et al, 1994), cellular migration and cell proliferation (Folkman and Klagsbrun, 1987). Increased expression of bFGF has been noted from the time of injury throughout the healing process and is mainly produced by fibroblasts at the wound edge (Chang et al, 1998). bFGF has been shown to increase type III collagen synthesis and cell proliferation *in vivo* (Chan et al, 2000). bFGF on a fibrin gel implanted into a medial collateral ligament defect, resulted in an increase in the early repair stages (Fukui et al, 1998). However, higher doses of bFGF had adverse effects and caused delays in the maturation of the scar tissue. Type I procollagen levels were also down regulated in all bFGF treated groups (Fukui et al, 1998). Other experiments using medial collateral ligament defects, confirmed the influence of bFGF on the early stages of repair, producing an increase in amount of granulation tissue with superior collagen orientation at 6-24 weeks (Kobayashi et al, 1997). This study also demonstrated that although bFGF only affects the early stages of ligament repair, the subsequent stages have increased speed and efficiency (Kobayashi et al, 1997). VEGF is active slightly later than other growth factors in tendon repair. Its expression is seen to increase during the proliferation and remodelling stages and is a known stimulator of angiogenesis (Jackson et al, 1997).

Platelet-derived growth factor

PDGF levels are elevated from early stages of tendon repair and this growth factor is known to induce the synthesis of IGF-1 (Lynch et al, 1989) and TGF β (Pierce et al, 1989). It has been shown to stimulate collagenous and non-collagenous protein synthesis, and through IGF-1 stimulation, increase cell proliferation (Abrahamsson, 1997). Interestingly, combined PDGF and TGF β treatment results in poor healing in ligaments (Hildebrand et al, 1998). Additionally, using defects in the medial collateral ligament of rabbits, PDGF treatment increased the rate of healing in a dose dependant manner (Hildebrand et al, 1998). Experimental work concentrating

on other methods of growth factor delivery has used gene transfer of PDGF on ligamentous tissue, resulting in an increase in matrix synthesis (Nakamura et al, 1998).

1.8 CELL AND TISSUE CULTURE AND TISSUE ENGINEERING

Reports of tissues being grown outside of the body began to appear in scientific literature in the mid-1880's. A report by Roux (1885) is the most commonly used citation; he described the maintenance of chick embryonic tissue in warm saline. Two years later Arnold described the survival and migration of frog leukocytes in saline, and then in 1903, Jolly showed the survival and division of salamander leukocytes, in what he described as 'hanging-drop cultures'. Harrison (1907) and Carrel (1912) developed tissue culture as a method of studying animal cells free from systemic variations.

In later years, methods for supporting mammalian cells were developed, as their normal and pathological development was nearer to that of human cells. The use of human tissue was highly influenced by the demonstration that human tumours could give rise to continuous cell lines (e.g. HeLa: Gey et al, 1952). The period between early tissue culture experiments and the later culturing of human cells lines, was marked by important developments in tissue culture, including the first use of the proteolytic enzyme trypsin to suspend attached cells in culture (Rous and Jones, 1916). The development of dedicated culture flasks allowed developments in technique for cell culture scale-up and the microscopic evaluation of cells in culture (Carrel and Baker, 1931, 1932). After that came the development of the roller tube technique for cell suspensions (Gey, 1933) and of a chemically defined growth medium (Fischer, 1948). Later, important experiments saw the observation of contact inhibition, where cells mobility in a monolayer ceases when contact is made with adjacent cells (Abercrombie, 1954), and that human fibroblasts die after a finite number of passages in monolayer culture (Hatflick and Moorhead, 1961).

Tissue culture technology evolved rapidly with applications developing to meet a range of scientific and medical problems. Although tissue culture has limitations, its major advantages include the control of the external environment, whether it is pH; temperature; osmotic pressure; or O₂ or CO₂ content. When the contamination issues of early tissue culture had been resolved, investigators were able to apply themselves to the nutritional requirements of cells. As indicated above, tissue

culture media was developed (Fischer, 1948), honed from early experiments using buffered saline solutions or plasma clots. The discovery that nutrient requirements differ from cell type to cell type, led to the development of many different media, with a variety of supplements usually being necessary for reliable cell growth. The most common additive was, and still is, serum (Olmsted, 1967; Honn et al, 1965), although, serum-free fully defined systems have been developed, they tend to have very restricted applications (Birch and Pirt, 1971; Ham and McKeehan, 1978; Barnes and Wolfe, 1980).

As with all scientific procedures there are disadvantages. These include the constant difficulties in maintaining aseptic conditions, the relatively poor yield of cells from tissue and their phenotypic instability. It is also important to note that there are major differences in cell behaviour *in vitro* compared with the *in vivo* situation. The cells *in vitro* lack systemic influences and homeostatic regulation from the endocrine and the nervous system. This results in cellular metabolism being more constant. Cells are also no longer in a three dimensional (3-D) environment, and lack the cell-cell/cell-matrix interactions which occur *in vivo*. Two dimensional (2-D) culture systems can cause the differentiated properties of cells to be lost; this can make it difficult to compare cultured cells with the functionality of the cells as in the original tissue. Different culture methods are examined in more detail below, comparing standard 2-D with more sophisticated 3-D culture methods.

1.8.1 Monolayers

The primary method of growing cells *in vitro* is as a monolayer. It is a cheap and simple method of culturing cells and most cells can be grown in this way. Cells plated in a tissue culture dish need to adhere to a substrate in order to survive and proliferate, by mitosis, to cover the entire area of the dish. Each cell has contact with adjacent cells forming adhesive and communicating junctions.

The 2-D organisation of this culture system enables the studies of some aspects of cell behaviour, but these are often clearly abnormal. It is important to remember that these cells are in an alien environment - they have been taken from 3-D systemic surroundings, treated relatively harshly with enzymes and plated in a 2-D context. However, monolayers have their uses. They are convenient for cytological and immunological observations of cells, and ideal for cell multiplication and large scale expansion of cell numbers (Freshney, 1983).

Connective tissue cells often require more sophisticated systems if they are to behave in any way similar to the *in vivo* situation. This is most easily shown with chondrocytes, where the differentiated state of the cells is monitored by expression of type II collagen, aggrecan and sox9 (Benya et al, 1978; Grundmann et al, 1980; Kypriotou et al, 2003). Chondrocytes require 3-D culture systems if the phenotype is to be maintained (van Osch et al, 2001), plating them in a monolayer leads to a rapid loss of the key markers. In high cell density culture this de-differentiation is prevented (e.g. Lemare et al, 1998; Schulze-Tanzil et al, 2002). This is likely to be true of other connective tissue cells where the phenotype is more difficult to monitor. For example, it is not possible to tell if plated fibroblasts in monolayer are undergoing any type of de-differentiation. What is the differentiated state of fibroblasts in a monolayer culture? There are no clear markers, neither extracellular, cell surface nor intracellular, although, some changes in amounts of collagen production have been implied in fibroblast dedifferentiation (Herrmann et al, 1980; Quinones et al, 1986).

1.8.2 3-D Cultures

Connective tissue cells often respond well to 3-D culture systems, either simply placing large numbers of cells together in a cell pellet or high density spot, or by incorporation them into a 3-D matrix or scaffold structure. 3-D cultures generally enhance differentiated function in chondrocytes (van Osch et al, 2001; Schulze-Tanzil et al, 2002; Tallheden et al, 2004), and are applicable to other connective tissue cells *in vitro* as well (see below). Scaffold structures are many and varied, including aqueous gels of natural material (e.g. collagen, alginate, agarose and hyaluronic acid) or produced synthetically (e.g. poly-ethylene oxide, poly-vinyl alcohol and poly-acrylic acid) (Drury and Mooney, 2003).

3-D cultures and tissue engineering

3-D culture systems and scaffold structures lend themselves to tissue engineering applications. Tissue engineering is defined as 'The development and subsequent replacement of diseased or damaged living tissue with living tissue designed and constructed for the needs of each individual to restore, maintain and improve function' (Langer and Vacanti, 1993). A 3-D cell-seeded scaffold is therefore a possible repair method. This type of engineered construct has the longest history in developing skin replacements, and a number of products are available. Fibroblasts and keratinocytes are grown on a range of scaffolds to represent the complexity of the living skin *in vivo* (Bell et al, 1983); one layered skin

replacements; epidermal or dermal, and a composite of these, for example Apligraf® (Organogenesis Inc. FDA approval in 1998). An additional skin product, Myskin, uses cultured keratinocytes grown onto a chemically defined plasma polymerised surface and has been used with relative success for burns patients and non-healing diabetic foot ulcers (Higham et al, 2003; Moustafa et al, 2004).

More recently much effort has been aimed at producing tissue engineered cartilage for the repair of defects (e.g. Sams and Nixon, 1995; Standing and Langer, 1999; Allemann et al, 2000; Glowacki and Mizuno, 2001). Scaffold based systems have a number of advantages. The scaffold gives a degree of intrinsic strength to the tissue construct, aiding handling and allowing surgical implantation and rapid function. Scaffolds can also be created to modify the behaviour of cells within the construct, perhaps by incorporating growth factors within the material to control phenotype (Edelman et al, 1991; Malhoney and Saltzman, 1999) or to affect the interaction of the implant with the host, e.g. vascularisation (Peters et al, 1998; Epstein et al, 2001; Richardson et al, 2001). They also have disadvantages in that their breakdown products may be deleterious to the host, or they may promote unforeseen host responses, or immune responses.

Another approach is to use scaffold free systems, where connective tissue cells produce their own matrix, forming an 'endogenous' scaffold. The advantage of this is that no exogenous materials are introduced, but the downside is that it is likely to be more difficult to control the characteristics of the material. The simplest method of culturing cells in a 3-D culture system without a scaffold is the micromass. The vast majority of micromass experiments have been to investigate chondrogenesis. Developed in 1977, micromasses have been used *in vitro* to mimic the tight packing of embryonic cartilage (Ahrens et al, 1977; Paulsen et al, 1988; Daniels et al, 1996). Other non-scaffold based constructs have investigated various suspension and gyrating culture systems, using cells from the liver, chondrocytes and cardiac fibroblasts (Kelm et al, 2004). Similarly to micromass cultures, pellet cultures are generally used on chondrocytes or to induce chondrogenesis and are easy to manipulate (Ahrens et al, 1977; Solursh, 1982; Daniels and Solursh, 1991; Xu et al, 1996; Stewart et al, 2000).

1.8.3 Bioreactors and 3-D culture

Bioreactors are a recent method of culturing cells at a large scale with or without a scaffold for long periods under optimal conditions. Originally bioreactors were used in industrial fermentation, water treatment and food processing. Now they are

common in the production of various antibodies, vaccines, and growth factors (Fischer and Emans, 2000; Kipriyanov and Le Gall, 2004). An important feature lacking in the previously described systems is the difficulty to administer mechanical strain or compressive force to the systems. To grow functional tissues as replacements for damaged or diseased tissue, cells must experience mechanical loading and/or numerous chemotrophic factors similar to that occurring *in vivo* and some bioreactors have been designed to do this (Seidel et al, 2004; Gonen-Wadmany et al, 2004). Bioreactors also allow for continuous nutrition of cells and the removal of by-products (Martin et al, 2004). Although, cell-seeded scaffolds are widely used within bioreactors, there is also potential for pure cell suspensions to be added to the systems. Problems incurred with the use of bioreactors include long-term sterility and a limitation to the size of the cell construct.

Application of 3-D culture and tissue engineering to tendon and ligament

Tendon and ligaments are frequently injured, sometimes catastrophically, and slow to heal and thus are candidates for a tissue engineering approach to repair. Most works have addressed the Achilles tendon and anterior cruciate ligament (ACL). There are many reviews evaluating methods for anterior cruciate ligament or flexor tendon repair, including autograft, allograft or synthetic grafts (Wilson and Sammut, 2003; Sherman and Banffy, 2004). Many synthetic materials have been tried and tested in ACL repairs, but with limited long term success (Fu et al, 1993). A typical example is the Gore-Tex ligament, which has enormous strength and stabilises the knee in the short term, but deteriorates in the medium of long term (Fukubayashi and Ikeda, 2000). Most problems with permanent prostheses arise from poor attachment at the ligament/tendon-bone interface or a lack of host tissue ingrowth.

Various scaffolds have been tested to encourage tissue ingrowth using numerous types of polymers including carbon fibre. These constructs have a porous or filamentous structure in order to promote tendon or ligament ingrowth. As these scaffolds are not degradable and tissue ingrowth is relatively slow, a new problem is created. The scaffold material shields the potential re-growth tissue from sufficient mechanical loading, resulting in non-aligned fibrous tissue (resembling scar tissue) (Dunn et al, 1994). With mechanical load consistently borne by the implant the regenerated tissue will not remodel or mature (Yamamoto et al, 1993).

Consequently, biodegradable 3-D scaffolds for tendon and ligament reconstruction have become an expanding field. Tendon cells in 3-D have been shown to continue synthesis of tendon associated ECM molecules and retain their characteristic morphology at high cell density (Schulze-Tanzil et al, 2004). Collagen-based scaffolds are favoured, as collagen is the main constituent of tendons and ligaments. These collagen scaffolds have been shown to induce the deposition of ligamentous tissue *in vivo* (Bellincampi et al, 1998). Furthermore, to aid scaffold performance in tissue regeneration, fibroblasts have been seeded into the scaffolds (Dunn et al, 1995). Since fibroblasts play an important role in collagen turnover during healing, including them within the scaffold may stimulate earlier healing (Manske et al, 1984; Russell and Manske, 1990; Gelberman et al, 1992), and consequently improved tissue formation.

Tendon fibroblasts have been shown to migrate along engineered collagen threads at similar rates to native tendon threads (Cornwell et al, 2004), allowing enhanced integration between engineered tissue replacements and natural tissues. Scaffolds that degrade rapidly result in improved strength of the replacement tendon or ligament (Dunn et al, 1993). Alternatively, constructs degrading too rapidly cause the regenerated tendon to lose its natural mechanical stimulus. The regenerating tendon is unable to be remodelled and the tissue reaches only 23% of the natural tensile strength (Qin et al, 2004). Therefore, it is important to understand the degradation of scaffolds and the rate of collagen synthesis by grafted cells and tissue integration into the host.

With the clinical goal to promote early healing and to improve long-term mechanical performance, these fibroblast-seeded scaffolds could provide an alternative to synthetic or even biological prostheses. Experiments using mesenchymal stem cells (MSC) as an alternative to fully differentiated cells showed an increase in repair upon implantation into window defects in the patellar tendon of rabbits. Additionally, if the cell-seeded constructs were put under tension before implantation there was a 50-60% level of stiffness compared to that of normal uninjured tendons (Butler and Awad, 1999).

AIMS

The overall aim of the work described in this thesis is to determine the importance of cell-cell interactions and 3-dimensional organisation of cells in matrix deposition by tendon cells *in vivo* and *in vitro* culturing systems. Specifically, the objectives are:

- To determine the timing of appearance of various types of cell-cell junction in developing tendons in relation to matrix deposition.
- To compare cell interactions in different 2-dimensional and 3-dimensional culture systems with reference to matrix secretion and deposition.
- To determine the effects on growth factors on qualitative and quantitative aspects of cell organisation and matrix deposition in a 3-D culture environment.
- To develop a novel large scale high density 3-dimensional culture system to examine cell interaction, matrix organisation in a system that could be upgraded to tissue engineering applications.

MATERIALS AND METHODS

2.1 Source of material

To acquire tendinous material, flexor tendons were dissected from the feet of 50-day old Broiler chickens acquired from Tibbits Poultry farm, Castleton, South Wales. To prevent infection each chicken foot was washed with 70% alcohol and a longitudinal ventral incision made in the third phalange. The flexor digitorum longus tendon was aseptically dissected from the claw as far as possible into the palm of the foot. Between 6-10 tendons were used at each harvest.

For embryonic material chick flexor digitorum longus tendons were taken at various stages from white leghorn developing chicks. Fertilised eggs were acquired from Hollyditch Farm, UK, and incubated at 37°C, 100% humidity for 6-19 days. Embryos were removed from the eggshell, separated from the remaining yolk and killed by decapitation. At embryonic day 10-19 the tendons were dissected from the embryonic limbs. In earlier embryonic limbs the tendons were not dissected out.

Cartilaginous material was acquired from the metaphalangeal joint of 7-day old bovine animals, acquired from F. Drury and Sons Ltd, the abattoir, UK. At each harvest 10-20 full depth explants were used.

2.2 Cell isolation and culture

Cells were isolated from adult chicken tendons using a method modified from Banes et al. (1988). The epitenon was removed from adult tendons because they are known to act differently compared to fibroblasts from tendon proper *in vitro* (Banes et al, 1988). The epitenon was removed by treating the tendon in 10ml of 0.5% collagenase (3min;Sigma), 10ml of 0.25% trypsin (15min;Sigma) and then scraped with a blunt scalpel. The tendon was cut into small pieces and sequentially digested with 10ml of 1% protease (1hr;Sigma) and 10 ml of 0.5% collagenase (3hrs) at 37°C. The digested tendinous material was centrifuged (MSE Minstril 1000) at 12 000 rpm for 5 minutes, the supernatant removed and cells washed in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen/GIBCO) containing 5% heat inactivated Foetal Bovine Serum (FBS), 1% antibiotic/antimitotic and L-glutamine (all tissue culture reagents from Invitrogen/GIBCO). This step was repeated and fibroblasts were plated on 100mm dishes (Costar). Incubated at 37°C, with 100% humidity in 5% CO₂ in air

atmosphere. Cells were grown to confluence and passaged after removal with trypsin/EDTA. Cells for experiments were used at passage 3.

Cells were also isolated from the tendons of 17 day embryos. It was not possible to remove the surrounding epitenon of the embryonic chick flexor digitorum longus tendons; they were sequentially digested in 5ml of 1% protease (30 minutes) and 5ml of 0.5% collagenase (1 hr) at 37°C. Tendon fibroblasts were washed twice in DMEM as above and plated into 60mm dishes (Costar). Cells were grown and passaged as above.

For comparative purposes full depth chondrocytes were extracted from explants taken from the metaphalangeal joint of 7-day old animals. Using a modification of the above methods of cell isolation, using sequential digestion, 10 ml of 0.1% protease for 1 hour and 10ml of 0.04% collagenase for 8 hours (overnight) at 37°C. Chondrocytes were centrifuged, washed and plated as above in DMEM+F-12 containing 10%FBS, 1% gentamycin (Invitrogen/GIBCO), 0.5mg/ml ascorbate (Sigma) and 1mg/ml glucose (Sigma). Articular chondrocytes were grown and passaged as above.

2.3 Fixation procedures

Special culture methods-Tendon material/cells were cultured in various ways including monolayers, micromass and pellets see chapters 4 and 5, short-term suspension culture and 3-D long term suspension roller cultures, see chapter 6 and complete embryonic specimens, see chapter 1. Each of these methods required differences in fixation procedures.

Tissue specimens, embryonic and adult, were prepared for labelling procedures by fixing in 95% industrial methylated spirits (IMS), at 4°C for 5-30 minutes dependant on size. Specimens were washed in phosphate buffer solution (PBS) at 4°C and frozen onto the cryostat chuck using Bright Instruments Cryo-M-Bed (Cryo-M-Bed, Ltd) compound on dry ice (-78°C). These were transferred to a Bright OTF 5000 cryostat and sections were cut at 8-20µm on disposable knives. Sections were collected on histabond slides (RA Lamb).

Cell cultures used for immunolabelling procedures were fixed in 95% alcohol, at 4°C for differing time intervals. Fibroblast monolayers and micromasses were washed in PBS and fixed for 2 minutes, excess alcohol was poured off, then left to evaporate before starting the labelling procedure (stored at -80°C). Pellets and 3-

D long term suspension roller cultures were removed from the media and fixed for 5-15 minutes dependant on size. They were washed in PBS and frozen onto the cryostat chuck with dry ice (see above). In short-term suspension cultures a cell sample was taken at specific time intervals and fixed in alcohol immediately. These cells were then dropped onto histobond slides and the alcohol evaporated off before starting the labelling procedures.

2.4 Immunohistochemistry

Water repellent rings were drawn around sections and selected cell cultures using a DAKO pen (Dako Cytomation), the specimens were re-hydrated in PBS/tween. Sections were preblocked using 1:20 goat serum in PBS/tween 20 (Sigma) (20mins) and labelled with various primary antibodies (See table 2.1). All primary antibodies were diluted in PBS/tween and incubated at room temperature for 1-2 hours. Sections were washed in 3 changes of PBS/tween and then incubated with goat anti mouse or rabbit as appropriate. Alexafluor488 conjugated goat anti-rabbit immunoglobulin (Sigma) was used to detect type I collagen label. Alexafluor488 conjugated goat anti-mouse immunoglobulin (Sigma) was used for all monoclonal antibodies (see table 2.1). Secondary antibodies were incubated at room temperature for 1 hour and washed in PBS/tween (x3). Phalloidin was used to directly label filamentous actin at a concentration of 5µg/ml in PBS/tween, incubation time 1 hour followed by washing PBS/tween as above. Labelled sections were mounted using Vectashield containing propidium iodide as a nuclear counterstain (Vector Laboratories). Sections were examined on a Leica Laborlux 12 microscope equipped with a coolsnap digital camera (Roper Scientific) or an Olympus BX 61 microscope with F-View camera and SIS AnalySIS® software (Soft imaging system).

Controls-Two types of negative control were carried out in these investigations for all primary antibodies used. Control sections were processed by replacing the primary antibody with dilutant, thus demonstrating no non-specific binding of the secondary antibody is occurring. Further control sections were processed with mouse immunoglobulin, at 10 µg/ml (Sigma) replacing the primary antibody to demonstrate antibody specificity.

2.5 Biochemical Analysis

Hydroxyproline assay- Hydroxyproline is found in collagenous amino acid sequences and is used as an indicator of collagen content (Creemers et al, 1997).

Preparation for hydroxyproline assay- Taken directly from culture, cell aggregates were digested in 125µg/ml papain overnight at 60°C. 500µl of digested material was hydrolysed with 500µl of hydrochloric acid, at 110°C overnight. Proteins were precipitated from media samples by addition of 50% ammonium sulphate (Sigma) relative to the sample volume, at 4°C overnight, with the addition of phenylmethanesulphonylfluoride (PMSF) (Sigma), ethylenediamine tetraacetic acid (EDTA) (Fisher Scientific) and N-ethylmaleimide (NEM) (Sigma) protease inhibitors. Samples were centrifuged at 21000 rpm for 30 minutes. The pellet formed was dissolved in 1ml PBS and 500µl were hydrolysed in 500µl of HCL (6N) (Fisher Scientific), overnight at 110°C. Acid was removed by vacuum drying and samples were reconstituted in 500µl de-ionised distilled water. Samples were microcentrifuged for 10mins removing any insoluble particulate matter that remained.

Onto a 96 well plate, triplicate 30µl aliquots of sample or standard were pipetted. Followed by 70µl of diluent (100ml propan-2-ol (Fisher), 50ml water) and 50µl of oxidant (0.7g chloramine-T, 10ml water, 50ml Stock Buffer: Stock buffer (500ml): 28.5g sodium acetate trihydrate, 18.75g tri-sodium citrate dihydrate, 2.75g citric acid (all reagents from Sigma), 200ml propan-2-ol. Solids were dissolved in 250ml water first, then add propan-2-ol (Fisher), then make up to final volume of 500ml with water). Plates were placed on a shaker at room temperature for 5mins and 125µl of colour reagent (4-Dimethylaminobenzaldehyde) (Sigma) were added and mixed on a plate shaker and incubated at 70°C for 10-20mins. Samples were read immediately on a plate reader at an absorbance wavelength of 540nm.

Hoechst DNA assay- This is a method of determining DNA concentrations in aqueous buffers that is not affected by contaminating protein or RNA (Cesarone et al, 1980; Downs and Wilfinger, 1983). Hoechst 33258 dye solution (Sigma)(1mg/ml) was diluted in buffer solution to a final concentration of 100µg/ml. Buffer solution consisted of 1mM sodium acetate (Fisher Scientific) and 0.5mM sodiumEDTA (Fisher Scientific). A DNA calibration curve consisted of a series of DNA standards (0-20 µg/ml of plasmid DNA) prepared in buffer solution. Duplicate 50µl papain digested sample or standard were added to 1ml Hoechst dye solution. The DNA was measured in the fluorometer at A₃₅₀ nm excitation and A₄₅₀nm emission.

Antibody	M/P	Working concentration	Dilution Factor	Enzyme	Antigen	Specificity	Reference	Source
Col-1	P	17ng/ml	1:2000	HC	Type I collagen	Broad	J. Cell Biol. 111: 1685 (1990)	Sigma
CIIC1	M	10 mg/ml	1:5	HC	Type II collagen	Ch, Hu, M, rat	J. Cell Biol. 129: 881-892 (1995)	DSHB
3B2	M	10 mg/ml	1:5	HC	Type III collagen	Ch	Matrix. 11:56-65 (1992)	DSHB
M1-B4	M	10 mg/ml	1:1	None	Tenascin	Ch	Cell 47: 131-139 (1986)	DSHB
Cx32	M	1ng/ml	1:10	None	Connexin 32	Rat, Hu, M, Ch	J. Cell Biol. 107: 1817 (1988)	Chemicon
Cx43	M	4ng/ml	1:50	None	Connexin 43	Ch, Hu, rat, M, Ca	J. Cell Biol. 111:2077-2088 (1992)	Chemicon
hVin1	M	60ng/ml	1:400	None	Vinculin	Ch, Hu, M, Bo, dog, rat	Proc. Natl. Acad. Sci. (USA) 87:5667 (1990)	Sigma
CB-1	M	10 mg/ml	1:1	None	Decorin	Ch	Matrix. 11:412-427 (1991)	DSHB
JLA20	M	10 mg/ml	1:4	None	Actin	Ch, Rat	Cell Mot. Cyto. 12:216-224 (1989)	DSHB
ACAM	M	250ng/ml	1:100	None	N-cadherin	Ch, Hu, rat, M	Dev. Biol. 139: 314 (1990)	Sigma
CH-19	M	70ng/ml	1:500	None	Pan-cadherin	Ch, Hu, rat, M	Development 102: 639 (1988)	Sigma
AMF-17b	M	13 mg/ml	1:1	None	Vimentin	Ch, Hu, Bo, Dog	J. Biol. Chem. 264: 17953-17960 (1989)	DSHB
M-38	M	10 mg/ml	1:4	None	Pro-collagen	Ch, Hu,	J. Mol. Biol. 211, 581-594 (1990)	DSHB
B3/D6	M	10 mg/ml	1:4	None	Fibronectin	Ch	Dev. Biol. 139, 383-395 (1990)	DSHB

Table 2.1. Primary antibodies

Abbreviation: M, monoclonal antibody; P, polyclonal antibody; HC, antibody requiring hyaluronidase pre-treatment, DSHB, Developmental Studies Hybridoma Bank.

Antibody	Abs*	Em*	Source	Working Dilution
Alexa Fluor® 488 goat anti-mouse IgG	495	519	Molecular Probes	1-10µg/ml-used at 5 µg/ml
Alexa Fluor® 488 goat anti-rabbit IgG	495	519	Molecular Probes	1-10µg/ml-used at 5 µg/ml

Table 2.2. Secondary antibodies

* Approximate absorption (Abs) and fluorescence emission (Em) maxima in nm.

CHAPTER THREE
Matrix deposition and cell-cell interactions in
tendon development

3.1 INTRODUCTION

Tendon development in the chick limb begins with the formation of the dorsal and ventral mesenchymal laminae. These tenascin-rich ECM lamina lie beneath the basal lamina of the ectoderm. Rodent embryos lack a mesenchymal lamina, but the equivalent region of mesenchyme express a number of genes, including *six* and *eya*, involved in tissue morphogenesis in other parts of the embryo, including the eye (Heanue et al, 1999; Oliver et al, 1995; Xu et al, 1997; reviewed Benjamin and Ralphs, 2000). Tendon fibroblasts in developing chick limb differentiate at embryonic day 7 (Ros et al, 1995) and begin to synthesise a distinct ECM (see development, chapter 1). Deposition of tenascin and type VI collagen are the initial ECM markers of developing tendon, type III collagen is faintly present at day 7 and precedes type I collagen deposition (Hurle et al, 1990). During development the rows of cells become pushed apart by the developing collagen fibres, though the cells retain contact through extended cytoplasmic processes (McNeilly et al, 1996). Complete tendon architecture including fascicular structure, is established by day 14 (Keene et al, 1991; Young et al, 2000; Schuppan et al, 2001), although tendons remain mechanically immature (Birk and Mayne, 1997). Described earlier in chapter 1, at around day 17 there are changes in matrix secretion with increased collagen deposition and decrease in synthesis of certain collagen-associated proteins, for example types XII and XIV collagen (Young et al, 2000; Zhang et al, 2003). This leads to an increase in the matrix to cell ratio (Nurminskaya and Birk, 1998), enabling the tendons to bear the appropriate mechanical strains.

Whilst the stages of collagenous matrix development are known (Hurle et al, 1990; Ros et al, 1995; summary table in Edom-Vovard and Duprez, 2004), there are significant gaps in our understanding of cell-cell and cell-matrix interactions (Hall and Miyake, 2000). Cell-cell interactions are important features of tendon structure (McNeilly et al, 1996; Ralphs et al, 2002) and have been reported at electron microscopic level (Greenlee and Ross, 1967). Both gap junctions and adherens junctions are important in adult tendon cell behaviour (Ralphs et al, 2002; Waggett et al, submitted+abstract) with adherens junctions linking cells longitudinally by connecting their actin stress fibres (Ralphs et al, 2002), and gap junctions controlling collagen synthesis in response to mechanical load (Waggett et al, submitted). In this chapter, the formation and differentiation of recognisable tendon precursors is monitored by observations of ECM deposition and related to the expression of protein associated with cell-cell interactions, in particular cadherins and gap junctions.

3.2 MATERIAL AND METHODS

To examine the developmental sequence of tendons within the chick hindlimb, specimens were taken from a range of developmental ages and ECM, cytoskeletal and cell junctional proteins were identified with immunolabelling techniques.

For tissue source and immunolabeling procedures see general materials and methods, (chapter 2). Fertilised eggs were incubated at 37°C, 100% humidity for 6-19 days. Embryos were removed from the eggshell, separated from the remaining yolk and killed by decapitation. The entire hindlimb was removed from 6-8 day embryos, and flexor tendons dissected from hindlimbs of 10-19 day embryos. Chicks at earlier stages of development, 6-8 days, had the entire hindlimb sectioned, rather than the tendons dissected out.

Tissue was either fixed in 95% IMS, at 4°C for 5-30 minutes dependant on size (as described in chapter 2), or frozen unfixed for cryosectioning. Specimens were washed in PBS at 4°C, orientated and frozen onto the cryostat chuck, using Bright Instruments Cryo-M-Bed compound, on dry ice (-78°C). These were transferred to a Bright OTF 5000 cryostat and sections were cut at 8-10µm on disposable knives. Sections were collected on microscope slides ready for immunolabelling.

Immunolabelling

Sections were labelled with antibodies to ECM components (type I, II and III collagen, tenascin), cytoskeletal components (actin by direct phalloidin label and vimentin) and cell junctional components (vinculin, pan-cadherin, n-cadherin, connexin 32 and connexin 43), see general materials and methods, chapter 2.

3.3 RESULTS

Tendons were easy to identify in cryosection from day 8 onwards, but earlier than this identification was difficult and positive identification was rare. In some cases small areas of obviously organised cells, in the vicinity of the mesenchymal lamina, formed elongate structures and these were assumed to be very early tendon primordia, although the precise tendon was not identified anatomically.

Identification and distribution of ECM components

Type I collagen – At 6 days small aggregations of cells and matrix were present within the mesenchyme, showing a prominent longitudinal organisation of type I collagen (fig 3.1 A). By 8 days, clear fibres of type I collagen were interspersed between rows of tendon fibroblasts (fig 3.1 B). The developing fibre bundles ran in a longitudinal fashion, and positive labels were present throughout the remaining period (fig 3.1 C, D, E, F), although its labelling pattern was never as clear as for 8 days.

Type III collagen – At 6 days there was enhanced labelling compared to that of the general mesenchyme in small orientated structures, which may have been tendon blastemas (fig 3.2 A). Fibres labelled with a predominantly longitudinal orientation were present in these structures. By 8 days, type III collagen label had become very prominent and highly organised (fig 3.2 B). Subsequently fibrous type III collagen became more spread out. In oblique section (fig 3.2 C), it was possible to identify areas of nuclei surrounded by type III collagen fibrils, rather than fibrils running in between all cells associated with the developing blastema (fig 3.2 C, D). By 15 days of development the type III collagen label was restricted to longitudinally running bands of label (fig 3.2 E), suggesting its association with the developing endotenon, surrounding fascicles of tendon fibroblasts and matrix, and not within the fascicles themselves. This pattern continued at 17 days of development (fig. 3.3 F).

Tenascin- Tenascin is known to be an early marker for tendon development (Hurle et al, 1990), and labelling occurred in day 6 blastemas (fig 3.3 A), as elongate fibres within aggregates of orientated cells. This fibrous distribution became more evident in subsequent developmental stages, with label being associated with the tendon cell rows, presumably at the cell surface (fig. 3.3 B, C, D, E, F).

Cytoskeletal components

Positive label for actin was present at all times, from the very early, tendon blastema at day 6, through all of the experimental stages examined. It took the form of bright, longitudinally running fibres within the blastema and developing tendon (fig 3.4 A-F). There was little obvious organisation at E6, but from E8 there were elongated cells labelling positively for actin filaments. Vimentin label was also present in the tendon cells at all ages, although the putative early day 6 blastemas were not observed in these sections. (fig 3.5 A-F).

Cell junctional components

Cadherins- Pan-cadherin and n-cadherin showed similar distributions at all stages, although the very early putative condensations that occur at day 6 were not encountered. Label was generally punctate in mesenchyme and day 8 tendon blastemas (figs. 3.6 and 3.7 A, B), but tended to be more diffuse in the developing tendon cells. There was a higher intensity of labelling in the tendon cells than in the surrounding mesenchyme (figs. 3.6 and 3.7 C).

Vinculin- Punctate label was present at all stages from day 6 onwards (fig 3.8 A-F), with labelling intensity becoming stronger at day 17, outlining the cells and consistent with the longitudinal orientation of the tendon (fig 3.8 F).

Connexins- Mesenchyme at 6 days labelled positively for connexin 32 , as widely distributed punctate spots of label between cells (fig 3.9 A). By 8 days, when prominent tendon blastemas were present, mesenchymal label was extremely bright surrounding the developing tendon (fig 3.9 B). Label was also present between tendon cells. With subsequent development label remained present between tendon cells and was especially bright in the epitenon (fig 3.9 C, D, E, F). Label for connexin 43 was distributed throughout the day 6 mesenchyme (fig 3.10 A), similarly to connexin 32, although no tendon primordia could be identified at this age. However, in tendon blastemas label was largely absent until 12 days (fig 3.10 D), when punctate label was present between tendon cells, this persisted at subsequent ages of development (fig 3.10 E, F).

Figure 3.1. Developmental sequence of tendon in chick hindlimb; **type I collagen** label from E6-E17; x40 objective lens, scale bar= 40µm.

A- 6day- There are areas in the developing mesenchyme that labelled positively for type I collagen, together with a degree of organisation.

B- 8day- Brightly labelled type I collagen fibrils are present together with a parallel and longitudinal orientation.

C- 10day- Type I collagen remains present, although the labelled fibrils are less clear than at 8 days of development. Insert- cell nuclei counterstained with propidium iodide.

D- 12day- Longitudinal organisation of type I collagen fibrils remains identifiable.

E- 15day- Type I collagen fibrils have a similar distribution as observed in the 12 day tendon.

F- 17day- Type I collagen is identified in tendons at this stage of development, together with longitudinal orientation of the fibres.

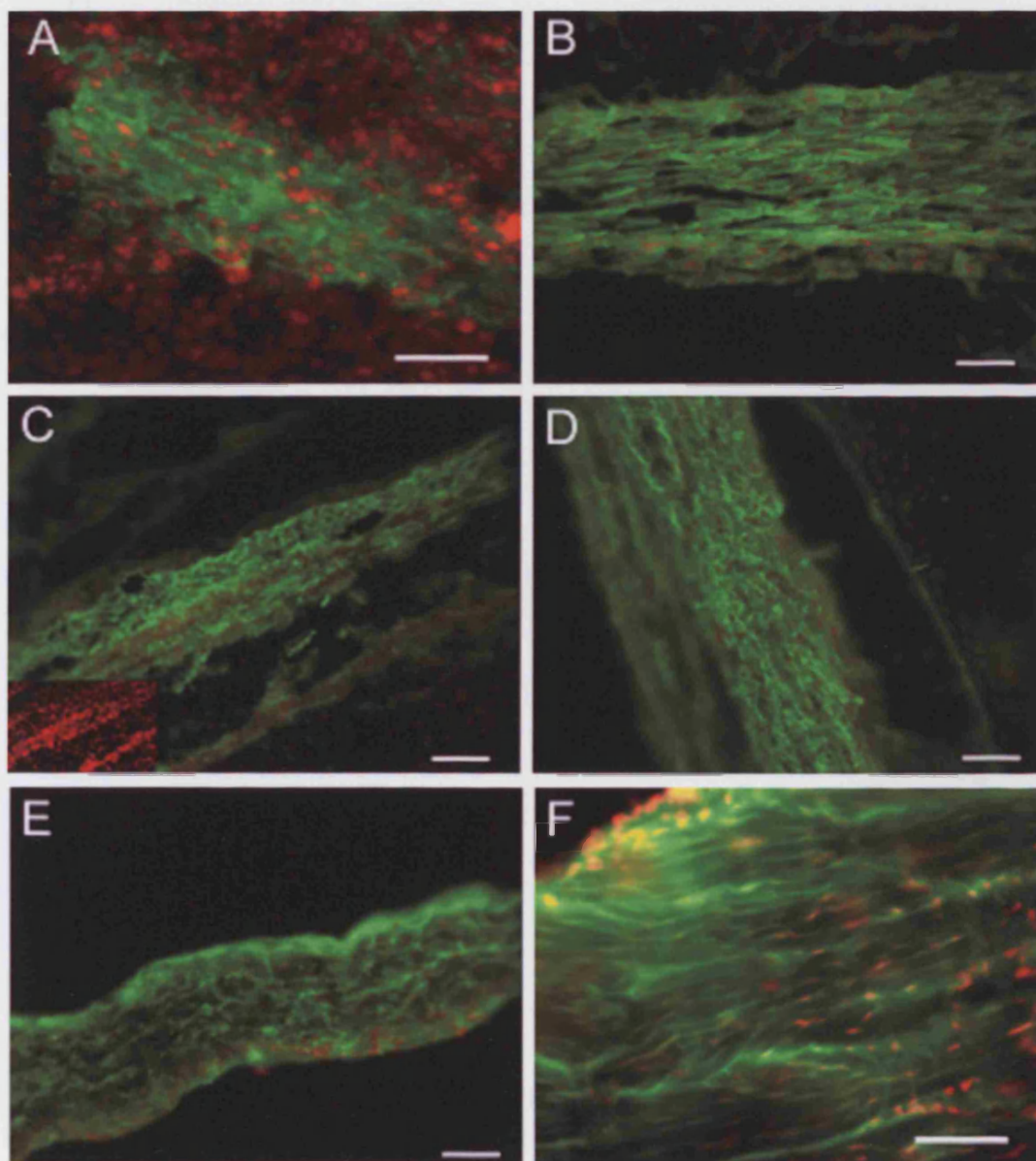


Figure 3.2. Developmental sequence of tendon in chick hindlimb; **type III collagen** label from E6-E17; x40 objective lens, scale bar= 40µm.

A- 6day- No clear tendon blastemas are seen in this section, but areas of apparently longitudinally orientated type III collagen are present.

B- 8day- Strong, highly orientated label is present between orientated rows of tendon fibroblasts.

C- 10day- Type III collagen fibrils present continue to orientate longitudinally and can be observed between cells rows. This oblique section shows areas of multiple nuclei surrounded by type III collagen fibrils.

D- 12day- The type III collagen remains fibrous and longitudinal in organisation, but the uniform distribution becomes restricted to areas of label, it is no longer between every cell row.

E- 15day- Reductions in type III collagen are possibly associated with developing tendon fascicles. The label is present between selected cell rows, but none is seen within them.

F- 17day- Similarly to developmental day 15 the type III collagen is present, although its expression remains restricted to the developing tendon fascicles.

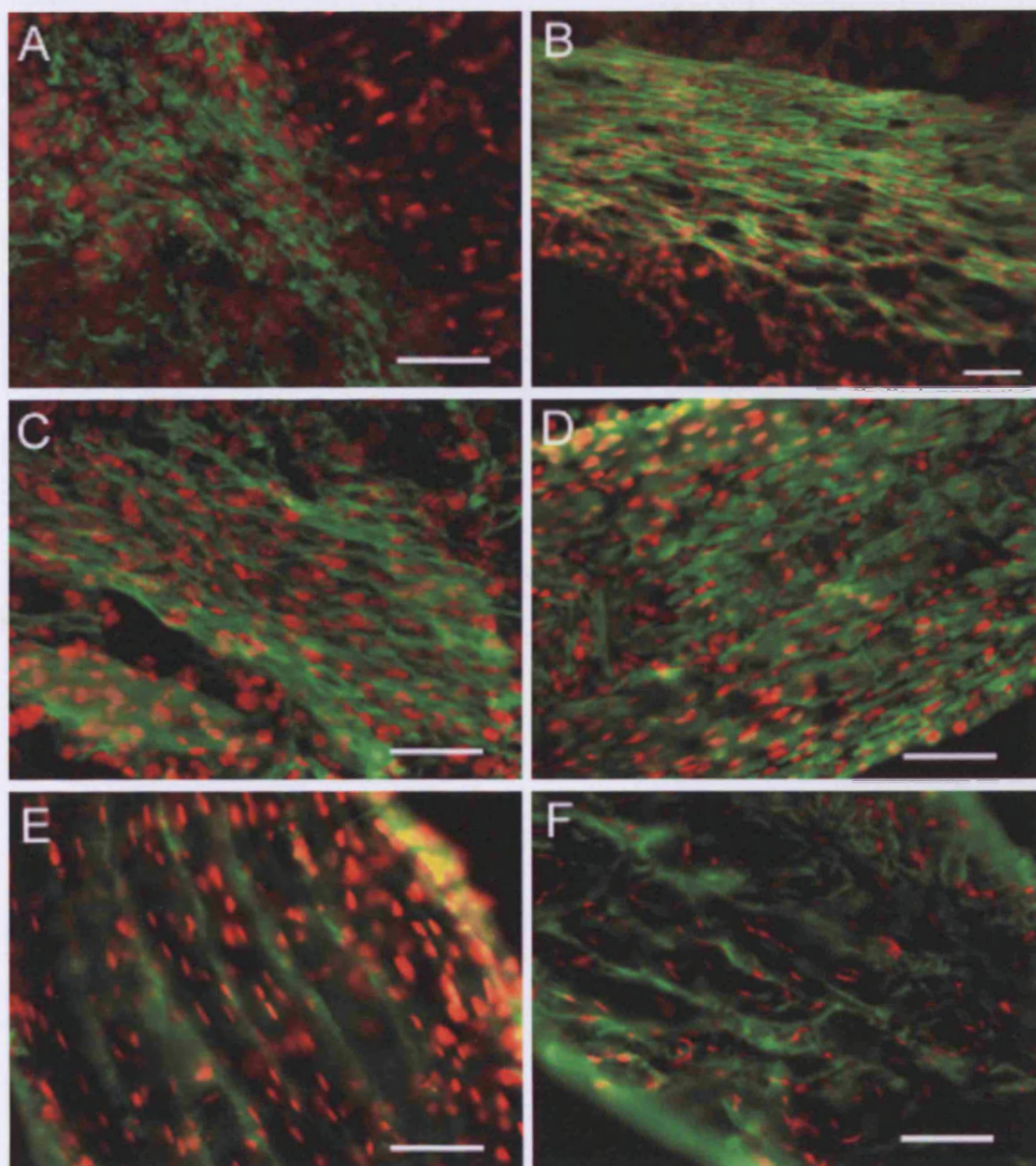


Figure 3.3. Developmental sequence of tendon in chick hindlimb; **tenascin** label from E6-E17; x40 objective lens, scale bar= 40µm.

A- 6day- Tenascin label is present, showing a slight organisation in a developing tendon blastema.

B- 8day- Longitudinally orientated tenascin fibrils are clear and associated with elongated cells of the developing tendon.

C- 10day- Longitudinally orientated tenascin fibrils are seen from day 10-17 in the developing tendons.

D- 12day- Tenascin

E- 15day- Tensacin

F- 17day- Tenascin

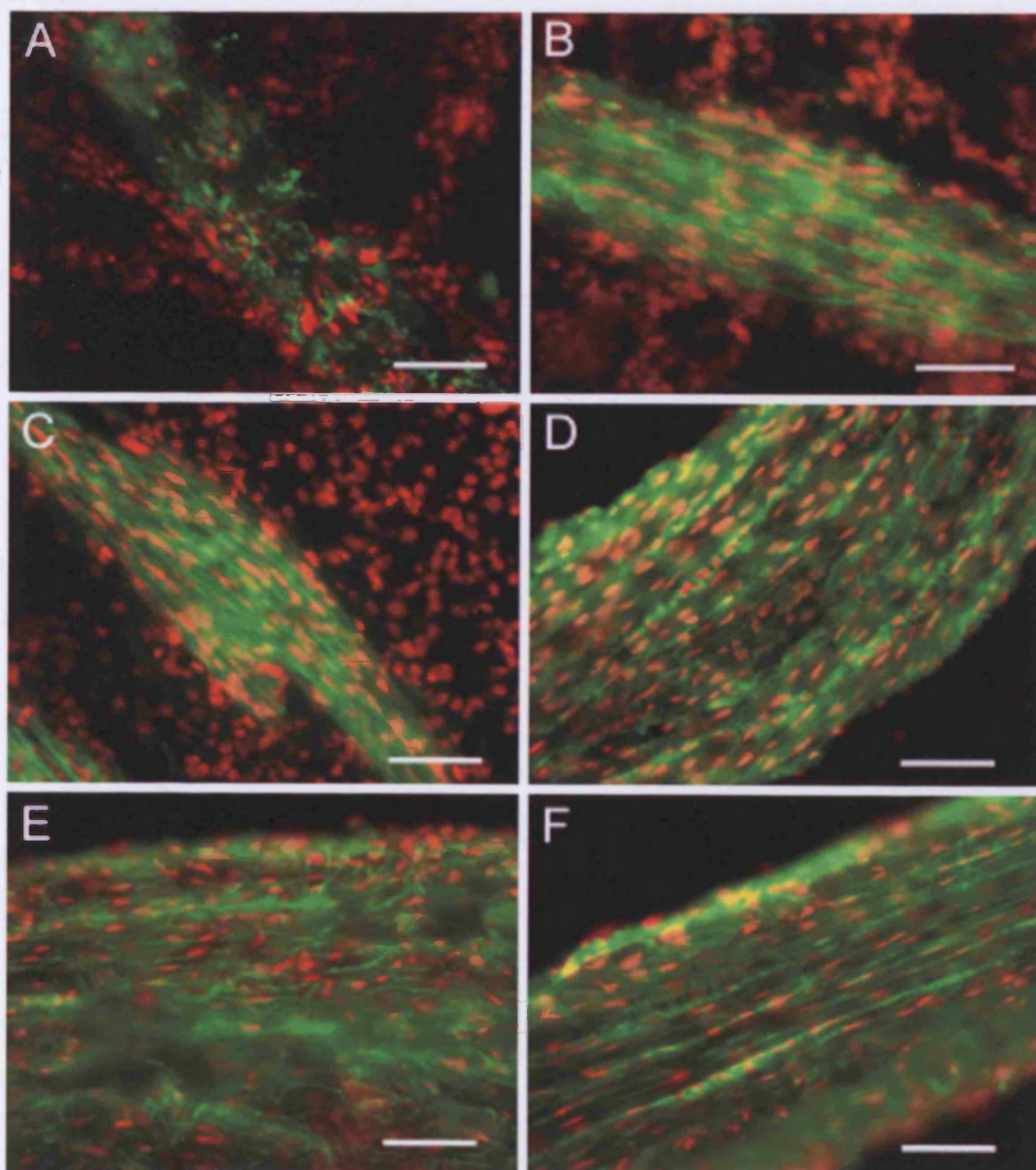


Figure 3.4. Developmental sequence of tendon in chick hindlimb; **actin** label from E6-E17; x40 objective lens, scale bar= 40µm.

A- 6day- Bright labelled foci of labelling show actin filaments are present from day 6 with an organisation.

B- 8 day- Actin filaments are identified running longitudinally within elongated tendon cells, distinctive from the surrounding mesenchyme.

C- 10day- Brightly labelled actin filaments are visible in E10 chick tendons, with a characteristic elongate and longitudinal orientation.

D- 12day- After 12 days of development the longitudinal orientation of the actin filaments remain within the developing tendon.

E- 15day- Continuing to label strongly, actin filaments are present in tendons at 15 days.

F- 17day- An apparent increase in labelling for the longitudinal actin filaments was observed in tendons and can be seen as dense green labelling in the section.

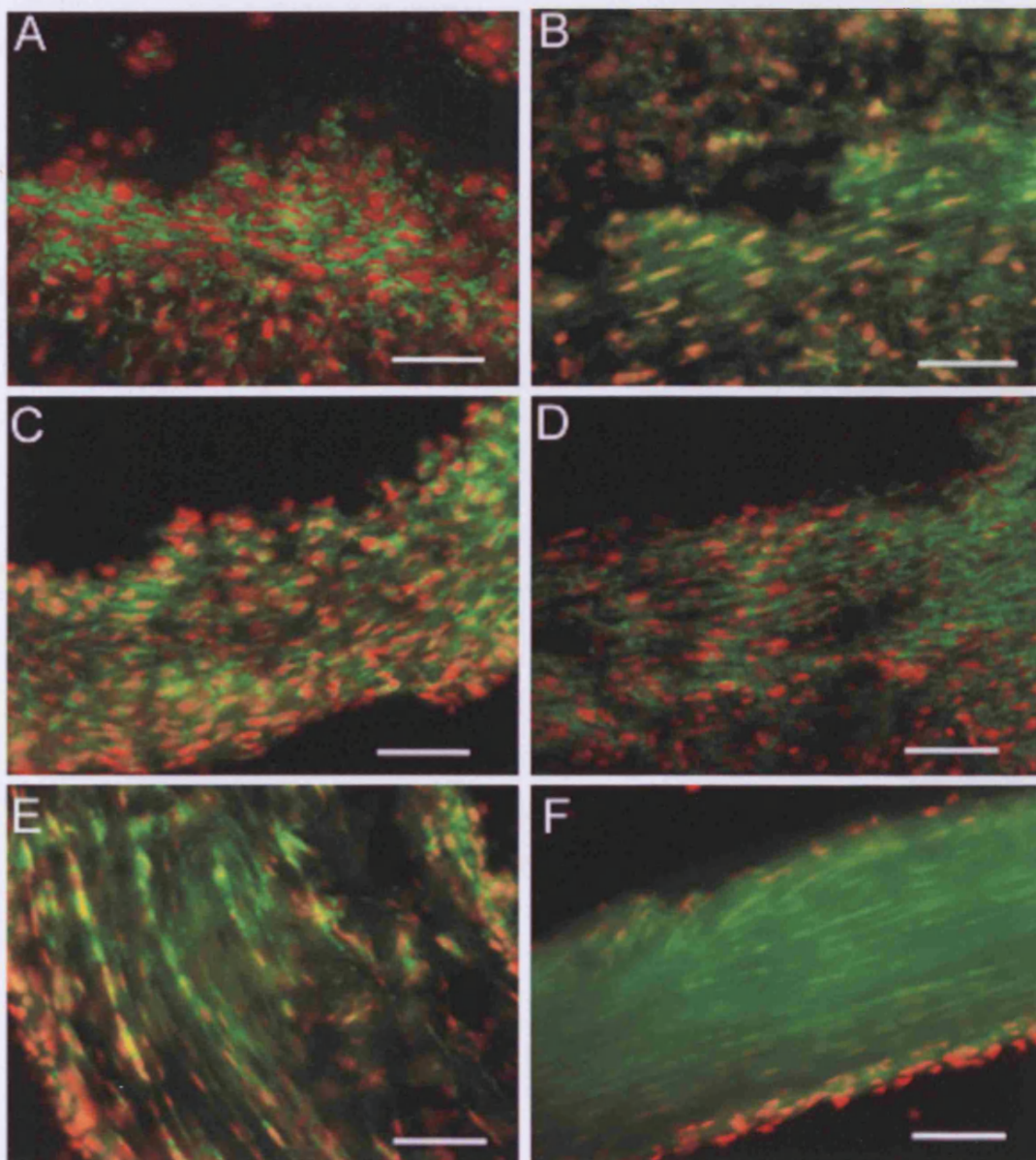


Figure 3.5. Developmental sequence of tendon in chick hindlimb; **vimentin** label from E6-E17; x40 objective lens, scale bar= 40µm

A- 6day- Developing tendon blastemas are unidentifiable at this stage however, vimentin is present throughout the developing limb, particularly in mesenchyme surrounding the cartilage blastema.

B- 8day- Bright foci of vimentin label are present in the early tendon.

C- 10day- Clearly labelled, vimentin begins to show a degree of longitudinal orientation.

D- 12day- Strongly labelled, with a longitudinal orientation the vimentin is present and remains associated with the developing tendon.

E- 15day- Subsequent developmental stages show vimentin labelling remaining longitudinal in orientation.

F- 17day- Vimentin

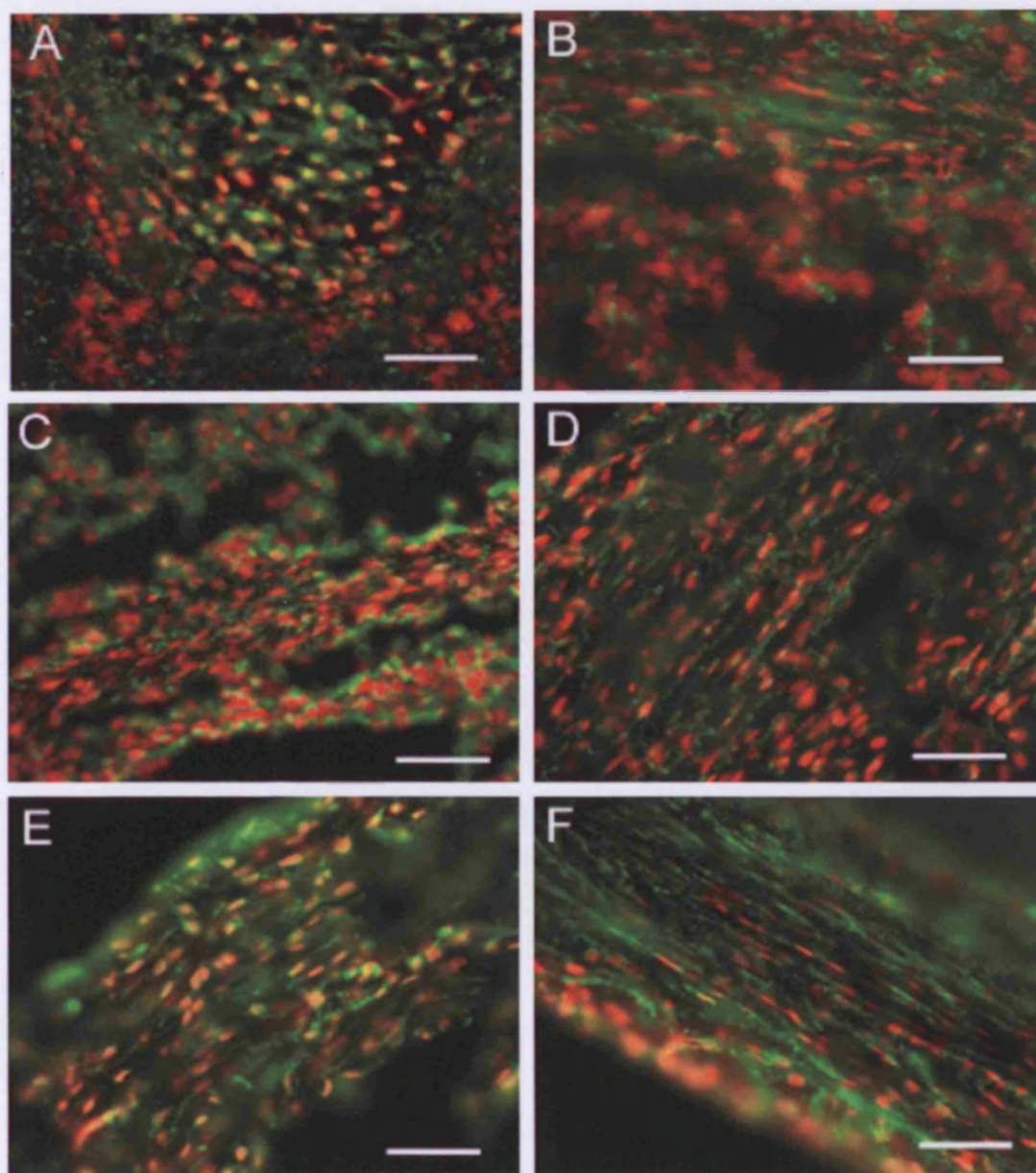


Figure 3.6. Developmental sequence of tendon in chick hindlimb; **pan-cadherin** label from E6-E17; x40 objective lens, scale bar= 40µm

A- 6day- Punctate labelling patterns of label are clearly observed in the mesenchyme of the developing limb, although no tendon blastemas were present.

B- 8day- In developing tendons, pan-cadherin labels strongly and some remnant of the spotted label can be observed. In other areas of the tendon the labelling is more diffuse.

C- 10day- Pan-cadherin labelling remains present and predominant in the tendon and the labelling pattern has become increasingly dispersed.

D- 12day- There was continual expression of cadherins within the developing tendon, although the labelling pattern remains diffuse it retained the overall longitudinal organisation of the tendon.

E- 15day- A continuation of the diffuse labelling is observed, also the edges of the section, the developing endotenon, labels less intensely than the tendon proper.

F- 17day- Pan-cadherin is present throughout the tendon following the longitudinal orientation of the tendon.

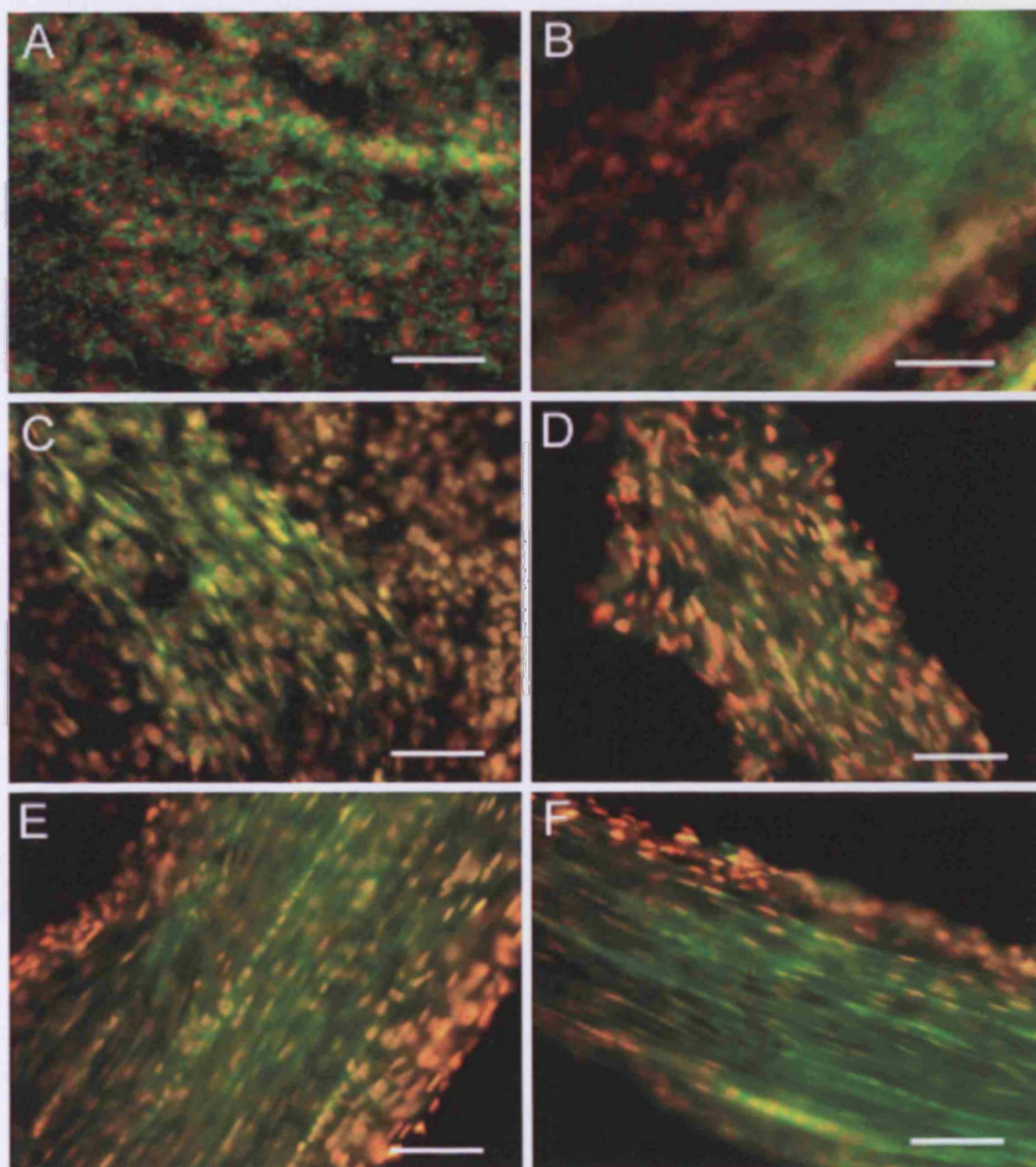


Figure 3.7. Developmental sequence of tendon in chick hindlimb; **n-cadherin** label from E6-E17; x40 objective lens, scale bar= 40µm.

A- 6 day- Following a similar pattern to the pan-cadherin label the n-cadherin labelling of the mesenchyme shows a clearly punctate label, with no tendon blastemas identified.

B- 8day- n-cadherin is present, and the spotted appearance of the label is particularly prominent here.

C- 10day- n-cadherin remains present, but as with pan-cadherin, the labelling pattern becomes increasingly diffuse.

D- 12day- Strong n-cadherin label is observed within the developing tendon, with the cells of the developing epitenon appearing to label negatively for n-cadherin.

E- 15day- n-cadherin labelling is present in subsequent ages of development, although the initial punctate labelling is not resumed.

F- 17day- n-cadherin

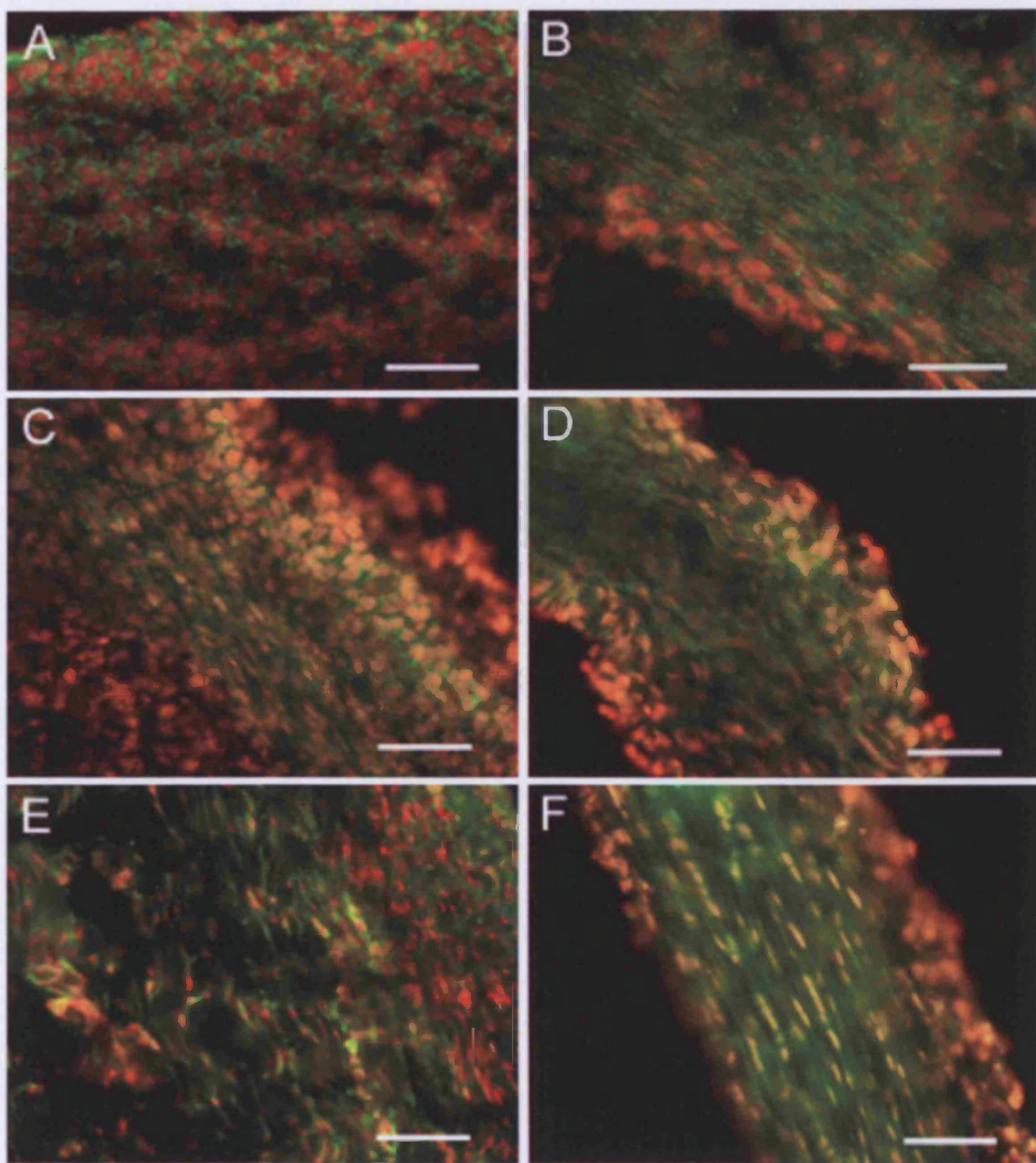


Figure 3.8. Developmental sequence of tendon in chick hindlimb; **vinculin** label from E6-E17; x40 objective lens, scale bar= 40µm.

A- 6day- Vinculin label is present in the mesenchyme, although none was associated with developing tendon blastemas.

B-8day- Elongated cells label positively for vinculin.

C- 10day- This section was cut obliquely so the characteristic elongated tendon cells appear more rounded, or stellate. Vinculin is present, although its expression pattern was unclear.

D- 12day- Also cut at a slightly oblique angle vinculin is shown to be present at the periphery of the cells.

E- 15day- Vinculin can be observed between cells in longitudinal cell rows of the developing tendon.

F- 17day- Vinculin label appears to have increased between 15 to 17 days of development and there is some spotted labelling.

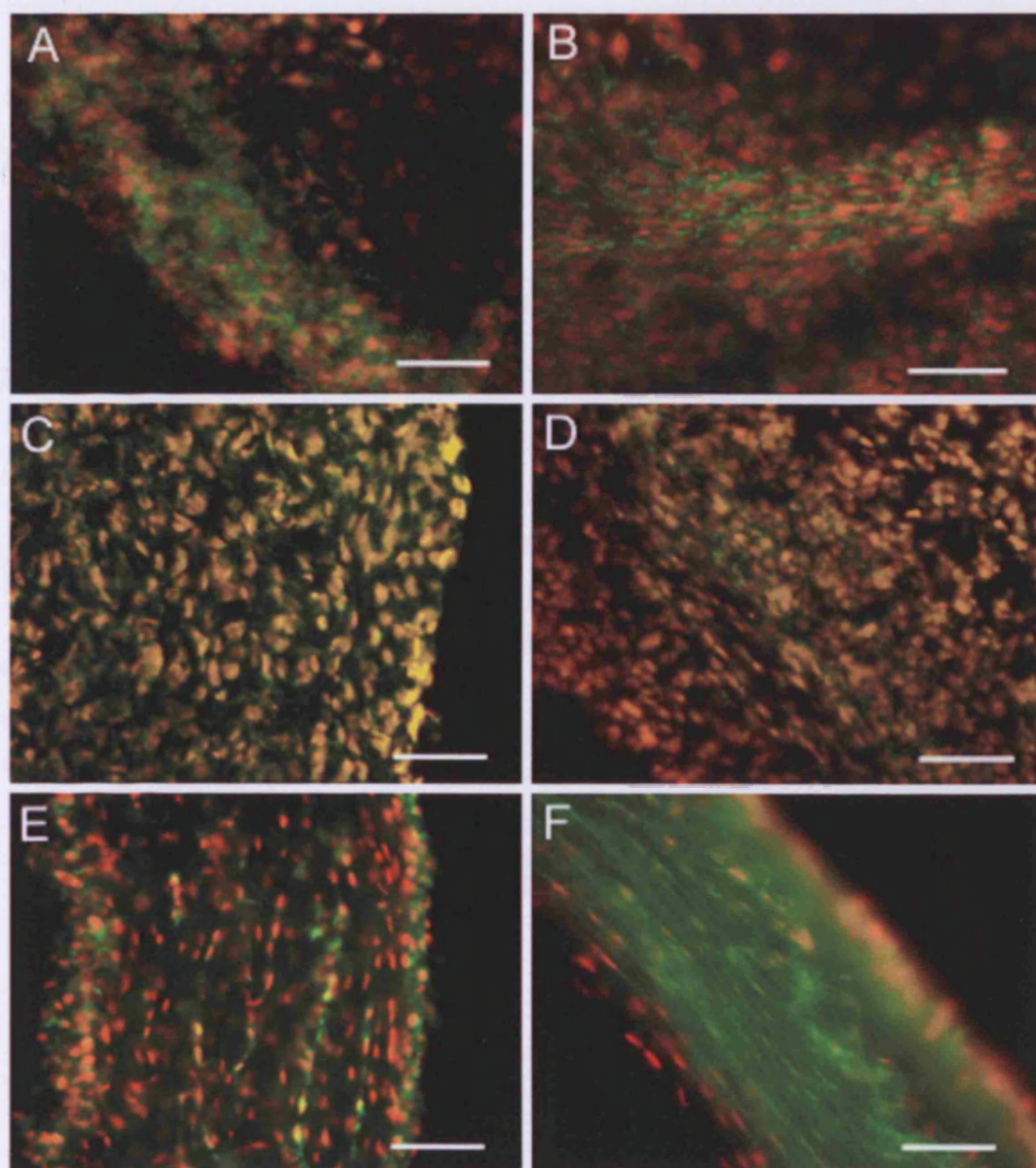


Figure 3.9. Developmental sequence of tendon in chick hindlimb; **connexin 32** label from E6-E17; x40 objective lens, scale bar= 40µm.

A- 6day- Connexin 32 is present in the mesenchyme as bright punctate labelling.

B- 8day- Tendons developing at day 8 showed connexin 32 is present between elongate cells, with labelling of the surrounding mesenchyme, directly associated with the tendon labelled strongly.

C- 10day- Connexin 32 remains present between the elongated tendon cells.

D- 12day- Connexin 32 continues to show positive labelling between tendon cells.

E- 15day- Connexin 32 labelling is observed within the developing tendon, although appears predominant at the periphery of the tendon section in the developing epitenon.

F- 17day- The connexin 32 labelling described at 15 days of development was maintained at day 17, the surrounding epitenon cells label with an increased intensity, affecting the exposure and causing difficulty in the identification of connexin 32 within the tendon.

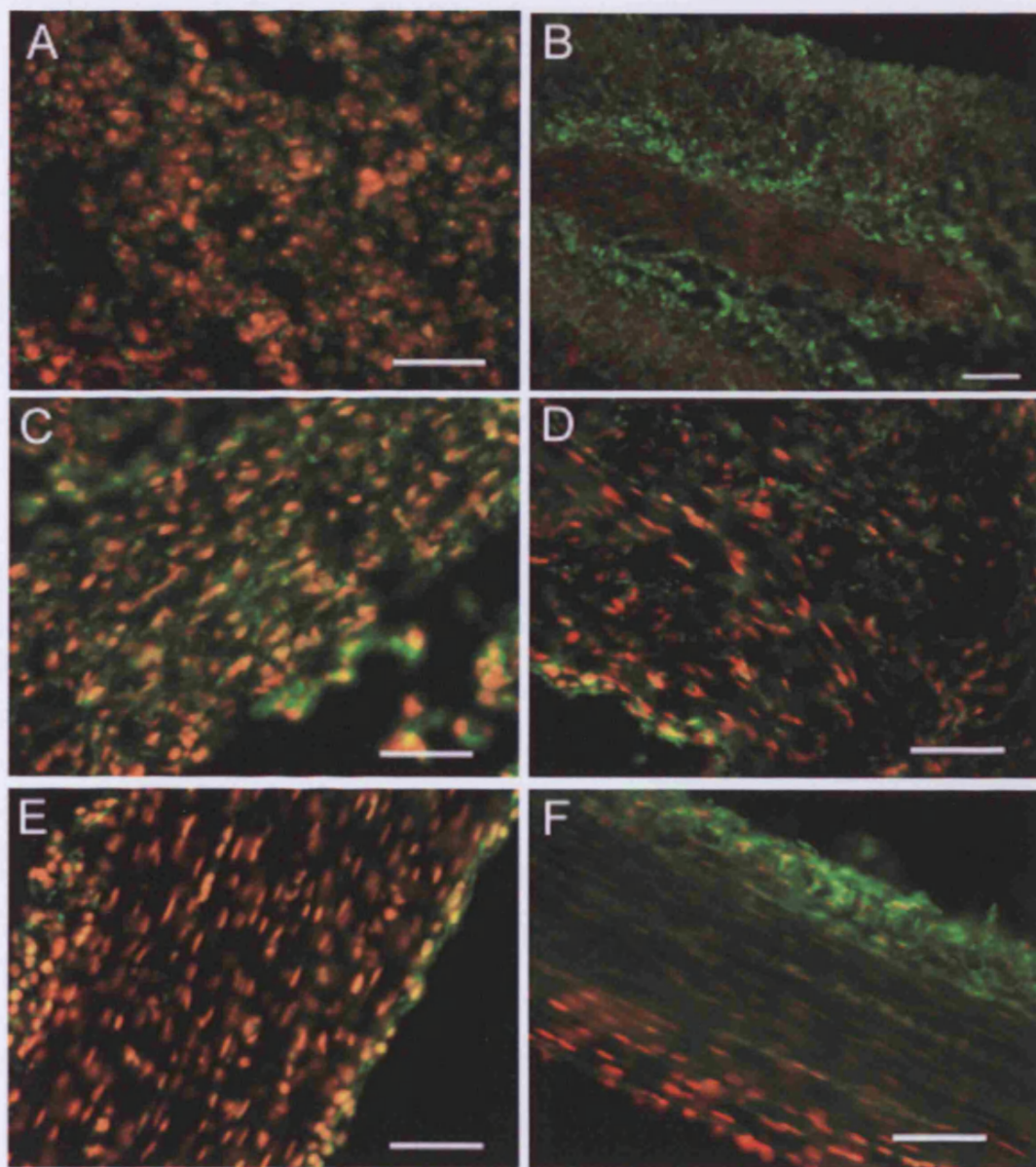


Figure 3.10. Developmental sequence of tendon in chick hindlimb; **connexin 43** label from E6-E17; x40 objective lens, scale bar= 40µm

A- 6 day- Connexin 43 is present in the mesencyme in small, but bright foci of label.

B- 8 day- Tendon blastema with longitudinally orientated cells were identified, but remain negative for connexin 43.

C- 10day- The labelling for connexin 43 remained largely absent in the developing tendons.

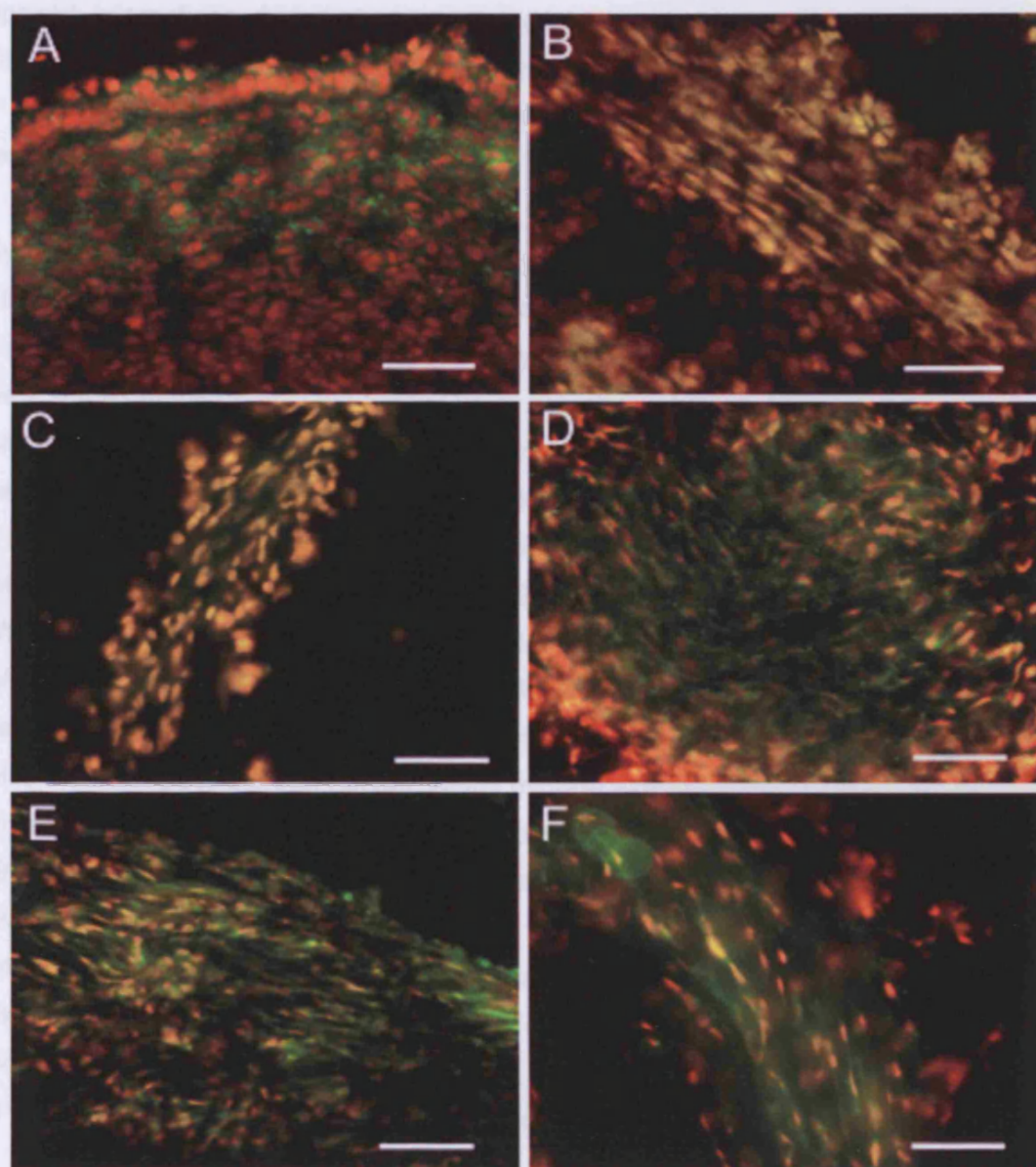
D- 12day- The presence of connexin 43 show dotted labelling, similarly to the labelling mesenchyme at day 6.

E- 15day- Connexin 43 labelling follows the longitudinal orientation of the tendon.

F- 17day- Connexin 43 is present with its characteristic puntate pattern of labelling.

2.4 DISCUSSION

Photographs and higher magnification fluorescence images of the explants and grafts in combination with other assays or specific treatments and their



Type I collagen appears early in the development and is robustly induced at 8 days of development, indicating that basement membrane and extracellular matrix are difficult to modify. Type I collagen is a major component of the developmental response and is closely associated with the cell surface and the surface of each collagen that binds to the extracellular matrix, such as decorin, fibronectin and laminin [Kosher and Vlodavsky, 1997; Sponner et al., 2000]. It is known to be present in embryonic [Glick et al., 1995; Eiken et al., 2000] and is important in controlling assembly and turnover of collagen [Roth [Schmidt et al., 1999; Roth et al., 1995; Weber et al., 1998; Eiken et al., 2000].

3.4 DISCUSSION

Investigations into tendon development have concentrated on its progression and growth in comparison with either muscle or skeletal development, and their reciprocal interactions during development (Kardon, 1998; Oldfield and Evans, 2003; Edom-Vovard and Duprez, 2004; Brent et al, 2005). This chapter details cell-cell interactions and cytoskeletal components involved in early tendon cell differentiation.

Early identification of tendon blastemas at day 6 has not previously been reported, but they are described from day 7 (Hurle et al, 1990; Ros et al, 1995). It may be that in some specimens examined the embryos were at late day 6, and that the earliest stages of the developing tendon were actually visible, certainly the putative blastemas have appropriate structure, matrix and cellular markers. Tenascin remains the easiest extracellular marker for identifying developing tendon, but it is also expressed by developing ligament, perichondrium and by satellite cells of growing nerves (Chiquet and Fambrough, 1984; Martini and Schachner, 1991; Werhle and Chiquet, 1990 and Wehrle-Haller et al, 1991). In later stages of development these structures can be clearly distinguished, however, in early stages, such as day 6, when any tendon primordia are extremely early in their development it is possible that nerves, which migrate alongside the tendons, might be mistaken for developing tendon (Martini and Schachner, 1991; Werhle-Haller et al, 1991). It remains possible to distinguish these from developing tissues labelled positively for tenascin, as examination under polarized light shows the collagen ensheathing the nerves is birefringent, whereas the embryonic tendon is not. At later stages of chick development the nerves do not continue to label positively for tenascin (Wehrle-Haller et al, 1991). However, there was no obvious evidence of nerve fibres within the structures, although these were not specifically labelled.

Type I collagen appears early in the developing tendon and is intensely labelled at 8 days of development, the labelling then becomes less prominent and individual fibres are difficult to identify. Type I collagen labelling patterns in later stages of the developmental sequence are possibly due to the molecules associated with the surface of each collagen fibril. Small, leucine-rich proteoglycans, such as decorin, fibromodulin and lumican (Hedbom and Heinegard, 1993; Svensson et al, 2000) are known to be present in embryonic tendon (Birk et al, 1995; Ezura et al, 2000) and are important in controlling assembly and diameter of collagen fibrils (Schonherr et al, 1995; Birk et al, 1995; Weber et al, 1996; Ezura et al, 2000).

Although, the distribution of these in relation to collagen fibrils has not been thoroughly studied in earlier stages of development, fibromodulin mRNA has a 6-8 fold increase from day 14-19, and decorin mRNA increases threefold (Nurminskaya and Birk, 1996), indicating both are present and likely to bind the type I collagen fibrils present. Other molecules indirectly bound to type I collagen fibrils are the FACIT collagens types XII and XIV (Keene et al, 1991). The presence of all these molecules along a type I collagen fibril may be responsible for the reduced ability of the antibody to bind to the fibril, creating the diffuse labelling pattern observed in later stages of tendon development.

Type III collagen shows a possible orientation of label in the day 6 specimen. In this section it is closely associated with a developing cartilage blastema, suggesting that it is a developing tendon insertion. The longitudinal orientation of the type III collagen fibrils in day 8 clearly shows nuclei between the fibrils. When observed at day 10, in an oblique section, areas of more than one cell nuclei are encompassed by the type III collagen fibrils which are present. This is perhaps an early indication of the differentiation of endotenon cells in comparison with tendon fibroblasts and the establishment of the fascicular architecture of the tendon, type III collagen is associated with adult tendon (Kumagai et al, 1994; Murphy et al, 1997) and fascicular structure (Birk and Mayne, 1997). This differential expression of type III collagen persists through day 12, although with the angle of section it is more difficult to distinguish, into the clearly restricted expression seen at 15 days with the development of tendon fascicles (Birk and Trelstad, 1986; Keene et al, 1991; Birk and Mayne, 1997; Young et al, 2000; Schuppan et al, 2001). It is interesting to speculate that the type III collagen observed clearly in the 8 day tendons actually represents initial deposition of the fascicular structure and is followed by type I collagen expression of the tendon fascicles proper.

The presence of tenascin-C in developing tendons is well documented (Hurle et al, 1990; Kardon, 1998), and it seems likely that this is what is labelled here. Although tenascin has adhesive and anti-adhesive properties, it is the anti-adhesive properties that predominate in tenascin-C (Chiquet-Ehrismann, 2004a, b). Tenascin-C is known to inhibit cell adhesion to fibronectin (Chiquet-Ehrismann et al, 1986; Riou et al, 1992; Orend and Chiquet-Ehrismann, 2000), implying that it may interfere with cell interactions with one another via cell surface fibronectin, and with the wider ECM. Within the developing tendon, at the cellular level, there is a requirement for both cell adhesion and non-adhesion. The cells form spaces for

deposition of collagen fibres, with no cell-cell contact, bounded by regions of cell-cell contact (Trelstad, 1982; Trelstad and Birk, 1984; Birk et al, 1991; Birk and Zycband, 1993). Therefore, membranes need to be kept separate between points of contact. The longitudinally running tenascin could help to maintain such separation. Its up regulation by mechanical strain (Chiquet-Ehrismann et al, 1994; Fluck et al, 2000) indicates that it will become a consistent component of loaded adult tendons, as well as in the developmental sequence examined here.

Actin filaments were present from the early stages of tendon development, present as fibres with a longitudinal orientation. Actin fibres occur as longitudinal contractile stress fibres in adult tendon cells *in vivo*, linked by adherens junctions (Ralphs et al, 2002). It is possible that this pattern is being established as soon as the tendon differentiates. However, actin stress fibres are also associated with cell orientation and early deposition of orientated collagen matrix, for example in the annulus fibrosus of the developing intervertebral disc (Hayes et al, 1999). Here, actin stress fibres appear, orient cells, and in later stages disband once the orientation is established. It may be therefore, that the actin stress fibres are involved in cell and matrix organisation. Unlike the intervertebral disc, however, the fibres persist in tendon into adulthood. In adult tendon cell stress fibre components are upregulated by strain (Ralphs et al, 2002) and so it could be that in development, initial orientational fibres are replaced by strain regulated fibres. The longitudinal orientation of the actin filaments may be influenced by reciprocal interactions with developing muscle (Kardon, 1998; Edom-Vovard et al, 2001, 2002), indicating a level of strain present in the developing tendon. The actin characteristic of connective tissue cells is alpha smooth muscle actin (Darby et al, 1990; Kim and Spector, 2000; Premdas et al, 2001; Kinner and Spector, 2002; Kinner et al, 2002; Hastreiter et al, 2004; Desmouliere et al, 2004). Whilst in the studies here we could not distinguish this, as phalloidin binds to all filamentous actin, it seems probable that the actin fibres are made of this particular actin.

The vimentin intermediate filaments in developing tendons correlate with the actin filaments in having an elongated orientation. In monolayer the vimentin intermediate filaments have been shown to have a degree of orientation corresponding with the direction of the cell, similar to actin stress fibres (see chapter 4). Intermediate filaments are known to provide the cell with mechanical stability and can withstand greater forces than actin stress fibres (Janmey, 1991; Stewart, 1993). Vimentin is present in load bearing areas of tendon, mainly areas

of fibrocartilage formation (Ralphs et al, 1991; Rufai et al, 1992) and is present, but certainly not prominent in areas of tendon not under compressive forces (Ralphs et al, 1998). In cartilage, vimentin is predominant in deep zone chondrocytes, with complex distributions surrounding the nucleus and underneath the plasma membrane. Its function here suggests involvement in cytoskeletal responses to changes in mechanical conditions (Durrant et al, 1999). There is evidence that filamentous actin acts as a template for cytokeratin intermediate filaments (Weber and Bement, 2002), and it is possible that vimentin intermediate filaments can function similarly, hence their longitudinal arrangement *in vivo*. Therefore it is possible the function of vimentin-based intermediate filaments present in development tendon are involved in maintaining the mechanical stability of the cells at the onset of mechanical strain.

The punctate label of n-cadherin is suggestive of the presence of adherens junctions between cells in the early stages of tendon development. Label for pan-cadherin was subtly different, being more extensive at the cell surface, giving a less sharp punctate pattern. It is possible this is an indication of additional cadherins present at these stages of development. Cadherins are involved in adherens junctions, connecting the actin filaments of adjacent cells and are present in adult tendon (Ralphs et al, 2002). N-cadherin is expressed by tendon and ligament fibroblasts *in vivo* (Ralphs et al, 2002; Lin et al, 1999). N-cadherin has been identified in mesenchymal condensations (DeLise and Tuan, 2002 a, b) and is thought to play an important role in the subsequent differentiation of chondrocytes (Oberlender and Tuan, 1994). It is possible that n-cadherin has a role in cell-cell adhesion, early assembly and cell differentiation within tendon blastemas. This is discussed fully in chapter 6.

In later stages the labelling pattern of both cadherin antibodies became more diffuse, with prominent fluorescence associated with tendon cells, but no obvious punctate pattern. Since cadherins are organised into punctate regions between adult tendon cells longitudinally in their cell rows *in vivo* (Ralphs et al, 2002), it may be that this represents a stage of reorganisation of cell-cell contacts from an embryonic to adult phenotype. This could be associated with the onset of the loading of tendons by differentiated muscle. Certainly, these junctional components, including cadherins and vinculin, are mechanically regulated (Ralphs et al, 2002). Unlike the earlier stages, the identical labelling pattern for n-cadherin

and pan-cadherin suggests either that only n-cadherin is present, or that, if others are present they are distributed identically.

Expression of vinculin appeared to change during the developmental period examined. Early on it was rather less prominent than the cadherin label, but at the later stages it appeared more strongly with a punctate pattern of labelling, reminiscent of its pattern in the adult (Ralphs et al, 2002). Vinculin is a component of focal adhesions between intracellular actin, cell surface integrins and the ECM (Critchley et al, 1999; Critchley, 2004; Nayal et al, 2004) and cell-cell actin linking adherens junctions (Knudsen et al, 1995; Rimm et al, 1995; Watabe-Uchida et al, 1998; Weiss et al, 1998). It would appear that at the early stages no prominent focal adhesions or adherens junctions were being formed, and that the cadherins are involved in weak cell-cell interactions and signalling, not involving large actin structures. Later, the more adult pattern of strong structural attachment appears to be formed. Cells may be involved in various migratory and orientational events earlier on with their position becoming more stabilised at later stages of development. Vinculin expression is mechanically regulated (Ralphs et al, 2002), so this could be related to increased muscle contraction in development (Ros et al, 1995; Kardon, 1998).

Gap junction distribution has been studied in developing tissues, with particular reference to cartilage condensations, and roles for connexin 43 have been demonstrated (Schwab et al, 1998; Zhang et al, 2002). Roles for connexin 32, however, have not been shown to date. Here, the results show connexin 32 to be a prominent component of interactions between mesenchymal cells, along with connexin 43, but they show that connexin expression changes with tendon differentiation. In developing tendon connexin 32 is present from when the blastemas first occur, whereas connexin 43 only becomes prominent later on, at around 12 days onwards. Both are present in adult tendons (McNeilly et al, 1996; Ralphs et al, 1998) and in the periodontal ligaments (Yamaoka et al, 2000), recent studies show that they are important in the response of tendon cells to mechanical load (Waggett et al, submitted; Waggett et al, 2001; personal communication). Connexin 32 is associated with increasing collagen secretion in response to strain, whereas connexin 43 inhibits it. The two connexin types work together to control tendon cell response to load.

Here the connexin 32 stimulatory pathway appears predominant in early tendon, with connexin 43 only becoming obvious as the tendon ECM becomes well established. This may correspond to the onset of mechanical loading from muscles, as the expression of connexin 43 is mechanically regulated (Banes et al, 1995). Interestingly, there is enhanced label for connexin 32 at the periphery of the tendon blastemas and in the immediate surrounding mesenchyme at 8 days. This would appear to relate to the separation of the tendon from its surroundings and is indicative of coordinated cell behaviour, possibly in the formation of the epitenon and early tendon sheath.

CHAPTER FOUR

**Matrix deposition by tendon cells in culture: A
comparison of 2-D and 3-D culture methods**

4.1 INTRODUCTION

The use of various cell culture systems with connective tissue cells has been discussed in chapter 1. Here, the behaviour of tendon cells is examined in monolayer and at high density to determine details of cell growth, matrix synthesis and matrix deposition. The aim is to determine the extent to which 3-D organisation of cells is necessary for the deposition of functional tendon matrix.

Monolayer cultures are routinely used to grow many different cell types. So far as connective tissue cells are concerned, whilst monolayers are an effective way to increase cell numbers, they are not associated with differentiated function, i.e. the ability of cells to deposit a functional extracellular matrix. Previous studies have shown that fibroblasts in monolayer continue to synthesise collagens, but release an overwhelming majority into the culture medium, rather than depositing it into a matrix within the cell layer (Herrmann et al, 1980; Freiburger et al, 1980).

At present there is comparably little data on tendon fibroblasts in monolayer cultures and their differentiated state is relatively unknown. Studies have shown that tendon fibroblasts in culture continue to produce extracellular components, characteristically type I and type III collagens (Herrmann et al, 1980). There is evidence to suggest that tendon cells in monolayer do de-differentiate, the rationale being that they lose their ability to synthesise large amounts of collagen relative to other proteins (Schwarz et al, 1976; Quinones et al, 1986; Bernard-Beaubois et al, 1997). It was also discovered that if the lactate ion concentration was raised, the tendon fibroblasts could resume the initial level of collagen synthesis that occurs *in vivo* (Klein et al, 2001). However, the majority of collagens and other extracellular matrix components produced by fibroblasts in monolayer are at reduced levels to that originally synthesised *in vivo* and most are released into the media, rather than being retained by the cells (Herrmann et al, 1980). This investigation also noted that as the number of passages increased, so did the amount of cells expressing type III collagen. Further studies on collagen polypeptides also showed evidence of de-differentiation. Type I collagen is comprised of $\alpha 1(I)$ and $\alpha 2(I)$ chains in a 2:1 ratio (Karsenty and Park, 1995). In primary cultures of tendon fibroblasts, however, this ratio did not remain constant, but increased with trimers of $\alpha 1(I)$ chains being produced simultaneously with the heterogenic trimers (Quinones et al, 1986). The transcription factor scleraxis has more recently been used to identify the differentiated state of tendon fibroblasts. Scleraxis expression has been researched *in vivo* and various *in vitro* systems

(Cserjesi et al, 1995; Schweitzer et al, 2001; Schulze-Tanzil et al, 2004), although there are no reports of scleraxis expression in tendon fibroblast monolayer to date. Monolayer cultures have major interactions with the culture substrate, along with a degree of cell-cell interaction, with the two being impossible to separate.

Connective tissue cells often retain their phenotype when grown under conditions of high cell density: this is particularly evident with chondrocytes (Aherens, 1977; Solursh, 1982); and osteogenic cells (Gerber and ap Gwynn, 2002). When large numbers of chondrocytes are centrifuged to form a pellet, they deposit a typical cartilage matrix (Moscona and Moscona, 1952; Moscona, 1963; Solursh, 1982), whereas if they are grown in monolayer they do not, but instead de-differentiate and stop synthesising cartilage specific molecules (Grundmann et al, 1980; Brodtkin et al, 2004). Similarly, when osteoblasts are initially plated as monolayers, a reduction in type I collagen expression is observed. No bone forms until large, highly cellular nodules had formed within the cultures (Gerber and ap Gwynn, 2002). A variant of the high density cell culture is the micromass (Ahrens et al, 1977; Solursh, 1982; Archer et al, 1982, Daniels and Solursh, 1991). In these systems, a high density spot of cells is plated into a culture dish, the cells are allowed to adhere and then the dish is flooded with medium. The high density spot allows high cell densities with small numbers of cells and has been previously shown to successfully produce cartilage formation from mesenchymal precursors (Mello and Tuan, 1999; Carlberg et al, 2001; White et al, 2003; Hatakeyama et al, 2003), differentiated chondrocytes (Seghatolleslami et al, 1995; Enomoto-Iwamoto et al, 1997; Kameda et al, 2000) and even dermal fibroblasts, in the presence of lactic acid (Nicoll et al, 2001). In micromasses the cells maintain their rounded nature and phenotype, therefore this system is predominantly used to study chondrogenesis. Consequently, micromasses have been important in researching various aspects of precartilage condensation: the molecular and genetic expression of cartilaginous markers, cell-cell and cell-matrix interactions and influences of growth factors (Oberlender and Tuan, 1994; Tavella et al, 1994; Gluhak et al, 1996; Stott et al, 1998; Nicoll et al, 1998; Mello and Tuan, 1999). Micromass cultures have not been applied to tendon research, but they offer the opportunity of investigating 3-D cell cultures with small numbers of cells. However, in a micromass, cells do still interact with the culture dish as well as one another.

To remove the cell-substrate interaction factor and concentrate on the interaction of cells with each other, another simple high-density culture system can be used.

The pellet culturing system was developed to investigate cell aggregation from a range of tissues. It has been extensively used with chondrocytes (Moscona, 1963; Solursh, 1991) and is easy to use and to manipulate. This system lends itself to experimentation including the effects of growth and transcription factors and mechanical strains, generally compression (Lennon et al, 2000; Indrawattana et al, 2004; Bai et al, 2004; Huang et al, 2004; Bosnakovski et al, 2004). A potential disadvantage of pellet cultures is that the central regions of the pellet have restricted access to the nutrients when compared to the outer layers. This is probably why this system is useful for chondrocytes as they have a relatively low metabolic rate (Archer and Francis-West, 2003; Urban and Roberts, 2003), although tendon cells also respond to lactate (Klein et al, 2001; see above) suggestive of an extent of anaerobic metabolism in their normal environment.

A new method of culturing cells at high density, initially developed to investigate chondrocytes and mesenchymal cells, has recently been applied to tendon fibroblasts. This system aims to retain tendon fibroblast phenotype and maintain the high levels of matrix synthesis *in vivo* (Schulze-Tanzil et al, 2004). Tendon fibroblasts were expanded (P10) and suspended at a high cell density. Cells were placed onto a membrane filter (pore diameter 0.2µm), on top of a stainless steel grid at the medium-air interface in a petri dish, and incubated for 14 days. The conclusions drawn were that this culturing system did indeed maintain the differentiated state of tendon fibroblasts, in that they retained the ability to produce matrix characteristic of tendon and positively expressed scleraxis (Schulze-Tanzil et al, 2004). This system remedied the possible nutritional problems encountered in central zones of pellet cultures, but as with pellet cultures this does not address matrix organisation. The tendon fibroblasts continue to synthesise the correct elements but the order and structure of these components remains difficult to influence. It is interesting that this system fails to recognize the known benefit of lactate levels in tendon cell ECM synthesis (Klein et al, 2001) indicated above. Also, scleraxis is characteristic of embryonic, differentiating tendon/ligament rather than adult ones (Schweitzer et al, 2001), although scleraxis is known to be present in mature tissues (Tsai et al, 2003).

The experiments outlined below were performed to determine whether cell density and cell-cell interaction were important in the deposition of matrix by tendon cells *in vitro*. Monolayers allow cell-cell interactions, but have large elements of cell-substrate interactions, they will in all likelihood be used as a preliminary stage of

most tissue culture procedures, for purposes of cell expansion; micromasses have enhanced cell-cell interactions, but retain some cell-substrate interactions, therefore this system is only suitable for some cell types; pellet cultures have almost exclusively cell-cell interactions until the onset of matrix deposition, as cells do not adhere to the walls of the tubes used to contain them.

4.2 MATERIALS AND METHODS

Studies on the effects of cell density and 3-D cell organisation on matrix deposition were conducted using monolayers, micromass and pellet cultures.

Culture procedure

For tissue source, cell isolation and maintenance in detail refer to general material and methods (chapter 2).

Monolayer cultures- Tendon fibroblasts isolated from 50 day old broiler chickens were grown in monolayer to passage 3 (P3) for 7 days, fed with DMEM/5%FBS every 3 days, incubated at 37°C, with 100% humidity in 5% CO₂ in air atmosphere. At 7 days, the tendon fibroblasts were fixed with 95% alcohol at 4°C (see materials and methods, chapter 2).

Micromass cultures- Tendon fibroblasts, (source as above), were removed from P3 monolayer cultures, the cell suspension adapted to 2×10^7 cells/ml and 10µl aliquots (2×10^5 cells) spotted in the centre of 30mm culture dishes (Costar). The cells were allowed to adhere for 30 minutes and then the cultures flooded with DMEM/5%FBS and 1mg/ml ascorbate (Sigma). Cultures were maintained for 1 hour to 7 days and incubated as above.

Pellet cultures- Cell suspensions of P3 tendon fibroblasts were adjusted to 1×10^6 cell/ml in DMEM/5%FBS and 1ml of suspension aliquoted into sterile polypropylene eppendorf tubes. The cells were gently centrifuged (2000 rpm) allowing pellets to form at the bottom of the tube. The pellets were fed with 1ml DMEM/5% FBS, 12.5mM HEPES buffer (Gibco) and 1mg/ml ascorbate (Sigma) every 3 days. Tendon fibroblast pellets were incubated for time periods of 1 hour to 7 days as above.

Analysis

At termination the monolayers, micromasses and pellets were prepared for immunohistochemical labelling or electron microscopy. For immunohistochemical analysis, the monolayers, micromasses and pellets were fixed in 95% IMS at 4°C and labelled for ECM components (types I, II and III collagen and tenascin), cytoskeletal components (actin by direct phalloidin label, vimentin) and cell junctional components (vinculin, n and pan-cadherin and connexins 32 and 43) as described in chapter 2.

Electron Microscopy

Transmission electron microscopy- Pellet cultures were fixed immediately in 2.5% glutaraldehyde, in 0.1M sodium cacodylate buffer (pH 7.4) for 2 hours at 4°C. Specimens were then washed in 0.1M sodium cacodylate buffer (3 x 10 mins) and postfixed in 1% osmium tetroxide in 0.1M cacodylate buffer, for 1 hour at 4°C. Specimens were washed in distilled water (4 x 5 mins) and stained *en bloc* with 0.5% uranyl acetate for 1 hour at 4°C in the dark, washed again and dehydrated through an ethanol series. The dehydrated specimens were infiltrated with Spurr resin (Agar Scientific Ltd.) overnight before being orientated in fresh resin and polymerised overnight at 60°C.

Semi-thin (1-2µm) longitudinal and transverse sections were cut and stained with toluidine blue for 1-2 minutes on a hotplate, for orientational purposes, and then ultra-thin sections (60-90nm, silver-gold) were cut and collected on 200 copper mesh grids. Sections were stained in 2% aqueous uranyl acetate and Reynolds lead citrate (Reynolds, 1963), washed twice in distilled water and dried on filter paper. Sections were examined on a Philips TEM 208 electron microscope at an accelerating voltage of 80kV.

Scanning Electron Microscopy-Pellets were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer pH 7.2-7.4 for 24 hours. Samples were washed with 0.1M phosphate buffer (4 X 15 mins), rinsed with distilled water (3 X 5 mins) and dehydrated in a graded series of ethanol, critical point dried (Balzers CPD 030) and mounted on an aluminium stub with double stick carbon tape. Samples were then sputter coated (Sputter coater S150B, Edwards) with gold and examined on a Philips scanning electron microscope XL20 operating at an accelerating voltage of 20kV.

Some samples were sectioned directly after fixation in 2.5% glutaraldehyde to examine the central regions of the pellet, processing was then resumed as above. To investigate the identity of matrix deposited at the pellet surface pellets were treated with 0.5% collagenase (Sigma; see chapter 2) for time periods of 1, 2 and 5 minutes. The pellets were directly fixed in 2.5% glutaraldehyde and processed as above.

4.3 RESULTS

4.3.1 Monolayers

Extracellular matrix components

Cells in monolayer appeared elongated and flattened on tissue culture substrate. This environment is ideal for cell expansion, and allowed rapid cell growth. Immunolabels for extracellular proteins showed type I and type III collagen to be present in monolayers. Type I collagen had a streaky appearance and in some areas appeared extracellular (fig 4.1. A), whereas, type III collagen remained intracellular (fig 4.1. C). Type II collagen was not present in these monolayers (fig 4.1 B). Tenascin labelled at the periphery of cells outlining their boundaries (fig 4.1 D).

Cytoskeletal components

Prominent actin fibres were present throughout the monolayers with a longitudinal and parallel orientation relative to the orientation of the cells (fig 4.1 E). Vimentin was present and, similarly to actin, showed a degree of longitudinal orientation governed by the orientation of the cell (fig 4.1 F).

Cell junctional components

Pan-cadherin and n-cadherin were present, seen at cell-cell borders throughout the monolayer (fig 4.2 A, B). Their appearance was spotted and showed signs of following a longitudinal orientation in conjuncture with cell orientation. Vinculin labelling was also observed at cell-cell boundaries (fig 4.2 C). Small collections of vinculin organised in a streaky parallel fashion were observed between cells and in areas over the superficial surface of the cell nuclei. This suggested that the cells were not only forming cell-cell junctions but were also involved in cell-matrix junctions with the substrate. Gap junctional protein connexin 32 labelled positively across the monolayer (fig 4.2 D). The pattern of labelling was streaky and followed the orientation of the cell membranes. Connexin 43 was present (fig 4.2 F), although its labelling was weaker in comparison to the connexin 32, and was punctate in nature.

4.3.2 Micromasses

Micromasses enabled cells to be studied at high density. In a micromass the central region is the densest, with a less dense region of cell outgrowth at the periphery. Culturing fibroblasts in micromasses for long periods (5-7 days) lead to

cell migration away from the high density spot, which resulted in circular monolayers rather than high cell density, multilayered spots.

Collagens

Type I collagen was present in micromasses at all time points. At 1 hour of culture (fig 4.3 A) it appeared predominantly intracellular. The intensity of the type I collagen labelling appeared to increase after 7 days in culture when cells within micromasses became monolayers and labelling appeared extracellular (fig 4.3 E). Type III collagen was present, but barely detectable after 1 hour in micromass culture (fig 4.3 B). At 1 day in culture there was an apparent increase in label and some appeared to be extracellular (fig 4.3 D), this continued into 7 days of culture (fig 4.3 F).

Actin

Actin fibres were present and were particularly predominant in central regions of the micromass after 1 day (fig 4.4 A). The actin fibres had no obvious orientation within the culture, but were seen following the spreading membrane of the cells.

Cell junctional components

Pan-cadherin and n-cadherin were present in 1 day micromass cultures, with labelling generally restricted to the cell periphery (fig 4.4 B, C). Vinculin was present throughout the cells within the micromass cultures at the cell periphery with an even appearance rather than punctate, at all time points (fig 4.6 A, B, C). Gap junctional protein connexin 32 was present at 1 hour predominantly located in the central zone of the micromass (fig 4.5 A). At 1 day, connexin 32 label appeared to increase in intensity (fig 4.5 C), this label remained after 7 days in culture (fig 4.5 E). Connexin 43 was possibly present after 1 hour, although label was faint and difficult to identify (fig 4.5 B). At 1 and 7 days connexin 43 was present, with a clear punctate labelling pattern (fig 4.5 D, F).

4.3.3 Pellet cultures

To investigate the behaviour of cells at high cell density with no culture substrate, tendon fibroblast pellets were established.

Extracellular matrix labelling

Type I collagen was present throughout the tested time intervals. It was present after 1 day of incubation and distributed as small foci of label, in the main part of

the pellet away from the periphery (fig 4.7 A). Type I collagen became more extensive after 2 days in culture (fig 4.7 B) and the fibrous network was prominent centrally in the pellet. Cells at the periphery of the pellet, orientated perpendicularly to the surface, showed weaker labelling for type I collagen and formed a cellular capsule around the pellet. Following the 2-day time period there appeared to be no obvious difference in type I collagen distribution. At 3, 7, 9 and 10 days, type I collagen remained extensively fibrous and evenly distributed in the pellet (fig 4.7 C- F). After 14 days in culture label had diminished in the central region of the pellet (fig 4.7 G).

Type III collagen was present from 1 day through to 14 days in pellet culture and appeared to increase in intensity of labelling over time. The label was extracellular and fibrous after 1 day in culture (fig 4.8 A) and became extensively fibrous after 7 days in culture (fig 4.8 B). After 14 days in culture, type III collagen remained intense and widespread (fig 4.8 C). At the periphery, fibrous label appeared to run circumferentially (fig 4.8 B).

Label for type I pro-collagen was present throughout the pellet at all time points (fig 4.8 D-F). Not all cells labelled positively and there was no recognisable pattern to the labelling. After 14 days in culture when the type I collagen present was waning, type I pro-collagen remained positive (fig 4.8 F).

It was decided to label fibronectin as it is highly involved in extracellular collagenous and proteoglycan interactions, and is involved in cell growth, differentiation and migration. Fibronectin was present in cell pellets from each time point. The label was fibrous and extracellular (fig 4.8 G), but became restricted to the pellet periphery in 7 day pellet cultures (fig 4.8 H). After 14 days in culture fibronectin was still detectable, although it was restricted to localised areas (fig 4.8 I).

Cytoskeletal labelling

Positively labelled filamentous actin was observed in pellets at each time point (fig 4.9 A-C), the 'hazy' green effect seen suggesting a gel-like filamentous organisation was present within the cells. No prominent actin fibres could be seen at this time, although in some central regions of the pellets, rounded cells had strong actin label at their periphery (fig 4.9 C). Vimentin was present throughout the pellets and at each time interval (fig 4.9 D-F). There was an apparent decrease

in the labelling intensity of vimentin in 14day pellet cultures, although positive label could still be observed (fig 4.9 F).

Cell junctional labelling

Immunolabelling for n-cadherin and vinculin was positive at cell boundaries throughout the pellet cultures (fig 4.9 G-L). The punctate pattern of labelling for n-cadherin is clear at 1 day (fig 4.9 G). Connexin 32 was present at all time intervals and seen widely distributed throughout the pellet (fig 4.10 A-C). At 7 days it was possible to distinguish labelling polarity in the cells (fig 4.10 B), although this was not present in 1 or 14 day pellets (fig 4.10 A, C). There appeared to be a decrease in immunolabelling intensity for connexin 32 after 14 days in culture (fig 4.10 C). Connexin 43 was present although it remained sparse and appeared strongest between 1 and 7 days in culture (fig 4.10 D, E).

Electron microscopy

4.3.4 Scanning electron microscopy

Scanning electron microscopy (SEM) enabled the observation of the general structure of pellets at increased levels of magnification. The general shape after 1 day (fig 4.11 A) showed that the superior surface of the pellet was concave and contained many rounded cells. Flattened cells were found elsewhere at the pellet periphery and extensive cell-cell contacts could be observed (fig 4.11 B). There were areas where matrix connections between cells were evident (fig 4.11 C), and cellular blebs in other regions suggesting possible cell death. After 7 days in culture the concave superior surface was less obvious (fig 4.12 A), it had a 'smoother' appearance compared to the 1 day pellet. Outgrowths developed from the original pellet (fig 4.12 A). There was increased fibrous matrix deposition generally at the pellet surface (fig 4.12 B, C). Cells had many intricate cell processes and extensive cell-cell contacts (fig 4.12 C). After 2 weeks in cultures the outgrowths had increased in size and number (fig 4.13 A). It was evident there was some degree of orientation of the cells at the cell periphery (fig 4.13 B). Cellular contacts were still prominent (fig 4.13 C), although there was a considerable amount of debris of unclear origin at the pellet surface (seen in the right hand corner).

Sectioned pellets

SEM images of sectioned 7 day pellets showed that some pellets contained a small void in their upper central region, apparently formed by growth and folding

over of the 'rim', seen in the earlier pellet. Apart from this region, cells were distributed throughout the pellet. The cells covering the pellet surface appeared smooth like a capsule enveloping the cells underneath (fig 4.14 B). Deposition of ECM is not only present on the surface (fig 4.14 A), but areas of distinctly fibrous ECM were found within the pellet (fig 4.14 C).

Collagenase digested pellets

To assess the nature of the surface ECM deposition, pellets were digested in collagenase. After 1 minute digest there was little difference in the surface fibrous matrix and areas of highly aligned deposited, matrix remained (fig 4.15 A, B). Following two minutes of collagenase digestion however, partial degradation of the ECM was observed and the once taut matrix appeared relaxed and loose (fig 4.15 C, D). After 5 minutes in collagenase digest the surface ECM of the pellets was greatly affected and remnants of digested fibrous matrix remained at the pellet surface (fig 4.15 E, F).

4.3.5 Transmission electron microscopy

Transmission electron microscopy (TEM) allowed the study of the cellular morphology and matrix deposition in greater detail.

TEM images were taken of pellets after 1, 2, 5 and 7 days in culture. After 1 day cells were closely packed with cell processes visible varying in size and in regions of the pellet (fig 4.16 A-D). There were prominent mitochondria (with well-defined cristae) within the cells (fig 4.16 C), and large cytoplasmic glycogen stores (fig. 4.16 A). Fat droplets were also visible, together with lysosomal areas and regions of rough endoplasmic reticulum (fig. 4.16 B). There were areas within the pellet where it was possible to see fibrous material being deposited into intracellular spaces created by the cells (fig. 4.16 D).

At 2 days the TEM images show many cell processes within intercellular spaces (fig 4.17 B). There was a general increase in intercellular space and collagen was deposited into some, but not all intercellular spaces (fig 4.17 B). In some areas these cell processes contacted one another and cell junctions were formed (fig 4.17 B), although it was difficult to characterise which cell-cell junction had actually formed. Collagenous matrix was laid down between cells perpendicular to the cell membrane and appeared to be associated with more than one cell (fig 4.17 A). In some areas there appeared to be some longitudinal orientation of the collagen

fibres. There was a dramatic increase in collagen deposition compared to 1 day pellets with areas of parallel and longitudinal orientation (fig 4.17 C-D).

Five day pellets showed that cell processes remained and as before, in some areas there was no deposition of matrix in intercellular spaces (fig 4.18 B). Collagen fibres associated with 2 or more cells, which were high in glycogen stores, were present (fig 4.18 C). The collagen fibres radiated perpendicular to the cell membrane and an increased amount of collagen fibres were deposited in some intercellular spaces. In these intercellular spaces the collagen seemed to have little or no overall organisation, although there were small areas showing a parallel orientation of collagen fibrils in bundles (fig 4.18 A). In areas where cell membranes became close junctional, complexes were observed with cytoskeletal association (fig 4.18 D).

After 7 days of incubation elongated cell processes were present and were associated with adjacent, orientated collagen fibres (fig 4.19 A), although some collagen fibres were still organised perpendicularly to the cell membrane (fig 4.19 C). Associated with these depositions was an organised cytoskeletal component intracellularly, most likely to be actin (fig 4.19 C). Increasingly large amounts of collagen fibres were deposited in intercellular spaces, again with only small areas of parallel organisation (fig 4.19 B, D).

Figure 4.1- Tendon fibroblasts monolayers, at confluence at passage 3.

Immunohistological labelling of ECM and cytoskeletal molecules present in tendon fibroblast monolayers. Scale bars measured in micrometres.

A-Cell monolayer. Type I collagen label

Type I collagen is present showing streaks of label, possibly between cells.

B- Cell monolayer. Type II collagen label

There is no type II collagen label.

C- Cell monolayer. Type III collagen label

Type III collagen is present, although labelling appears mostly intracellular.

D- Cell monolayer. Tenascin label

Tenascin label occurs at cell surfaces, outlining the tendon cells.

E- Cell monolayer. Phalloidin label

Filamentous actin is present with prominent longitudinal orientation of the actin fibres within the cells, probably actin stress fibres.

F- Cell monolayer. Vimentin label

Vimentin is present throughout the monolayers with a degree of longitudinal orientation, in conjuncture with the orientation of the cells.

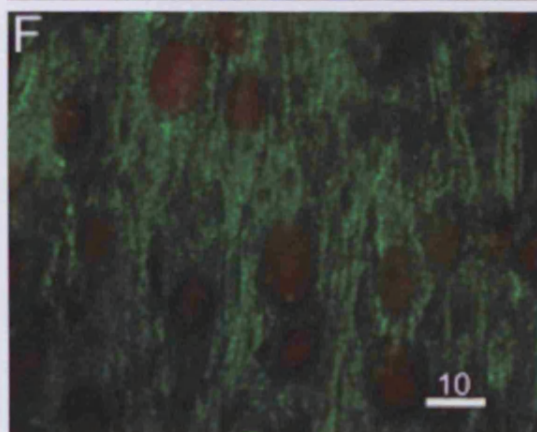
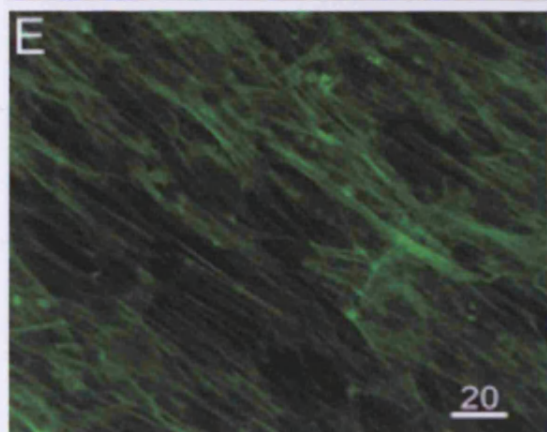
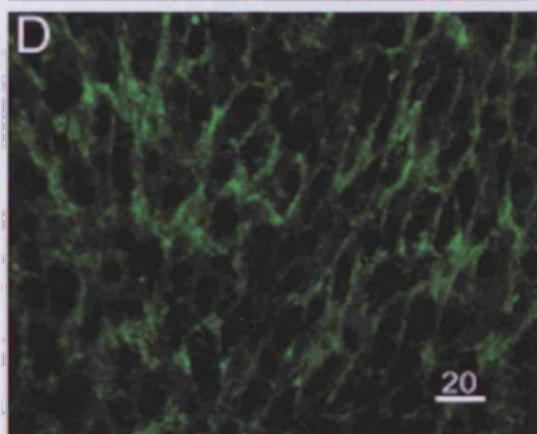
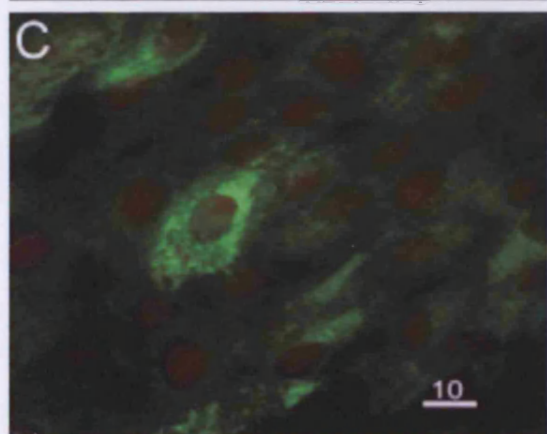
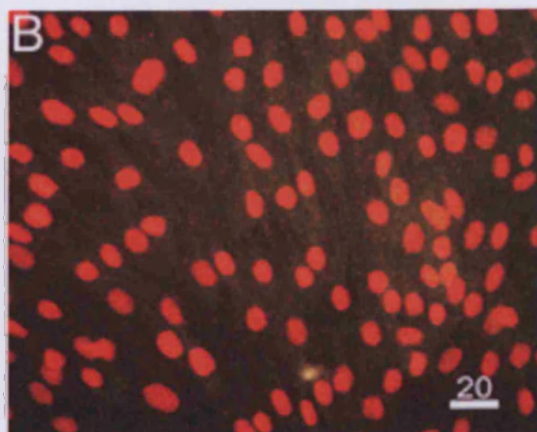
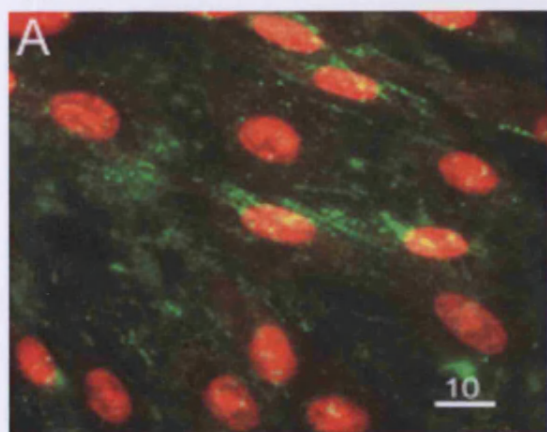


Figure 4.2- Tendon fibroblasts monolayers, at confluence at passage 3.

Immunohistological labelling of associated cell junctional molecules present in tendon fibroblast monolayers. Scale bars measured in micrometers.

A- Cell monolayer. pan-cadherin label

Pan-cadherin is present and concentrated at the cell borders.

B- Cell monolayer. n-cadherin label

n-cadherin is present with a similar distribution to pan-cadherin; concentrated at the cell boundaries and spotted in appearance.

C- Cell monolayer. Vinculin label

Vinculin is present and clustered into areas of short and longitudinally orientated parallel streaks between cell membranes. There are thin streaks seen on the superior surface of the monolayer indicating both cell-matrix adhesions and cell-cell adhesions.

D- Cell monolayer. Connexin 32 label

Connexin 32 is present, with wisps of longitudinally orientated label throughout the monolayer.

E- Cell monolayer. Connexin 43 label

Positively labelled connexin 43 is present in patchy areas, with individual foci being apparent.



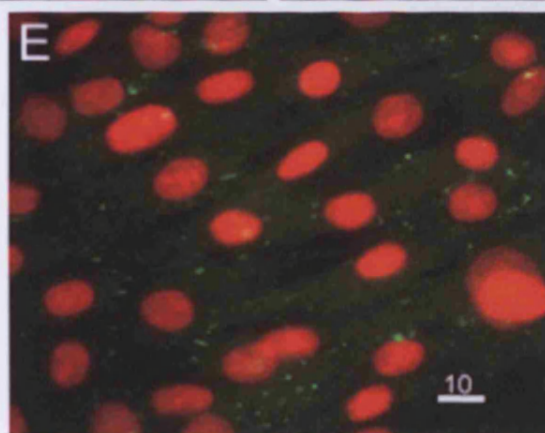
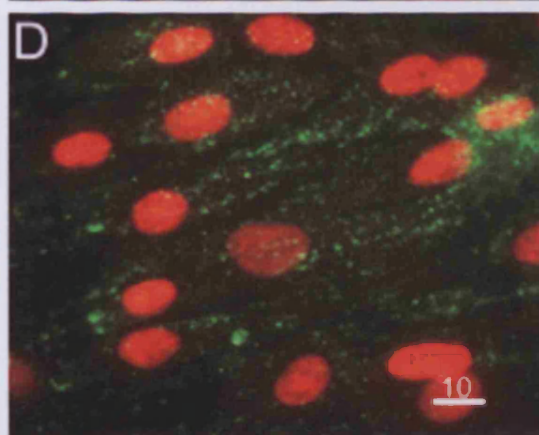
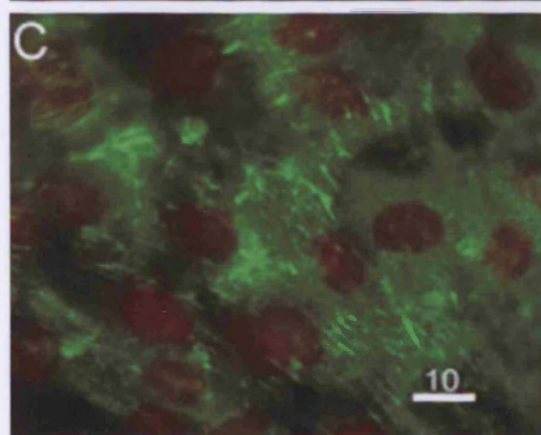
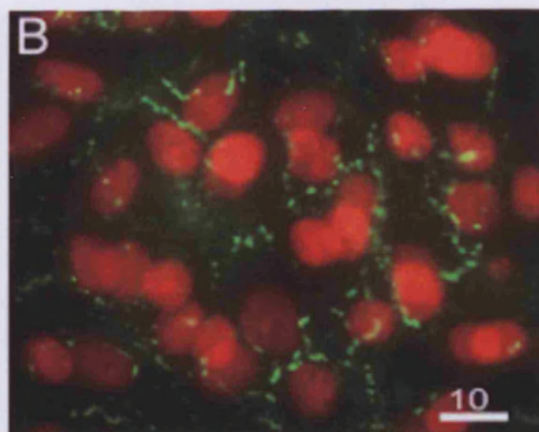
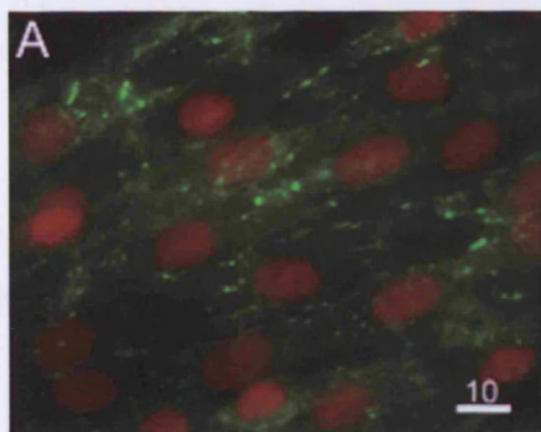


Figure 4.3- Micromass experiments. Collagens present in tendon fibroblast micromass culture systems. Scale bars = 50µm, unless otherwise stated.

Type I collagen- A, C, E

A- 1 hour -Type I collagen is present at all time points, at 1 hour the positive label is intracellular.

C- 1 day-After 1 day type I collagen is present but labelling was hazy.

E- 7days- Cells in culture over 7 days are increasingly flattened but still labelled positively for type I collagen.

Type III collagen-B, D, F

B- 1 hour-Type III collagen is faintly present in some cells within micromass culture.

D- 1 day- Positively labelled type III collagen appears to increase in intensity.

F- 7 days- The flattened fibroblasts resemble a monolayers culture, and the cells label positively for type III collagen.

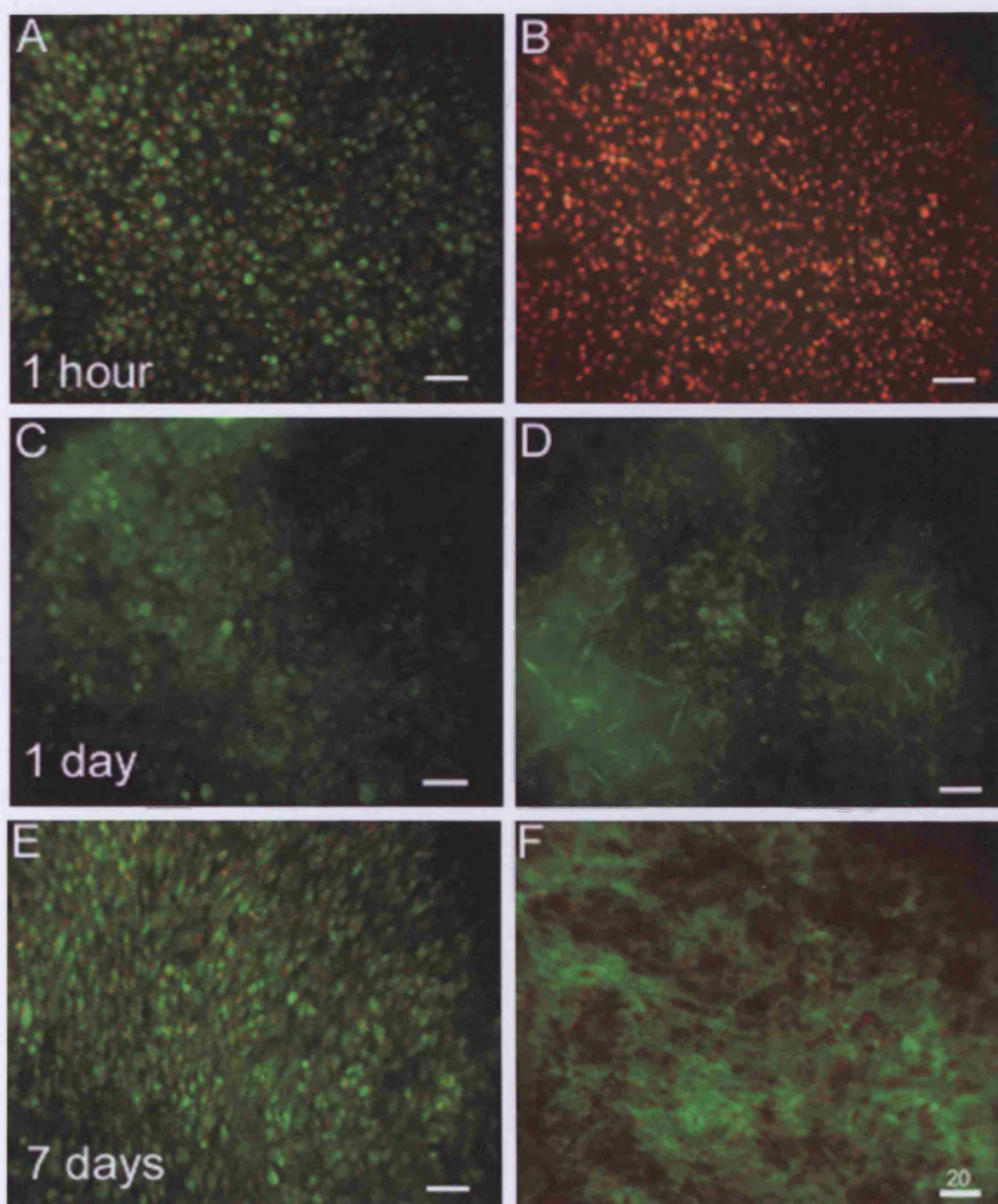


Figure 4.4- Micromass experiments. Actin, pan-cadherin and n-cadherin present in tendon fibroblast micromass culture systems.

A- Actin label. 1 day -Actin stress fibres are present, particularly in central regions of the micromass where they had a no clear orientation. Scale bar = 40µm

B- Pan-cadherin. 1day -Pan-cadherin is present and label is seen predominantly at the cell boundaries. Scale bar = 50µm

C- N-cadherin. 1day -n-cadherin is present, following a similar pattern to pan-cadherin, with abundant label at the cell edge. Scale bar = 50µm

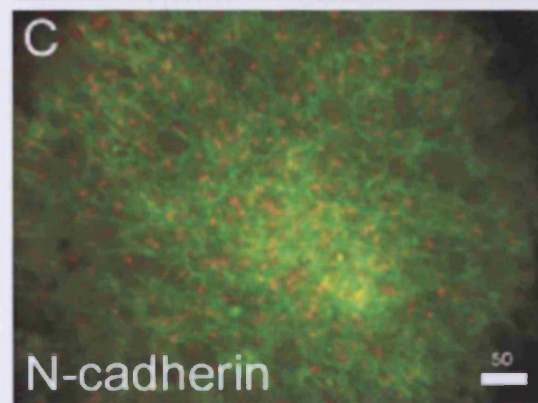
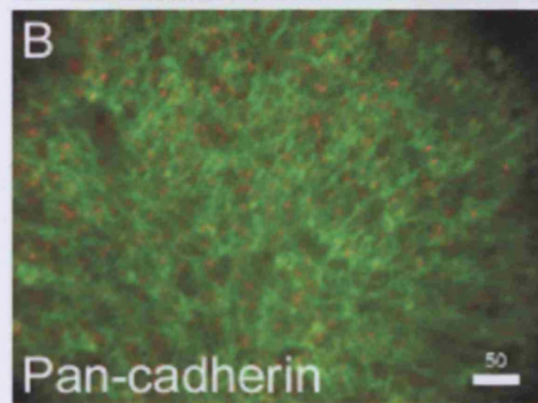
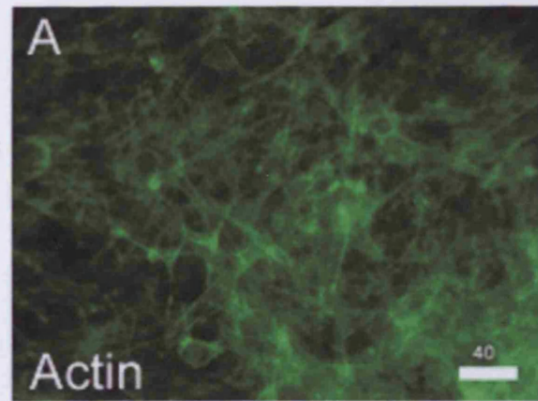


Figure 4.5- Micromass experiments. Gap junctional molecules present in tendon fibroblast micromass culture systems. Scale bars = 50µm, unless otherwise stated.

Connexin 32 label A, C, E

A- 1 hour -Connexin 32 is faintly present.

C- 7 day - The positive connexin 32 appeared to increase in intensity.

E- 1 day -Connexin 32 label continues to label positively.

Connexin 43 label B, D, F

B- 1 day- Connexin 43 is present after 1 hour in micromass culture.

D-1 hour-There appeared to be an increase in the connexin 43 present, with clearly identifiable punctate foci.

F-7 day -Connexin 43 continues to label positively.

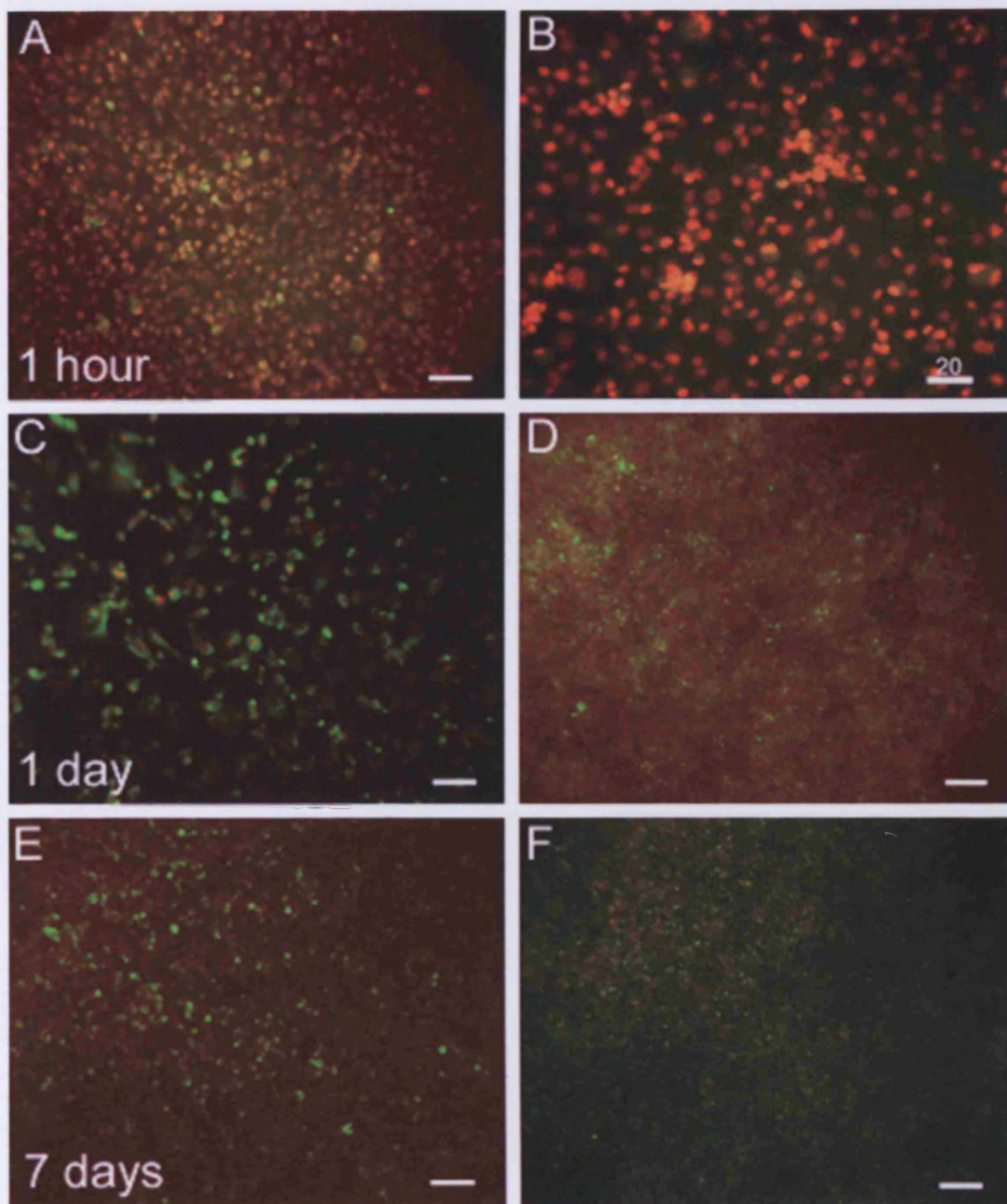


Figure 4.6- Micromass experiments. Vinculin present in tendon fibroblast micromass culture systems. Scale bar = 50µm.

Vinculin label A, B, C.

A-1hour -Vinculin is present, with labelling characteristically focused at the cell periphery.

B-1day -Positive labelling for vinculin was observed.

C-7day - Cells in micromass culture for 7 days label positively for vinculin.

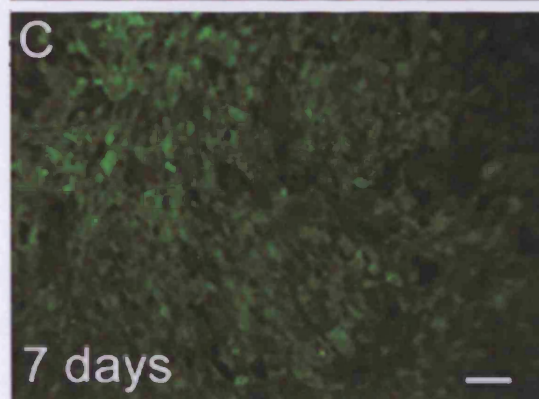
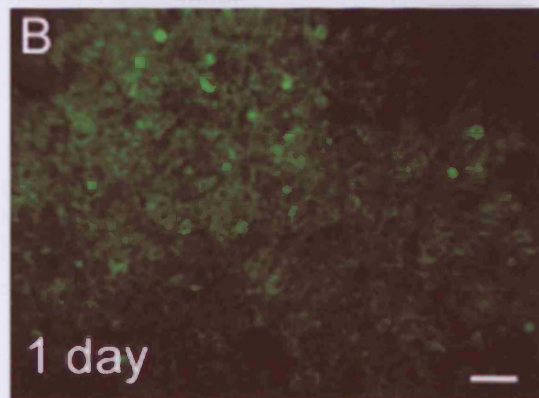
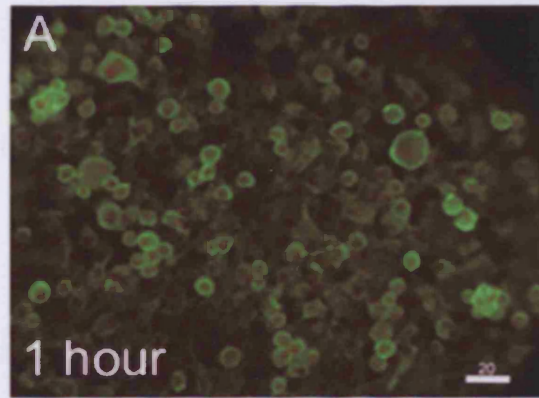


Figure 4.7 Tendon fibroblasts in a pellet culture, treated with 5% FBS/DMEM.

Scale bars = 50 μ m.

Type I collagen

- A- 1day pellet.** Positively labelled type I collagen has a fibrous nature, and areas at the periphery show a reduction in type I collagen.
- B- 2day pellet.** An apparent increase in labelling intensity for type I collagen is seen in the centralised region of the pellet, with a cellular rim labelling sparingly for type I collagen.
- C- 3day pellet.** Type I collagen is ubiquitously distributed throughout the pellet and appeared extensively extracellular and fibrous.
- D- 7day pellet.** There is little difference in the type I collagen distribution between 3 and 7 days in pellet culture, label is predominantly extracellularly.
- E- 9day pellet.** The fibrous nature of type I collagen remains consistent in the 9 day pellet cultures.
- F- 10day pellet.** Type I collagen is present throughout the cell pellet, elongated cells at the periphery continues to show lesser type I collagen labelling compared to the central zone of the pellet.
- G- 14day pellet.** Type I collagen is sparsely present in the centre of the cell pellet, the general appearance shows a deterioration of type I collagen in the central zone of the pellet.

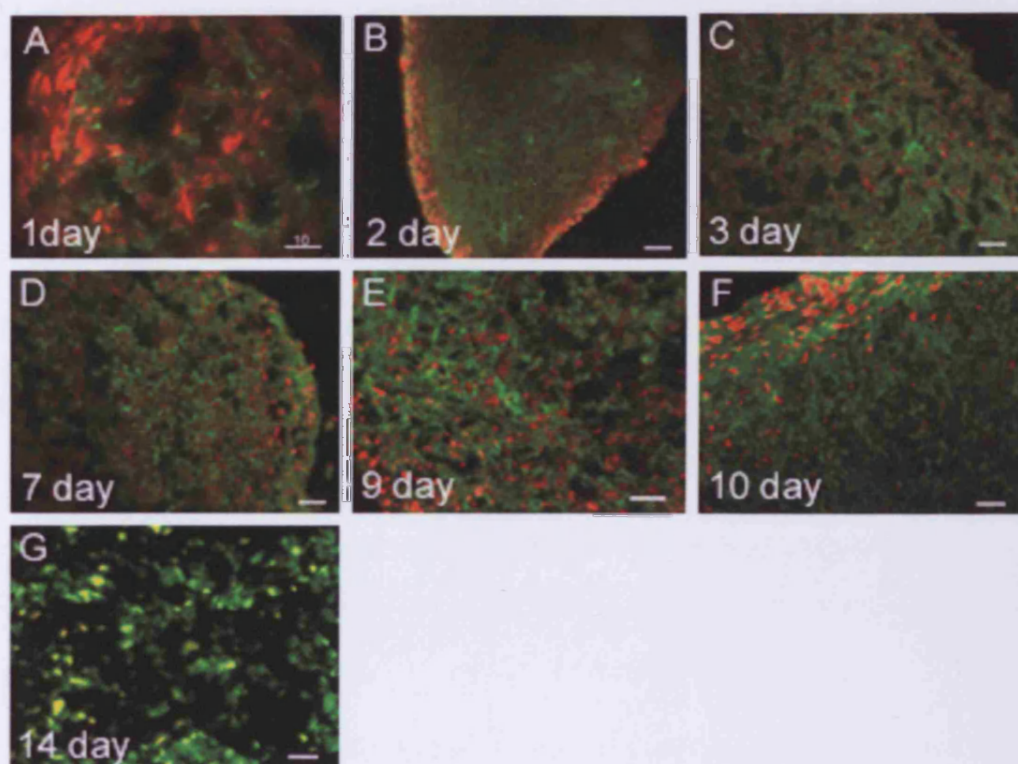


Figure 4.8 Tendon fibroblasts in a pellet culture, treated with 5% FBS/DMEM.
Scale bars = 50 μ m.

Type III collagen

A- 1day- Type III collagen showed extracellular, fibrous labelling patterns after 1 day in culture.

B- 7day-Positively labelled type III collagen continued to show extensive extracellular labelling and an apparent increase in intensity at the pellet periphery.

C- 14day-Type III collagen remained extracellular and widespread in the pellet.

Type I pro-collagen

D- 1day-Isolated cells within the pellet cultures label positively for type I pro-collagen. The sporadic labelling showed no pattern or differences between central and periphery zones.

E- 7day-Infrequent type I pro-collagen remained consistent after 7 days in culture.

F- 14day-After 14 days in pellet culture individual cells are consistently labelling positive for type I pro-collagen, even in the central region of the pellet.

Fibronectin

G- 1day- Fibronectin shows positive extracellular labelling after 1 day in culture.

H- 7day- Fibronectin is present predominantly in the peripheral regions of the pellet, maintaining a fibrous appearance.

I- 14day- Areas of fibronectin remain in the 14 day pellets, showing similar patterns of extracellular labelling.

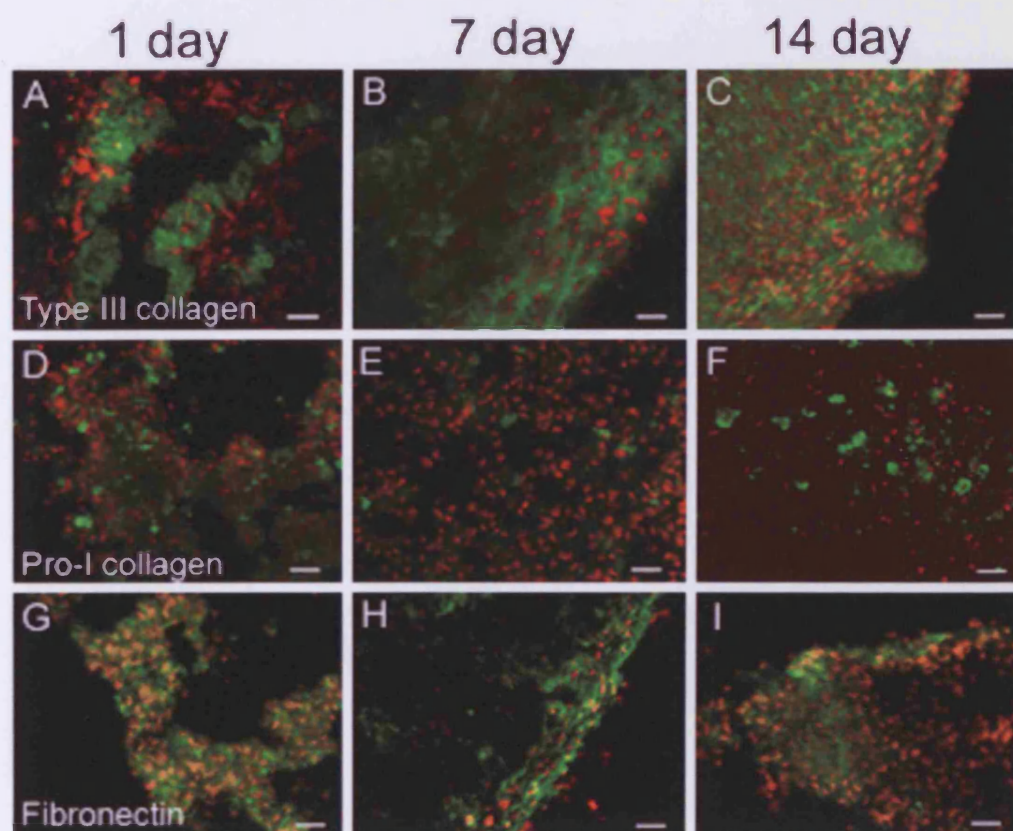


Figure 4.9 Tendon fibroblasts in a pellet culture, treated with 5% FBS/DMEM.

Scale bars = 50 μ m.

Actin

A- 1day-Actin is present and shows a green hazy background, indicating a gel-like filamentous structure of actin.

B- 7day-Only selected regions of the pellet shows the 'cloudy' green label, demonstrating the gel network of actin present in the cell.

C- 14day-Rounded cells label strongly for filamentous actin, particularly in central regions of the pellet.

Vimentin

D- 1day-Vimentin is present from 1 day in incubation and is extensively observed throughout the pellet.

E- 7day- A consistent level of vimentin can be observed in the pellet.

F- 14day- There are indications that vimentin label has diminished, although areas of vimentin remain distinguishable.

n-cadherin

G- 1day-n-cadherin is clear in pellets cultured for 1 day, the labelling pattern is predominant at the cell boundaries.

H- 7day- n-cadherin is present and widespread throughout the culture.

I- 14day-n-cadherin appears to have been reduced in 14 day cultures, although remnants of label are present

Vinculin

J- 1 day-Vinculin can be observed focused at the periphery of the cells throughout the pellet culture.

K-7 day- Vinculin shows consistent and extensive labelling.

L- 14 day-Vinculin is present after 14 days, although a possible reduction in label can be observed.

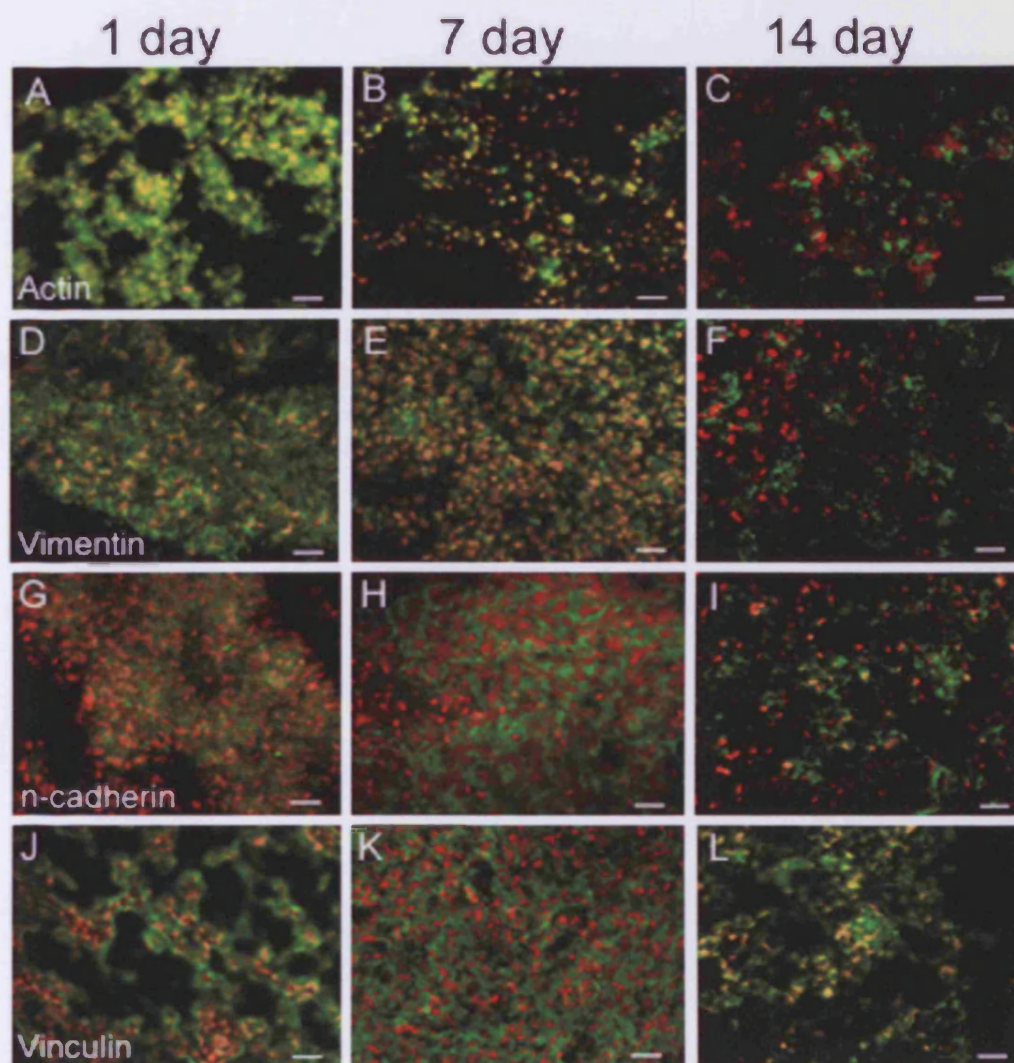


Figure 4.10 Tendon fibroblasts in a pellet culture treated with 5% FBS/DMEM.
Scale bars = 50 μ m.

Connexin 32

A- 1day-Connexin 32 can be identified, but is predominant at the cell periphery.

B- 7day-Connexin 32 is present in cells showing extensive label, many cells label positively for connexin 32 showing a degree of cellular polarity.

C- 14day-After 14 days cells continue to label positively for connexin 32, a focus of connexin 32 at the pellet edge can be identified.

Connexin 43

D- 1day-Sporadically labelled connexin 43 could be observed in areas of the cellular pellet. There is no pattern and the areas of labelling positive are infrequent and difficult to identify.

E- 7day-Small areas of connexin 43 were identified and are predominantly found in the pellet periphery.

F- 14day-There is little or no connexin 43 present in the pellets after 14days in culture.

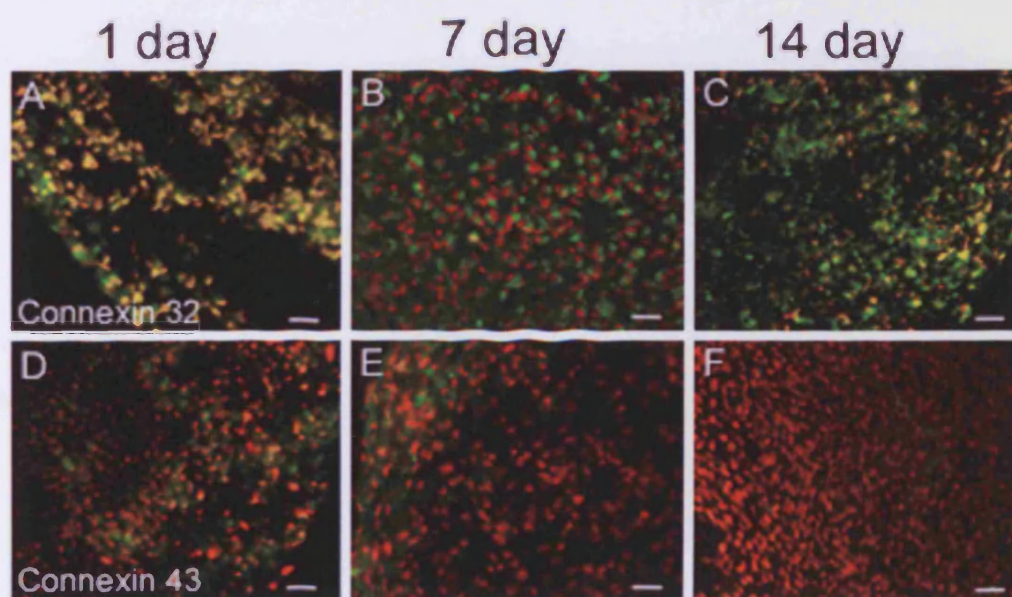


Figure 4.11 Scanning electron micrographs

A- 1day tendon fibroblast pellet

The general pellet shape after 1-day in culture shows clear rims where the cells have flattened and a concave superior surface containing more rounded cells.

Scale bar 500 μ m, magnification x140

B- 1day tendon fibroblast pellet

The flattened cells at the rim show a small degree of elongated orientation.

Scale bar 50 μ m, magnification x1000

C- 1day tendon fibroblast pellet

There is evidence of cell-matrix contacts between the more rounded cells.

Scale bar 5 μ m, magnification x10 000

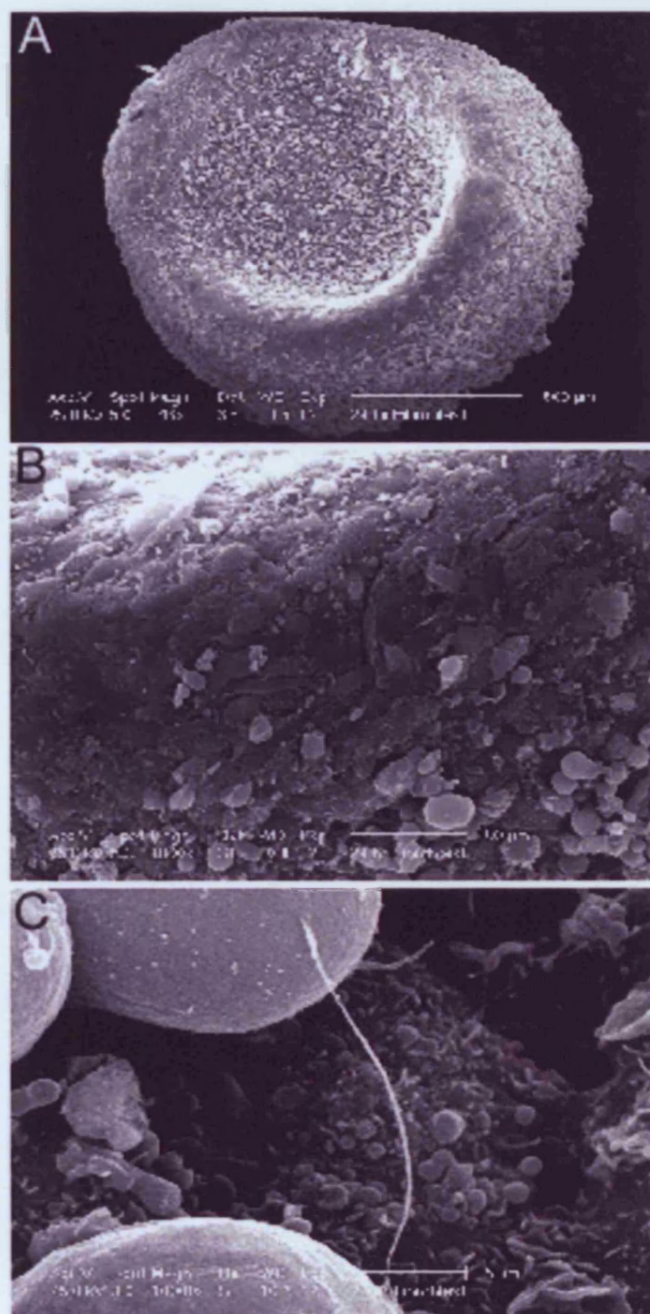


Figure 4.12 Scanning electron micrographs

A- 7day tendon fibroblast pellet

The pellet shape after 7 days in culture, cellular growths begin to appear and the concave appearance of the superior surface has begun to fill. Small outgrowth can be detected projecting from the pellet surface (top right).

Scale bar 200 μ m, magnification x51

B- 7day tendon fibroblast pellet

Closer inspection shows fibrous matrix deposited at the cell surface and elongated cells.

Scale bar 10 μ m, magnification x1000

C- 7day tendon fibroblast pellet

Elongate cell processes and contacts have developed.

Scale bar 10 μ m, magnification x1000

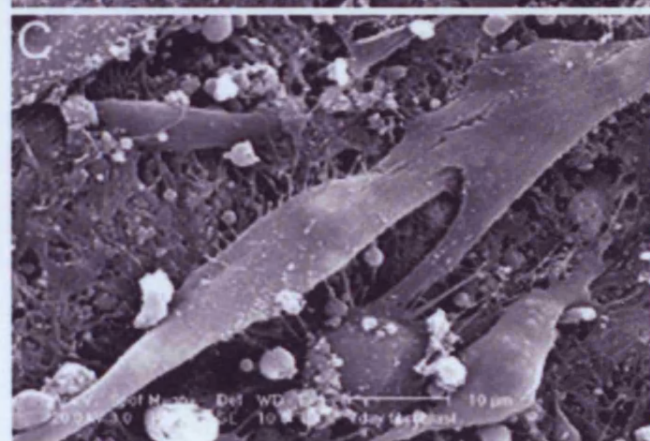
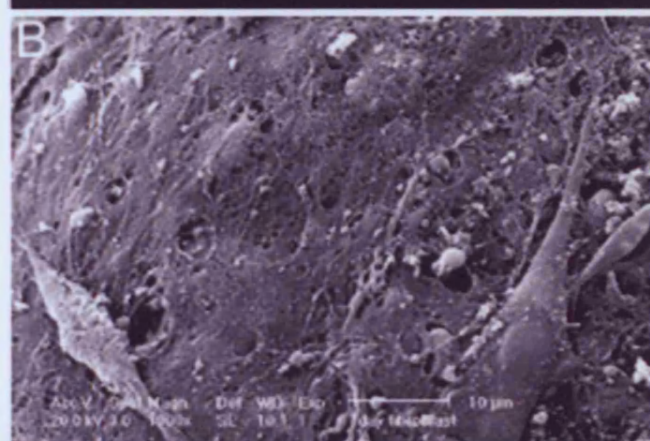
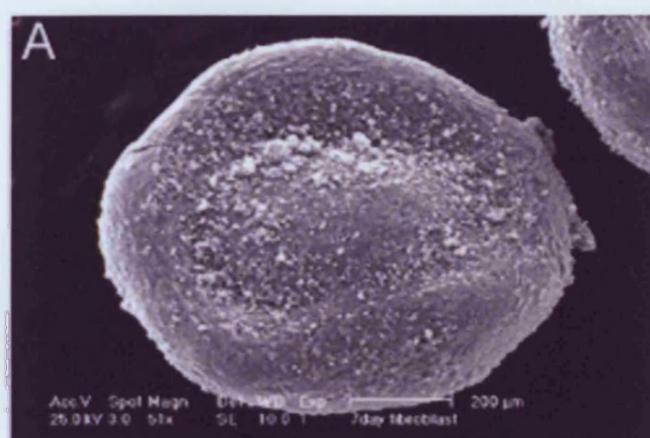


Figure 4.13 Scanning electron micrographs

A- 14day tendon fibroblast pellet

The pellet shape is less uniform, cellular outgrowths have increased in number and size.

Scale bar 200 μ m, magnification x65

B- 14day tendon fibroblast pellet

An increased orientation of the elongated cells is apparent, together with multiple cell processes.

Scale bar 20 μ m, magnification x800

C- 14day tendon fibroblast pellet

The flattened cell contacts are more established. Areas of debris are present, although the origin is unknown.

Scale bar 5 μ m, magnification x3500

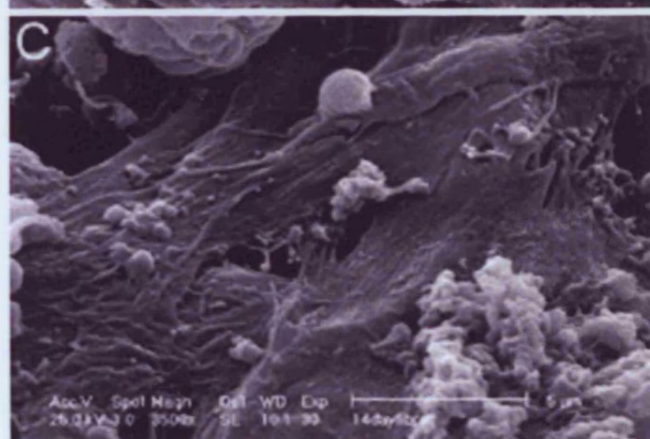
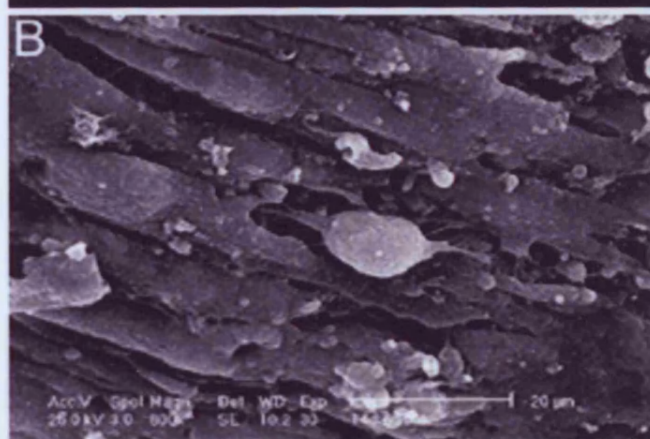


Figure 4.14 Scanning electron micrographs

A- 7day tendon fibroblast pellet, transverse section

The transverse section of a pellet after 7 days in culture shows a central cavernous area where the cellular growth has joined superiorly.

Scale bar 200µm, magnification x250

B- 7day tendon fibroblast pellet, transverse section

There are tightly packed cells within the pellet and the flattened cells only appear at the surface.

Scale bar 100µm, magnification x650

C- 7day tendon fibroblast pellet, transverse section

There is matrix deposition at areas within the pellet, not only at the surface (B).

Scale bar 5µm, magnification x12 000

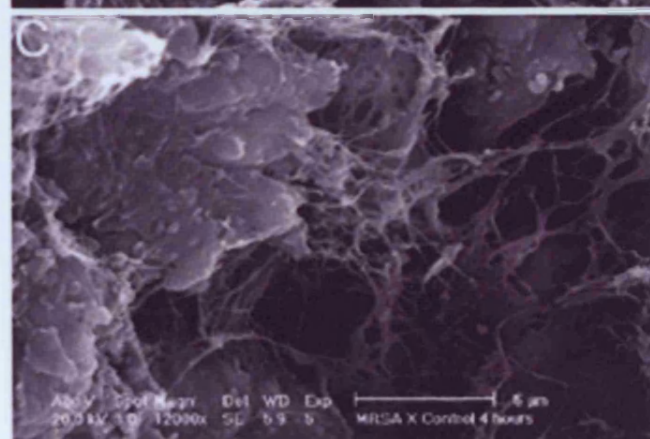
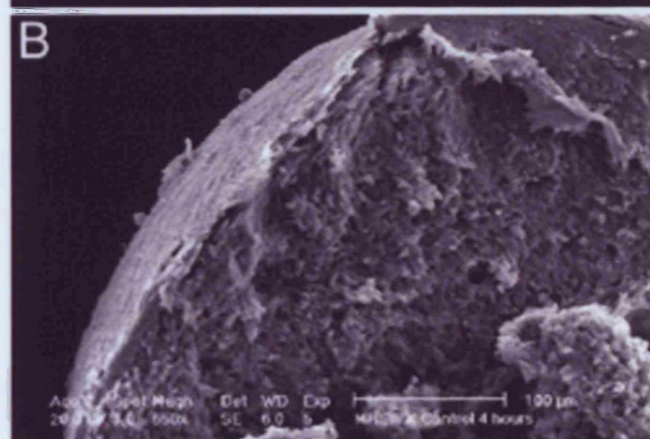
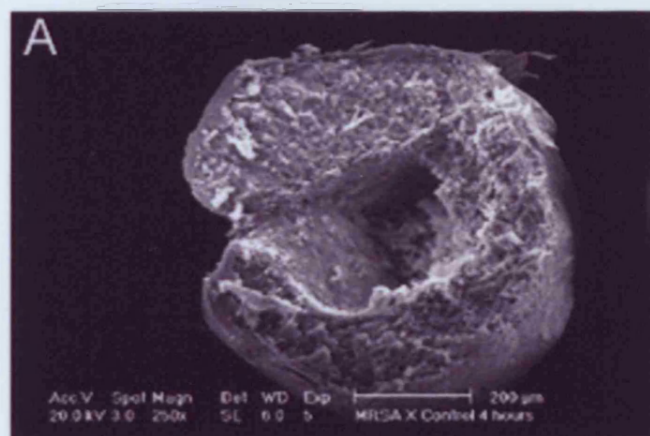


Figure 4.15 Scanning electron micrographs

A- 7day tendon fibroblast pellet. 1 minute collagenase treatment.

Elongate areas of cells and fibrous matrix remain present in the pellet despite collagen digestion.

Scale bar 10µm, magnification x5000

B- 7day tendon fibroblast pellet. 1 minute collagenase treatment.

Scale bar 5µm, magnification x10 000

C- 7day tendon fibroblast pellet. 2 minute collagenase treatment.

Scale bar 10µm, magnification x5000

D- 7day tendon fibroblast pellet. 2 minute collagenase treatment.

Elongate cells covered in fibrous matrices appear to have loosened and show less orientation after 2 minute collagenase digest.

Scale bar 5µm, magnification x15 000

E- 7day tendon fibroblast pellet. 5 minute collagenase treatment.

The fibrous matrix once covering the pellet surface has been removed, leaving matrix fragments covering the surface.

Scale bar 20µm, magnification x3500

F- 7day tendon fibroblast pellet. 5 minute collagenase treatment.

Small areas of matrix remain, although they appear heavily digested.

Scale bar 5µm, magnification x12 000

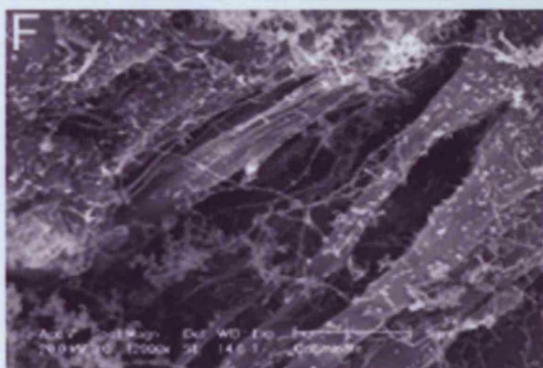
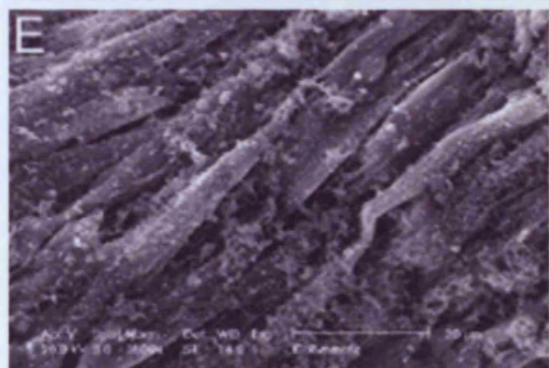
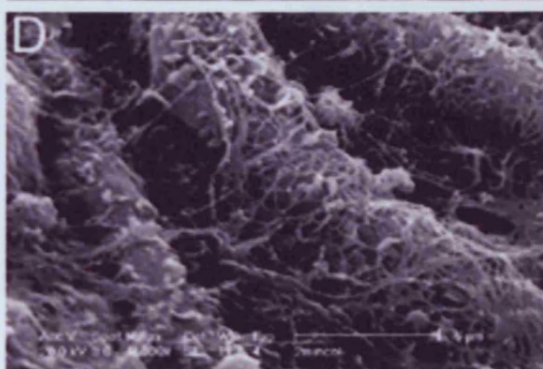
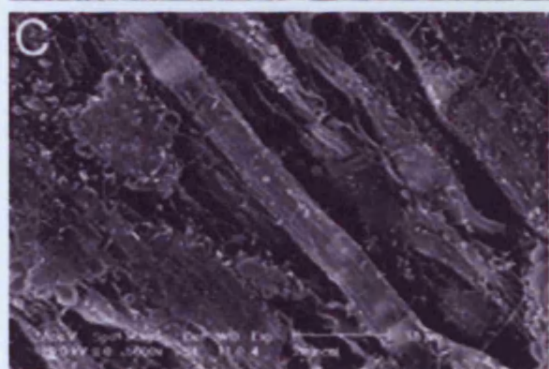
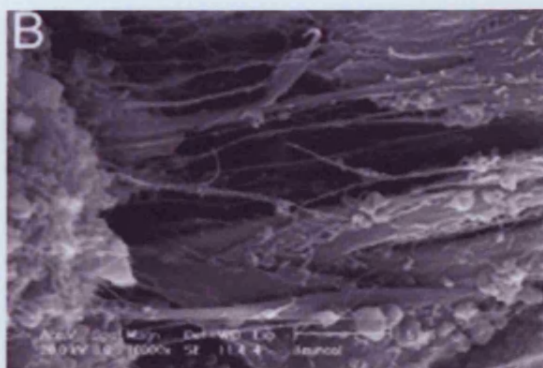
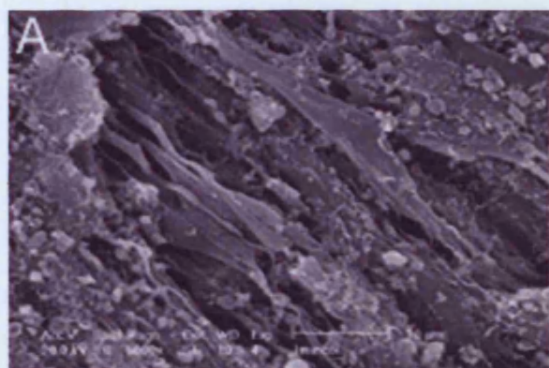


Figure 4.16 Transmission electron micrographs

A- TEM image. 1day pellet.

Large glycogen deposits are abundant within the tendon cells together with fat droplets. Cellular processes of varying widths pass between cells with intact plasma membranes.

Magnification x16K

Scale bar= 0.5µm

B- TEM image. 1day pellet.

Cellular processes within the tendon fibroblast cell pellet are clear (CP), together with large fatty deposits (F) Lysosomal bodies (L) are present and part of the rough endoplasmic reticulum is visible (RER). Areas of organised cytoskeletal components can be identified and a couple of mitochondria can be seen (M).

Magnification x16K

Scale bar= 0.5µm

C- TEM image. 1day pellet.

Numerous mitochondria are present, with well characteristically defined cristae (M). Surrounding matrices show fragments of possible fibrous matrix and proteoglycans present.

Magnification x16K

Scale bar= 0.5µm

D- TEM image. 1day pellet.

Cell processes (CP) and the deposition of fibrous matrix are suggested (F). Areas of intracellular glycogen deposits can be seen to the left and cytoskeletal components are visible running parallel with the cell membrane, in the cell on the right.

Magnification x63K

Scale bar= 0.16µm

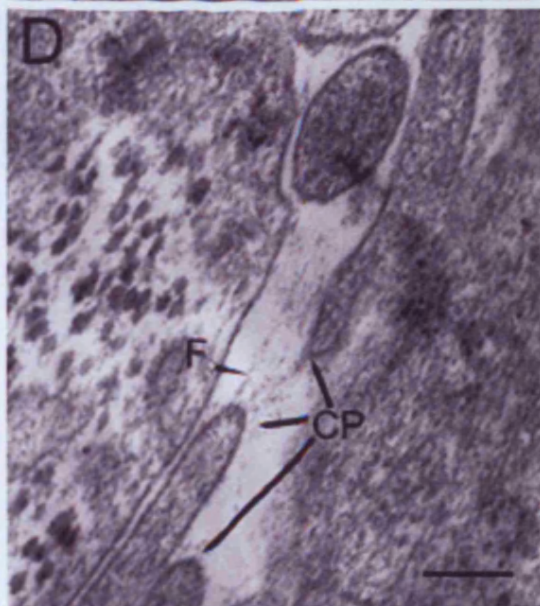
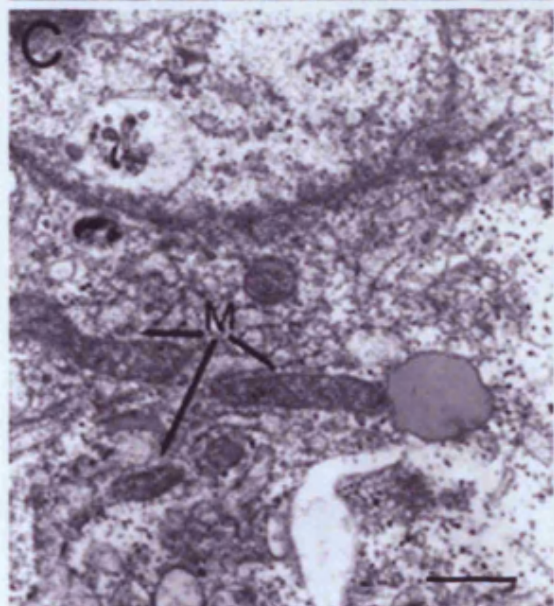
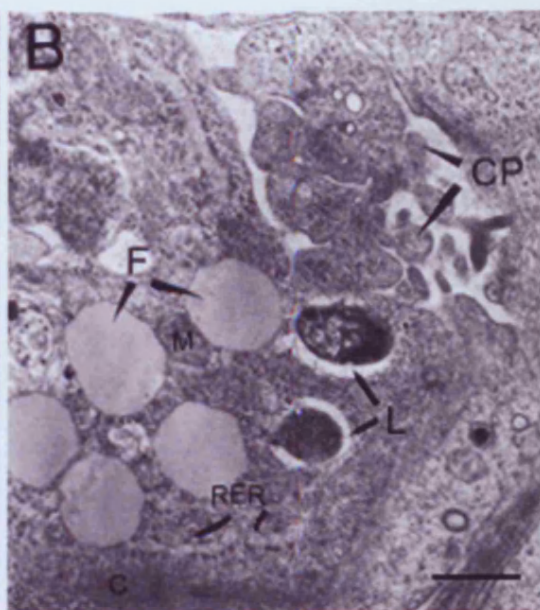
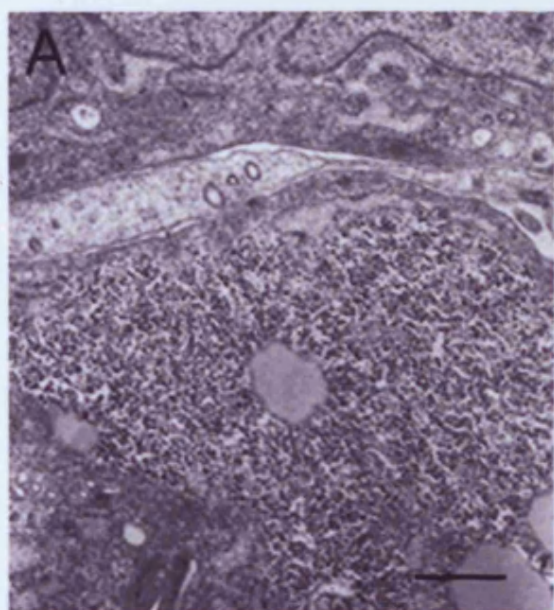


Figure 4.17 Transmission electron micrographs

A- TEM image. 2day pellet.

Deposition of collagenous fibres between adjacent cells can be observed (C). The orientation of these fibres is perpendicular to the cell surface and cytoskeletal components of the cell are visible near the cell membrane.

Magnification x25K

Scale bar= 0.42µm

B- TEM image. 2day pellet.

Cell processes are abundant and have various orientations (CP). There is no collagen deposition in the intercellular spaces in this area. Cell junctions (CJ) are present where cell membranes meet, though the junction type cannot be distinguished.

Magnification x32K

Scale bar= 0.33µm

C- TEM image. 2day pellet.

Areas of longitudinally orientated collagen fibres are present (C) and the characteristic banding of fibrillar collagens can be seen. The area surrounding the collagen fibres is heavily granular.

Magnification x63K

Scale bar= 0.16µm

D- TEM image. 2day pellet.

Increased magnification of C. The longitudinally orientated collagen fibres show characteristic banding of fibrillar collagens.

Scale bar= 0.08µm

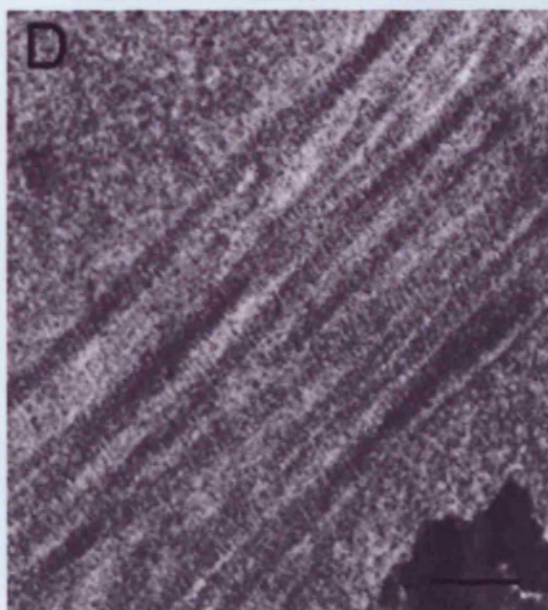
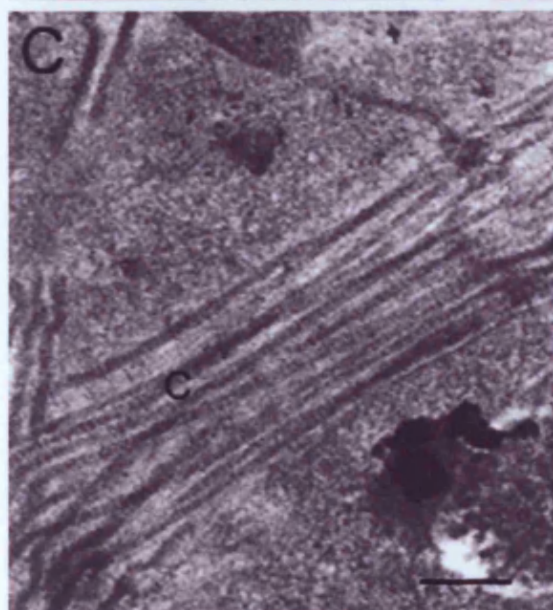
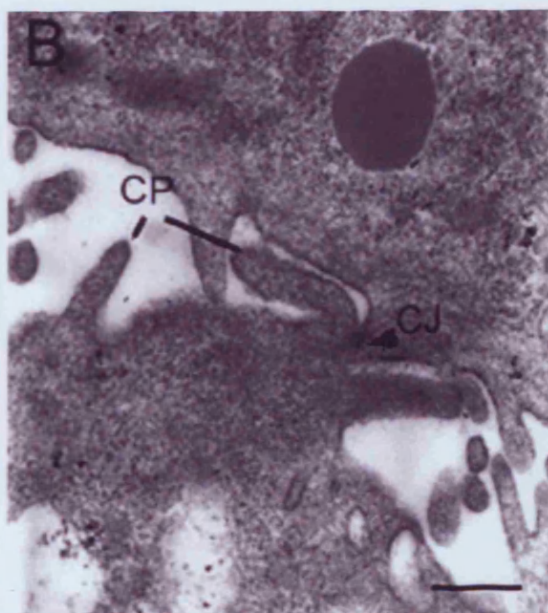
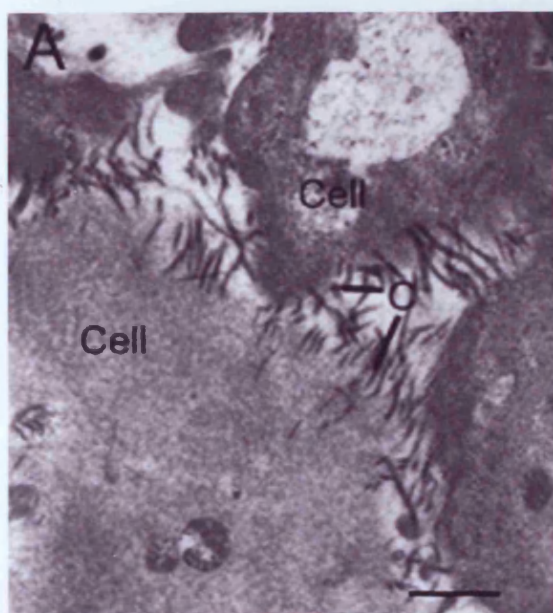


Figure 4.18 Transmission electron micrographs

A- TEM image. 5day pellet.

Deposition of collagenous fibres into large intercellular spaces is present (C). There is no general orientation of these fibres with respect to the cell surface, however parallel alignment is observed in small clusters of collagen fibres.

Magnification x13K

Scale bar= 0.85µm

B- TEM image. 5day pellet.

Cell processes are abundant and have various orientations (CP). There is no collagen deposited in this region though the intercellular spaces are abundant. The intercellular spaces are not totally void of material, though what type of material is deposited is unknown.

Magnification x40K

Scale bar= 0.25µm

C- TEM image. 5day pellet.

Collagen deposition is perpendicular to the cell surface (C) and large deposits of glycogen are found within the tendon cells. The left hand cell contains a fine cytoskeletal arrangement just underneath the cell surface.

Magnification x40K

Scale bar= 0.25µm

D- TEM image. 5day pellet.

Fibrous collagen (C) deposited between cells, with the general orientation being parallel to the to the cell surface. Cytoskeletal components (CS) are clearly visible in the cytoplasmic side of the plasma membrane. Large glycogen stores are present.

Magnification x32K

Scale bar= 0.33µm

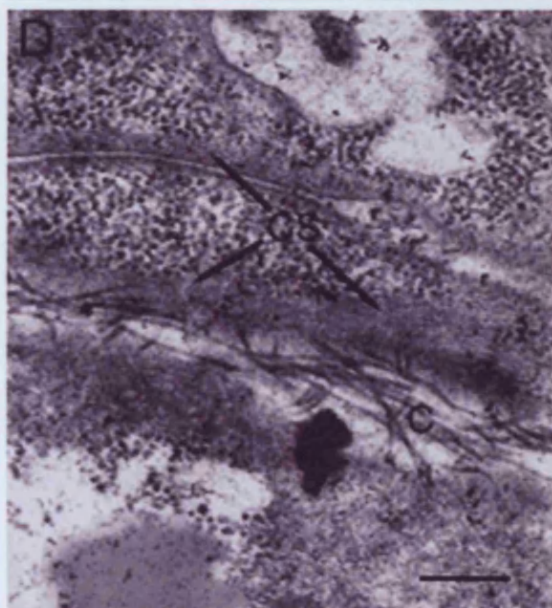
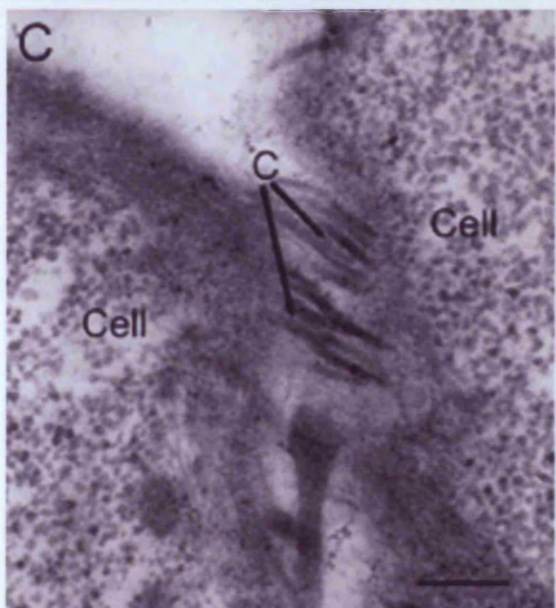
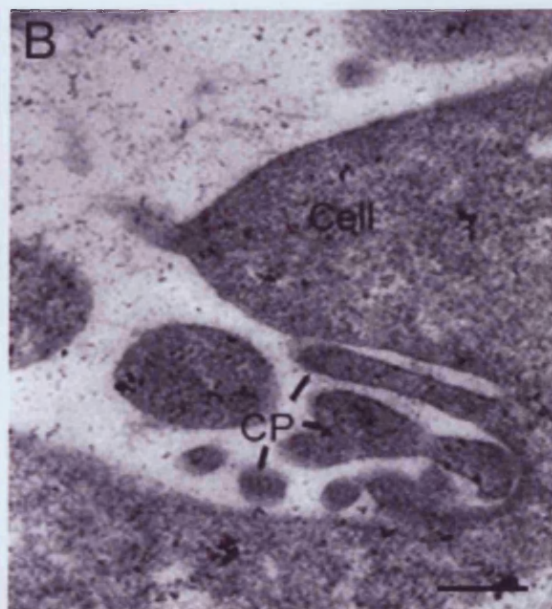
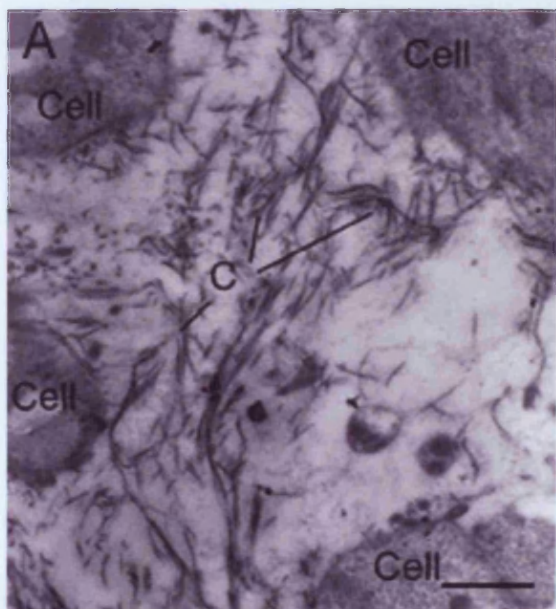


Figure 4.19 Transmission electron micrographs

A- TEM image. 7day pellet.

Cell processes are abundant and have areas of longitudinal orientation (CP). The collagen fibres near these cell processes also show longitudinal orientation (C).

Magnification x32K

Scale bar= 0.33 μ m

B- TEM image. 7day pellet.

Collagen fibres (C) deposited with associated cytoskeletal components (CS), in this section both share a common longitudinal orientation.

Magnification x40K

Scale bar= 0.25 μ m

C- TEM image. 7day pellet.

Collagen deposition is perpendicular to the cell surface (C) and highly ordered cytoskeletal components (CS) can be seen close to the plasma membrane arranged in a parallel fashion.

Magnification x50K

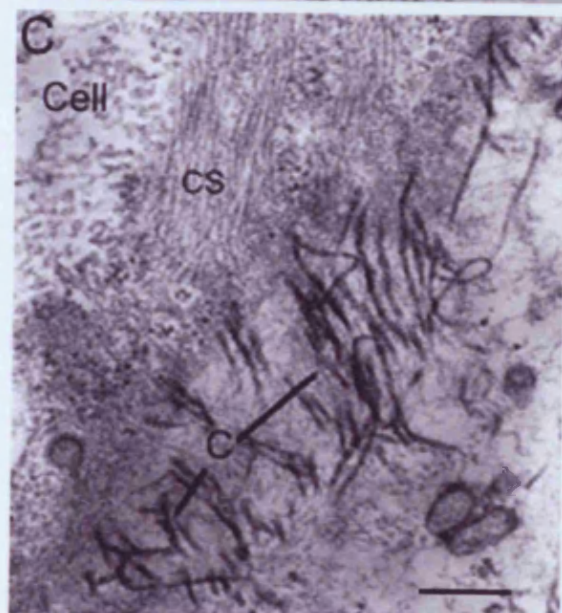
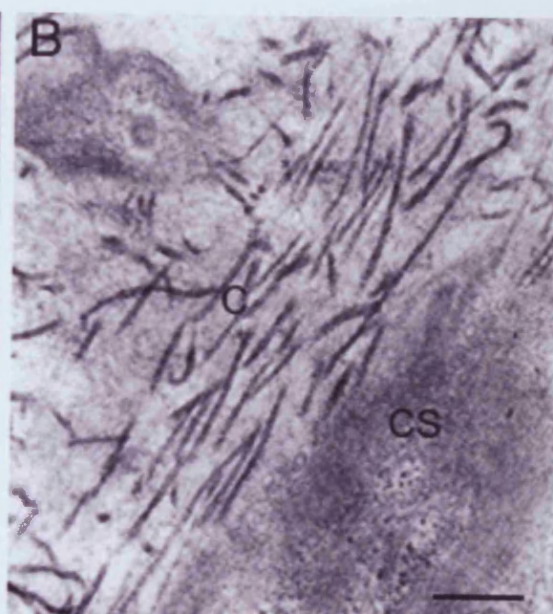
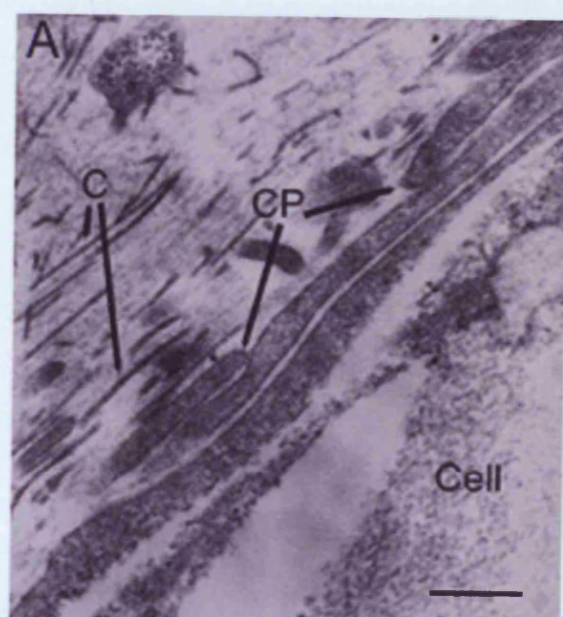
Scale bar= 0.2 μ m

D- TEM image. 7day pellet.

Deposition of collagenous fibres into large intercellular spaces is present (C). There is little orientation of these fibres with respect to the cell surface, they can be seen longitudinally in the plan of view and perpendicularly to it.

Magnification x25K

Scale bar= 0.42 μ m



4.4 DISCUSSION

There were clear differences in the ability of tendon cells to deposit collagen into an ECM in the three culture systems examined. Monolayers showed small amounts of what appeared to be type I collagen, micromasses showed more extensive deposition of types I and III collagen, and pellet cultures developed an extensive collagen rich matrix. This demonstrates clearly that a combination of high cell density and 3-D organisation of cells promotes matrix deposition, and that pellet cultures are better able to replicate the *in vivo* situation.

The results in monolayers are consistent with other studies, which indicate that 85-94% of collagen produced by tendon cells in monolayer is released into the culture medium (Freiberger et al, 1980; Herrmann et al, 1980; Waggett et al, submitted). It would appear that the small amount retained is either intracellular or deposited in the very small available regions between cells within the cell layer. Strikingly, it appeared that the deposited collagen was type I; type III collagen appeared intracellular suggesting either that cells were unable to secrete it on monolayer, or were unable to deposit it into the matrix. Other studies report synthesis of type III collagen by fibroblasts in monolayer (Herrmann et al, 1980), so it would appear that the cells could not deposit it, but lost it all to the media. In contrast tenascin is present and deposited, apparently associated with the cell surface (Chiquet-Ehrismann, 2004).

By comparison, cells in micromass cultures did deposit ECM containing both types I and III collagen, although the longer the cells were in culture, the more they began to resemble monolayers, as cell multilayers were apparently dispersed by cell migration from the periphery. However, it was clear that enhanced cell density, with a degree of 3-D cell organisation, was more effective at promoting collagenous matrix deposition when compared to monolayers. With other cell types, notably cartilage, micromass cultures allow deposition of extensive matrix (Aherens, 1977; Solursh, 1982); and osteogenic cells (Gerber and ap Gwynn, 2002), so while in this study they were a little better than the monolayers at matrix deposition, they cannot be regarded as especially successful.

Pellet cultures allowed extensive deposition of both collagen, along with tenascin and fibronectin. The reduced cell density in the centre of the oldest pellets suggest some nutritional problems, however, this did not cause serious problems over the timescale studied, although collagen label was reduced. Type I and III collagen

were deposited at the same time in pellet cultures, although type III collagen has a more peripheral distribution, together with fibronectin. This area has some degree of cell and matrix organisation, also seen in SEM images and is perhaps associated with a specialised function of these cells, possibly a developing endotenon or epitenon. Some aspects of this feature are reminiscent of other tissue types studied in this system. Chondrocytes cultured as pellets form a cartilaginous pellet with flattened cells at the periphery expressing type I collagen, resembling the perichondrium (Larson et al, 2002). These tendon cells could be replicating their own peripheral tissue organisation. The presence of tenascin everywhere is suggestive of a generalised function rather than a specific, positionally restricted function like fibronectin or type III collagen in the pellets.

The presence of actin filaments in monolayer are prominent, these filaments are likely to be actin stress fibres. Actin stress fibres are common in connective tissues (Defilippi et al, 1999) and *in vitro* are characteristic of chondrocyte dedifferentiation, associated with a fibroblastic phenotype (Li et al, 2003). Actin has a role in matrix deposition, as studied in the intervertebral disc (Hayes et al, 1999) and possibly in tendon development (see chapter 3). If actin filaments are associated with matrix organisation, they appear to be unnecessary in monolayer as no significant matrix is deposited. It may be that actin requires a 3-D organisation of cells to be able to function in this way.

Expression of prominent actin filaments occurred in the high cell density regions of the micromass, suggesting an association with cell-cell contacts as shown in other experimental systems (Wozniak et al, 2004). Whilst they are clearly present they are less extensive than in monolayer and so, perhaps, they represent an organisation closer to the *in vivo* situation. It may be related that these cultures, while they last, can deposit more matrix than cells in monolayer.

In the pellet cultures no clear actin fibres were present in early stages, they became present in some orientated regions at the periphery of the pellet. This suggests that the actin fibres are not particularly important in matrix deposition itself, but may be more important in matrix organisation, such as in the intervertebral disc and possibly in early tendon blastemas (Hayes et al, 1999; see chapter 3). There is evidence that suggests cadherins can induce actin stress fibre formation (Banes et al, 1995; Wu et al, 1999; Lee et al, 2000b). Certainly, actin stress fibres are important in reorganisation of collagen gels in experimental

systems (Lee et al, 2000a; Zaleskas et al, 2001; Spilker et al, 2001; Kinner and Spector, 2001) and in matrix reorganisation in scar and wound repair by myofibroblasts (Darby et al, 1990; Hinz and Gabbiani, 2003; Desmouliere et al, 2004).

Monolayer cultures expressed the full range of cell-cell junctional components examined, as did micromasses. The only difference between the three culture systems was that connexin 43 label is very sparse in the pellet cultures. In monolayers there was evidence of vinculin associated with cadherin-based adherens junctions, and with cell-matrix focal adhesions, as shown by streaks of label positioned away from cell-cell interfaces. Because of the culture organisation of micromass and pellet cultures it was not possible to draw similar conclusions as the two types of junction could not be distinguished in the 3-D systems at light microscope level. However, given that matrix was deposited it would be surprising if such junctions were not present, and structures suggestive of focal contacts were present in pellets examined under the electron microscope.

Connexin 43 is associated with the inhibition of tendon cell response to strain (Waggett et al, submitted, Waggett et al, 2001; see chapter 3). It is interesting that the only culture system that deposits a substantial matrix is low in this gap junction component. This is similar to the situation in early tendon blastemas (see chapter 3). Therefore, it appears in circumstances where matrix deposition is favoured, connexin 43 is reduced. Connexin 43 and 32 have different transmission characteristics (Goldberg et al, 2002) and form separate networks in tendons (McNeilly et al, 1996), although it is, as yet, unclear how these networks interface with systems controlling collagen production.

In images acquired through electron microscopy, in day 1 pellets there was evidence of matrix deposition, but not classical banded collagen fibrils, although there was some diffuse material in spaces between cells. These fine, beaded filaments had a similar appearance to fibrillin (Sherratt et al, 2001). By day 2, collagen was being deposited within the pellets, as in tendon and other fibrous connective tissues (Birk et al, 1991; Birk and Zycband, 1993). However, there was also other collagen organisation with fibres protruding straight out from the cell membrane, not organised in bundles or being passed into cellular channels (Birk et al, 1991; Birk and Zycband, 1993). These two appearances of collagen were generally not observed in the same place. This may be due to cells in certain

areas of the pellet behaving in a relatively normal fashion, and other areas containing cells that were not. These cells had no orientational cues from surrounding cells or matrix and therefore secreted collagen anywhere.

Frequently observed cell processes projected into intercellular spaces, it is possible these are fibroblasts, or their precursors, associated with matrix deposition (Canty et al, 2004). Often these projections appear in spaces that do not contain collagen, and as fibroblasts are short lived and associated with only the early stages of matrix deposition and orientation (Canty et al, 2004), it is possible these structures are present with the pellets.

There was evidence of cytoskeletal association with collagen under TEM. As indicated above, there appeared to be focal contacts with orientated actin approaching the plasma membrane, interacting with a dense plaque and at the outside of the membrane with collagenous matrix. It is unclear as to whether this is a simple adhesion to matrix or whether the cells are actively orienting it using actin and cell matrix junctions, as occurs in the intervertebral disc (Hayes et al, 1999) and as described above.

5.1 INTRODUCTION

Pellet cultures were developed to study chondrogenesis (Solursh, 1991), as indicated in chapter 4, they enable tendon fibroblasts to deposit a substantial collagen rich matrix over 7-14 days in culture. A particular advantage of this system is that the cultures can be prepared quickly and with relatively small numbers of cells, with the result that experiments can be performed rapidly and with a high number of replicates. In this chapter the behaviour of tendon cells in pellet culture is examined both in the presence or absence of growth factors, using qualitative analysis of matrix deposition and quantitative analysis of collagen synthesis.

Growth factors are diffusible polypeptide signalling molecules secreted by cells and whose major sites of action lie relatively close to the cells where they are synthesised (Lawrence, 1985; Roberts and Sporn, 1985; Gospodarowicz et al, 1987; Burgess and Maciag, 1989). In connective tissues, growth factors (GF) control cell proliferation, differentiation, matrix synthesis and degradation (James and Bradshaw, 1984; Rosso et al, 2004). They can interact with extracellular matrix components and become an integral part of the matrix (Taipale and Keski-Oja, 1997). In tendon, they are part of a complex and poorly understood communication system: which involves direct linkage of cells, with membrane contact and cell junctions (McNeilly et al, 1996; Ralphs et al, 1997, 2002), linkage via the ECM (Harwood et al, 1999; Martin-Bermudo, 2000; Ross, 2004) and growth factor signalling between cells. Whilst there is huge literature on growth factor effects on a wide range of cell types *in vitro* and in foetal development and tissue repair *in vivo*, the effects of growth factors on fibroblasts behaviour are of particular relevance here. Experiments on pellets were performed using TGF β , bFGF and PDGF, because these factors are known to have significant effects on tendon cell behaviour (Canalis et al, 1981; Roberts et al, 1985; Taipale et al, 1997; Holland et al, 1998; Tallquist et al, 2004). These factors are examined in detail below.

5.1.1 Transforming growth factor-beta (TGF β)

TGF β and its three mammalian isoforms (1,2 and 3) are widely expressed in adult and foetal tissue. TGF β 4 has only been reported in the chick (Jakowlew et al, 1988) and TGF β 5 only in the *Xenopus* (Kondaiah et al, 1990). The three mammalian isoforms of TGF β result from homodimeric and heterodimeric combinations of β 1 and β 2 subunits (TGF β 1, TGF β 2 and TGF β 1.2) (Cheifetz et al,

1987). There is a high degree of conservation across species, 99% between human and mouse indicating the critical biological roles of TGF β s (Massagué et al, 1987). TGF β has complex effects on cells, for example, it can stimulate or inhibit cell proliferation dependant on the cell type or cell environment (Tucker et al, 1984; Shipley et al, 1985; Roberts et al, 1985a; Massagué et al, 1985b; Moses et al, 1994; Klein et al, 2002; Lu et al, 2004; Tomlinson et al, 2004).

TGF β is released from cells in an inactive, latent complex which consists of active TGF β and a prodomain, called TGF β latency associated protein (TGF β -LAP) (Hyytiainen et al, 2004). At secretion, the active TGF β is cleaved from the propeptide, but the two remain attached through non-covalent interactions, the complex remaining latent (Gentry et al, 1988). Often this complex contains additional proteins associated with LAP, bound via disulphide bonds. These are collectively named latent TGF β binding proteins (LTBPs) (Taipale and Keski-Oja, 1997) and their function is thought to lie in secretion, extracellular storage or activation of the complex (Miyazono et al, 1993; Dallas et al, 1995; Keski-Oja et al, 1995; Taipale et al, 1996). Latent TGF β cannot interact with cell surface receptors (Lawrence et al, 1985) and requires activation. Methods of activation TGF β *in vivo* are complex and thought to involve numerous processes. There are two enzymatic processes that can yield activated TGF β . First, the latent complex is treated with glycosidases (Miyazono and Heldin, 1989) and second, there is a proteolytic degradation of LAP (Lyons et al, 1988, 1990). There are two predominant enzymes involved in this proteolytic degradation, namely, plasmin and cathepsin D. Each release TGF β from its latent complex by cleaving LAP at its n-terminal (Lyons et al, 1988, 1990). Plasmin can also release matrix-bound latent TGF β (Taipale et al, 1996), enabling it to travel in an inactive form to target cells (Gelizes et al, 1997). TGF β activation is blocked by plasmin inhibitors, or by the depletion of plasminogen (propeptide of plasmin) (Sato and Rifkin, 1989). However, cathepsin D may substitute for plasmin in plasminogen knock-out mice (Lyons et al, 1988; Bizik et al, 1996). In addition non-enzymatic activation can occur, latent TGF β can form a complex with thrombospondin-1, which causes TGF β activation (Schultz-Cherry and Murphy-Ullrich, 1993, 1994), although the precise mechanisms are unknown.

TGF β binds to specific cell surface receptors, forms a protein complex and activates the intracellular SMAD proteins (summarised in Heldin et al, 1997;

Massagué, 1998; Moustakas et al, 2001; Derynk and Zhang, 2003; ten Dijke and Hill, 2004). TGF β brings together two types of receptor kinase to influence cell behaviour, one kinase phosphorylates the other, which in turn phosphorylates the SMAD proteins. SMAD proteins are signal transducers that can pass through the nuclear membrane and commence transcriptional complexes of DNA binding (Massagué, 1998; ten Dijke and Hill, 2004). The genes targeted may encode proteins that affect cell cycle regulators or ECM components determining cell adhesion, positioning or movement (Massagué, 1998; ten Dijke and Hill, 2004). In primary human dermal fibroblasts *in vitro* an increase of TGF β will increase type I collagen transcription and SMAD 3 is known to transmit the signals (Chen et al, 1999). This study also showed SMAD 7 as a possible autocrine negative feedback in collagen regulation, discerning the complexity of the system.

Binding to ECM components can regulate TGF β , including type IV collagen, decorin, biglycan, fibronectin and thrombospondin (Massagué, 1998). Although the regulation of TGF β by ECM protein binding is vital for accumulation and quick release into the ECM, it is also possible that this binding prevents TGF β degradation, allowing the sustained release of TGF β , or could act as a TGF β clearance system (Massagué, 1998).

Throughout development TGF β and its influence is associated with numerous connective tissues (Heine et al, 1987). TGF β is known to contribute to the segmentation of the axial skeleton, and its subsequent skeletal development (Hill and Logan, 1992). TGF β induces a cartilaginous phenotype, namely type II collagen. Furthermore, it induces cartilage specific proteoglycan production in embryonic mesenchyme (Seyedin et al, 1985, 1986) and, in fact, promotes cartilage differentiation (Kulyk et al, 1989; Wang et al, 2003; Tuli et al, 2003; Rutherford et al, 2003; Hentschel et al, 2004). It has been suggested that TGF β has a role in early angiogenesis (Heine et al, 1987) and subsequent development of cardiovascular structures. *In vitro* experiments on endothelial cells in 3-D collagen gels, showed that treatment with TGF β enabled cells to form tubular structures (Madri et al, 1988a).

Cell adhesion and migration are influenced by multiple stimulants, including growth factors. It is the action of TGF β on cell adhesion mechanisms and the composition of the ECM that causes its influence to be apparent. An increase in cell adhesion

is seen coordinated with enhanced matrix synthesis and deposition upon treatment with TGF β (Hyytiainen et al, 2004). Additionally, a decrease in pericellular proteolysis can be caused by a TGF β -induced up-regulation of plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitor of metalloprotease (TIMP), both inhibitors of extracellular matrix degrading enzymes (MMPs) (Laiho et al, 1986, 1987; Edwards et al, 1987; Coletta et al, 1999; Ma et al, 1999; Eren et al, 2003; Jiang et al, 2003; Yamanaka et al, 2004; Nahm et al, 2004). As a result of TGF β treatment there is a decrease in the expression of collagenase (Edwards et al, 1987) together with a modification of cell surface molecules (Massagué, 1998). From this observation, TGF β has been shown to effect cell morphology, differentiation and migration, but is also significantly involved in wound healing (Ignatz and Massagué, 1985; Roberts, 1986; Grazil-Bilska et al, 2003; Schiller et al, 2004; Hsu and Chang, 2004; Saika, 2004; Vladimirov and Dimitrov, 2004).

The healing processes of tissues are highly regulated and growth factors are one of the most important molecular families involved. GFs involved in the repair cascade are described in chapter 1. It is known that TGF β has an anti-inflammatory influence (de Martin et al, 1987; Shull et al, 1992; Letterio and Roberts, 1998), but there are also high levels of TGF β stored in platelets (Assoian et al, 1983; Cheifez et al, 1987). TGF β has been shown to be active in all stages of tendon healing having a variety of effects (Chang et al, 2000). It can induce extrinsic cell migration, being a chemoattractant for monocytes and fibroblasts (Wahl et al, 1987; Postlethwaite et al, 1987), generate fibronectin-binding interactions (Wojciak et al, 1994), regulate proteinases (Bennett et al, 1993) and terminate cell proliferation or enhance it (Ashcroft et al, 1999; Zhu et al, 2001), plus stimulate collagen synthesis (Mauri et al, 1997). Ectopic induction of neotendon/ligamentous tissue was observed when growth and differentiation factors 5, 6 and 7 (GDF 5,6 and 7, members of the TGF superfamily) were implanted in an unnatural location (Wolfman et al, 1997). Using gene transfer techniques as a vehicle for GF delivery, improved tendon healing (Gerich et al, 1996; Nakamura et al, 1998; Lou et al, 2000; Review Zhang and Lineaweaver, 2002; Hildebrand et al, 2004). It is because of these actions that TGF β is widely used for experimental purposes *in vitro* and why it was chosen for use in the experiments conducted in this chapter.

5.1.2 Platelet-derived growth factor (PDGF)

Platelet-derived growth factor (PDGF) was discovered as a result of the observation that material released from platelets was the principal source of mitogens (a stimulant of mitosis) present in serum, and that this was responsible for the growth of many cells in culture (Ross et al, 1974). Three forms of PDGF were studied up until recently include PDGF-AA, PDGF-BB and PDGF-AB (Heldin, 1993). However, there are now known to be 2 additional isoforms, PDGF-CC and PDGF-DD (Li et al, 2000; Gilbertson et al, 2001; LaRochelle et al, 2001; Bergsten et al, 2001), both of which differ from the original isoforms, as they are secreted in an inactive form (Kazlauskas, 2000; Li et al, 2000; Gilbertson et al, 2001; LaRochelle et al, 2001; Bergsten et al, 2001).

The activity of PDGF is regulated through three related tyrosine kinase receptors, namely $\alpha\alpha$, $\beta\beta$ and $\alpha\beta$ (Ronnstrand and Heldin, 2001). PDGF $\alpha\alpha$ -receptors bind all isomers of PDGF, apart from PDGF-DD, with a high affinity (Claesson-Welsh, 1989), whereas the $\beta\beta$ receptor binds only PDGF types comprising of at least one B polypeptide chain and PDGF-DD (Claesson-Welsh, 1993). The $\alpha\beta$ -receptor will bind PDGF-AB, -BB and -CC (reviewed in Tallquist and Kazlauskas, 2004).

PDGF has a wide range of biological actions. Different PDGF chains exhibit different responses on cells but generally, PDGF isoforms are potent mitogens for connective tissue cells (Abrahamsson, 1997). Their effects include an influence on smooth muscle cells (Bornfeldt et al, 1994), chondrocytes (Lubinus et al, 1994), epithelial, endothelial cells (Brewitt and Clark, 1988; Kuwabara et al, 1995) and fibroblasts (Siegbahn et al, 1990). PDGF is also involved in the differentiation of chondrocytes (Stoog et al, 1990). Studying chondrogenesis in mesenchymal micromass cultures, PDGF-A can stimulate chondrogenesis in cells up to stage 24, but in any later stage it inhibits chondrogenesis, indicating very different roles dependant on stages of development (Ataliotis, 2000). PDGF is known to be essential for tendon fibroblasts as it enable them to proliferate under load (Banes et al, 1995).

Interestingly, PDGF show differing effects in the DNA and protein synthesis dependant on the areas of tendon used (Yoshikawa and Abrahamson, 2001). PDGF has been used in gene transfer experiments and its use resulted in an increase in collagen gene expression within the tendon (Wang et al, 2004). Important in proliferation of vascular smooth muscle cells and their migration,

PDGF plays an important role in vascularisation (Abedi and Zachary, 1995). Its role in migration may be associated with its ability to induce the expression of MMPs within tissue (Matrisian and Hogan, 1990; Laurent et al, 2003). PDGF is released from platelets, increasing its local concentration (Duffy et al, 1995). It has been shown to play a part in the early stages of healing because of its ability to induce the synthesis of other growth factors (Lynch et al, 1989) and its effects on DNA synthesis, collagen and non-collagenous protein synthesis (Pierce et al, 1991; Yoshikawa and Abrahamson, 2001). Through *in vivo* studies it has been shown that PDGF has its greatest effects in the initial stages of healing by triggering healing cascades during inflammation (Molloy, 2003).

5.1.3 Basic fibroblast growth factor (bFGF)

Basic fibroblast growth factor (bFGF), also known as fibroblast growth factor (FGF)-2, comes from an extensive growth factor family (23 members), but bFGF was the original molecule discovered from this superfamily (Gospodarowicz, 1975). It is a monomer containing 12 anti-parallel β -sheets (Moy et al, 1996) and its primary form is 18kDa, but it can also exist as 22kDa and 22.5kDa, 24kDa and 34kDa isoforms (Gospodarowicz, 1975).

After secretion bFGF is sequestered, either bound to the cell surface or to matrix GAGs (Folkman et al, 1988; Burgess and Bohlen, 1989). Its ability to bind heparan sulphate reflects a complex regulatory mechanism (Faham et al, 1996), and protects bFGF from increases in heat, any drop in pH or protease activity (Conrad, 1998). Heparan sulphate proteoglycans can also increase the affinity of bFGF for its receptors (Yayon et al, 1991). There are 4 basic receptors for bFGF, although many spliced variants are also known to exist. This enables these alternative forms to have different ligand binding or signalling qualities (Vainikka et al, 1992). Using these four high affinity receptors and heparan sulphate low affinity receptors, bFGF acts on cells via paracrine or autocrine mechanisms (Mignatti et al, 1991; Jouanneau et al, 1997).

Basic-FGF affects a wide variety of cells and does not solely stimulate growth (Bikfalvi et al, 1997). bFGF can affect the proliferation of endothelial cells, chondrocytes, smooth muscle cells, melanocytes and of course fibroblasts (reviewed Burgess and Maciag, 1989; Basilico and Moscatelli, 1992; Nugent and Iozzo, 2000). Its role in chondrogenesis is complex, as it has been shown to inhibit chondrocyte proliferation and differentiation (Iwasaki et al, 1995; Ogawa et al,

2003), but can work synergistically with TGF β in chondrogenesis (Schofield and Wolpert, 1990; Frenz et al, 1991, 1994) and is thought to accelerate vascularisation of the growth plate (Baron et al, 1994).

Basic-FGF also stimulates of cell migration and angiogenesis, both *in vivo* and *in vitro* (Folkman and Klagsbrun, 1987; Nugent and Iozzo, 2000; Okada-Ban et al, 2000). In rabbit flexor tendons bFGF levels are increased at the injury site, with the highest levels in intrinsic tendon fibroblasts and fibroblasts migrating from the epitenon along the edge of the wound (Chang et al, 1998). It has a dose dependant effect on the expression of type III collagen and cellular proliferation (Chan et al, 2000). Using bFGF in a dose dependant manner on ligament defects demonstrated that higher dosages can have adverse effects, with a decreased type I procollagen expression (Fukui et al, 1998). IGF-1 and bFGF are important in the proliferative and remodelling stages of tendon repair (Letson and Dahners, 1994; Molloy et al, 2003). *In vitro* wound models showed bFGF has a dose dependant effect on wound healing, although there is an optimum dosage (Chan et al, 1997). Basic-FGF is known to be a potent angiogenic factor (Basilico and Moscatelli, 1992; Norrby, 2002; Aviles et al, 2003; Suhardjia and Hoffman, 2003). All of these actions make it a major influence in wound repair and revascularisation.

5.2 MATERIALS AND METHODS

Pellets cultures were prepared as described in chapter 4. Tendon fibroblasts were isolated from 50 day old broiler chickens (see chapter 2), suspended at 1×10^6 cells/ml in DMEM (5%FBS, 1mg/ml ascorbate, HEPES buffer, 1% antibiotics), and 1ml of cell suspension aliquoted into sterile eppendorf tubes. The cells were gently centrifuged (2000 rpm) for 2 minutes (Boeco M-24 microcentrifuge, Germany) to form pellets at the bottom of the eppendorf. Once pellets were formed, supernatant was removed and replaced with the experimental medium. Experimental media were made up in DMEM/1%FBS/ascorbate/antibiotics as a base medium. Cultures were set up with this as the control; with 5%FBS; with TGF β (5ng/ml) (Sporn and Roberts, 1991), bFGF (10ng/ml) (Gospodarowicz et al, 1987) and PDGF (50ng/ml) (Pierce et al, 1989), with 10 replicates being set up for each treatment. Three replicates were used for qualitative analysis and 7 were used to quantify DNA and collagen. A further series of pellets were set up with combinations of growth factors, at the individual concentrations above. These were bFGF+PDGF, bFGF+TGF β and TGF β +PDGF. Cultures were maintained for 1 day, 7 days and 14 days, and fed with fresh medium every 3 days. The spent medium was retained and stored at -80°C for collagen analysis. Tendon fibroblast pellets were incubated at 37°C , with 100% humidity and 5% CO_2 in air atmosphere.

Qualitative analysis

Pellets were removed, fixed in cold 95%IMS for 5 minutes, cryosectioned and immunolabelled for ECM components (types I, II and III collagen, tenascin, fibronectin), cytoskeletal components (filamentous actin directly labelled with phalloidin and vimentin) and cell junctional associated proteins (pan-cadherin, n-cadherin, vinculin and the connexins 32 and 43). Details of these procedures are given in chapter 2.

Quantitative Analysis

For each time period, collagen content of the cell pellet and culture medium was measured using the hydroxyproline assay (Creemers et al, 1997; see chapter 2). DNA content of the pellets was measured fluorometrically using Hoechst 33258 dye (Cesarone et al, 1980; Downs and Wilfinger, 1983; see chapter 2). Collagen content was expressed relative to DNA content. Total collagen, cell pellet and culture medium collagen content and DNA content were compared using a one way ANOVA followed by the Tukey multiple comparison pair-wise test, with a 95% confidence limit.

5.3 RESULTS

5.3.1 Qualitative Studies

There are no obvious qualitative differences between the control pellets supplied with 1% serum, or pellets treated with 5% serum, single or combined growth factors. Results are summarised in table 5.1; detailed descriptions are present below.

Extracellular matrix labelling

Pellets treated with single growth factors or combinations, contained type I and III collagen at all time intervals (fig 5.1-5.2). Initially type I collagen was intracellular (fig 5.1 A, D, G, J), and by 7 days in culture prominent extracellular labelling was present (fig 5.1 B, E, H, K). Label intensity appeared reduced in 14 day pellets (fig 5.1 C, F, I, L). Type III collagen was present at all time points (fig 5.2), although labelling was weak and sparse in 1 day cultures (fig 5.2 A, D, G, J), it appeared at a higher intensity at the pellet periphery in later stages (fig 5.2 H, I, L). Type I pro-collagen was present at all time points, although only a proportion of cells within the aggregates were labelled (fig 5.3 A-L). Type I pro-collagen remained present in centralised regions of the pellet after 14 days (fig 5.3 C, F, I, L).

Other ECM molecules labelled include tenascin, which was seen extracellularly throughout the pellets at all time intervals and with each GF treatment (fig 5.4 A-L). Labelling for fibronectin was conducted due to its cell adhesive qualities and its association with collagen. Positive fibronectin label was absent in 1-day pellet cultures treated with either 1% FBS/DMEM, PDGF or TGF + PDGF pellets (fig 5.5 A, J). It was present in small amounts in FGF or TGF treated pellets (fig. 5.5 D, G) and in pellets treated with FGF + PDGF and TGF + FGF (not shown, see table 5.2). At 7 and 14 days all pellets contained fibronectin (fig 5.5 B, C, E, F, H, I, K, L), often showing elevated labelling at the periphery.

Cytoskeletal components

Filamentous actin was present in all pellet cultures at all time points. In 1 day pellets, generalised green intracellular label indicated the presence of a filamentous actin network within the cell (fig 5.6 A, D, G, J), but without prominent actin fibres. Cells in older pellets had a different distribution of labelling. Flattened cells at the periphery appeared to contain bright fibres (fig 5.6 E, H) and some cells in the central parts also labelled strongly (fig 5.6 L). This could occur in any

culture and was not specifically linked to any particular treatment. Vimentin was also present, again with no difference between culture treatments (fig 5.7 A-L).

Cell junctional components

Label for Pan-cadherin, n-cadherin, vinculin and connexins 32 and 43 were present. Labelling patterns were similar to that described in chapter 4 and together with the cytoskeletal components, appeared unaffected by growth factor treatment (figs 5.8, 5.9 5.10, 5.11 and 5.12).

5.3.2 Quantitative analysis

Single Growth factor treated pellets: DNA content (figure 5.13)

In experiments testing the effects of single growth factors, there was a general increase in DNA content of all cultures from 1 to 7 days, with levels dropping again at 14 days. These changes were all statistically significant ($p < 0.05$). Within the particular time intervals, small variations in DNA content with serum or growth factors treatments were not significant at 1-7 days ($p > 0.05$). At 14 days, there were significant differences between treatments: 5% serum increased DNA content compared to 1% serum, bFGF and TGF β ($p < 0.05$), and PDGF had a greater effect than TGF β ($p < 0.05$).

In experiments testing combinations of factors, some caused significant differences in DNA content between treatments, but no clear patterns emerged. Also the enhancement of DNA content at 7 days observed above did not occur even in serum treated and control cultures. There was, if anything, a decline in DNA content over time.

Combination growth factor treated pellets: DNA content (figure 5.14)

Pellets at 1 day showed no difference in DNA content between most treatments, only bFGF+TGF β treated pellets showed elevated DNA levels ($p < 0.05$). At 7 days, 1% controls, 5% serum treated and bFGF+TGF β treated cultures showed significantly reduced DNA content compared to 1 day ($p < 0.05$), the others being unchanged ($p > 0.05$). There were also differences between treatments at 7 days. All growth factor treatments showed enhanced DNA content compared to 1% serum ($p < 0.05$), but could not be distinguished from one another ($p > 0.05$). The bFGF+PDGF and TGF β +PDGF treated cultures also had increased DNA compared to the 5% serum treated cultures ($p < 0.05$). At 14 days, there was no difference to the 7 day cultures for 1% serum, 5% serum and bFGF+TGF β

treatment ($p>0.05$). bFGF+PDGF cultures appeared reduced ($p<0.05$) and TGF β +PDGF cultures were elevated ($p<0.05$). Within the time point, only TGF β +PDGF showed a significant difference from the other treatments, with elevated DNA content ($p<0.05$).

Effects of growth factors on collagen production

Statistical comparison of data on collagen content was made difficult by wide variations in the data, as shown by the error bars (see fig. 5.15, 14 day data especially). Generally, this variation was the result of 1 or 2 pellet measurements being greatly different from the others (see raw data, appendix). Some analysis was attempted omitting such values, but with n-values of around 7, this represented a selection of preferred data, especially when more than one value was involved, and it was decided not to continue in this manner. Where possible, statistical analyses of data are presented; with the others general trends are presented, but the lack of statistical validity is acknowledged.

Single factors: (figure. 5.15)

Cultures maintained in 1% and 5% serum containing medium synthesised collagen over the 14 day period, with around threefold collagen present at 14 days than at 1 day. There appeared to be less collagen at 7 days, but the quantities recovered and increased markedly by 14 days. This general pattern was also true of the growth factor treated cultures.

At 1 day of incubation (fig 5.15 (i)), there were some statistically significant differences between certain growth factor treatments and 5% serum treated cultures. Generally, where these were present, the growth factor treatments have less total collagen than the serum treatments. Also, noteworthy at this time, was that roughly equal amounts of collagen were found in the cell pellets as was released into the media.

At 7 days of incubation, collagen content relative to DNA content had reduced compared to that present at day 1 (fig. 5.15 (ii)); although this was influenced by the high DNA content at this stage (see fig 5.14 (ii)). TGF β treated cultures had significantly more collagen than sera treated cultures, with no detectable difference between the others. Generally, the ratio of collagen released to media compared to that retained in the pellet was higher in these cultures than those at day 1.

At 14 days of incubation, there were no statistically significant differences between treatments, although as the error bars indicate, the data was highly variable (fig 5.15 (iii)). The cultures had released the majority of the collagen they produced to the culture medium, with around 25% being retained in the pellet and 75% released to the media. This seemed to be unaffected by the culture treatment, although subtle changes would not be seen because of the noisy data.

Growth factor combination (figure. 5.16)

Cultures in these experiments demonstrated a general trend of increased collagen secretion with time in culture, with around a ten-fifteenfold increase in total collagen made by the cultures over the 14 day period compared to 1 day ($p < 0.05$). Most of this increase occurred in the second week in culture, as with the previous experiment.

Comparing different treatments, there is little difference at 1 day, with TGF β +PDGF apparently promoting slightly greater release of collagen to the culture medium than the others ($p < 0.05$), but all other treatments and parameter being similar.

At 7 days, cultures were again very similar, with the only significant differences being the amount of collagen incorporated into the cell pellet: it was greater in 5% serum culture than in the growth factor combination treatments ($p < 0.05$).

At 14 days, overall quantities for total collagen and medium collagen were much increased ($p < 0.05$), although amounts in the cell layer were similar to that at previous time points. There were some differences in treatments, 5% serum has more collagen than 1%, bFGF+PDGF and bFGF+TGF β treatments in total and released to the media. bFGF+TGF β and bFGF+PDGF were reduced compared to the TGF β +PDGF treated cultures.

Figure 5.1 Pellet cultures treated with growth factors, x40 objective, Scale bar= 40µm. Type I collagen.

1% serum treated controls.

A- 1day.

B- 7day.

C- 14day.

bFGF treatment

D- 1day. Type I collagen is present and intracellular.

E- 7day. There is an increase in type I collagen with an extracellular appearance.

F- 14day. Type I collagen has diminished compared to that present in the 7 day pellet.

TGFβ treatment

G- 1day. Type I collagen is present and intracellular.

H- 7day. Similar to FGF treated pellets there is an increase in label.

I- 14day. Type I collagen is clearly present after 14 days of culture.

PDGF treatment

J- 1day. Type I collagen is present and intracellular.

K- 7day. There is an increase in type I collagen from 1 day cultured pellets.

L- 14day. As with TGF treated pellets, there is type I collagen at 14 days.

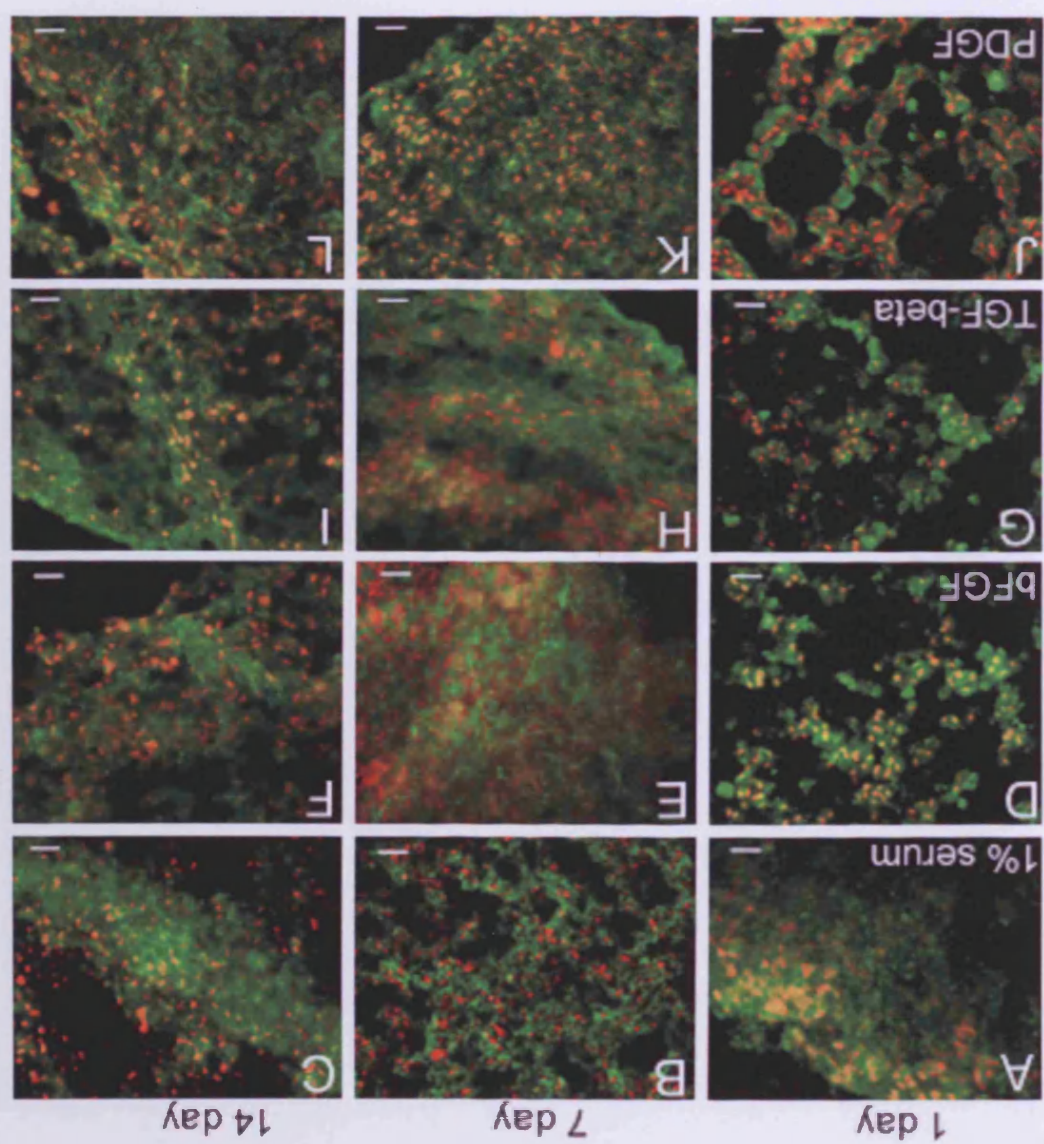


Figure 5.2 Pellet cultures treated with growth factors, x40 objective, Scale bar= 40µm. Type III collagen.

1% sera treated controls

A- 1day.

B- 7day.

C- 14day.

bFGF treatment

D- 1day. Type III collagen is thinly present.

E- 7day. There is an increase in type III collagen and it could be seen extracellularly.

F- 14day. Type III collagen is present and remains extracellular.

TGFβ treatment

G- 1day. Positive type III collagen label patterning is very similar to FGF treated pellet (D).

H- 7day. Type III collagen is present and extracellular.

I- 14day. Type III collagen label is present, but concentrated at the periphery of the pellet.

PDGF treatment

J- 1day. Type III collagen has a similar distribution to FGF and TGF treated pellets (D and G).

K- 7day. Type III collagen label increases in intensity and is extracellular throughout the pellet.

L- 14day. Type III collagen is abundant and concentrated at the pellet periphery.

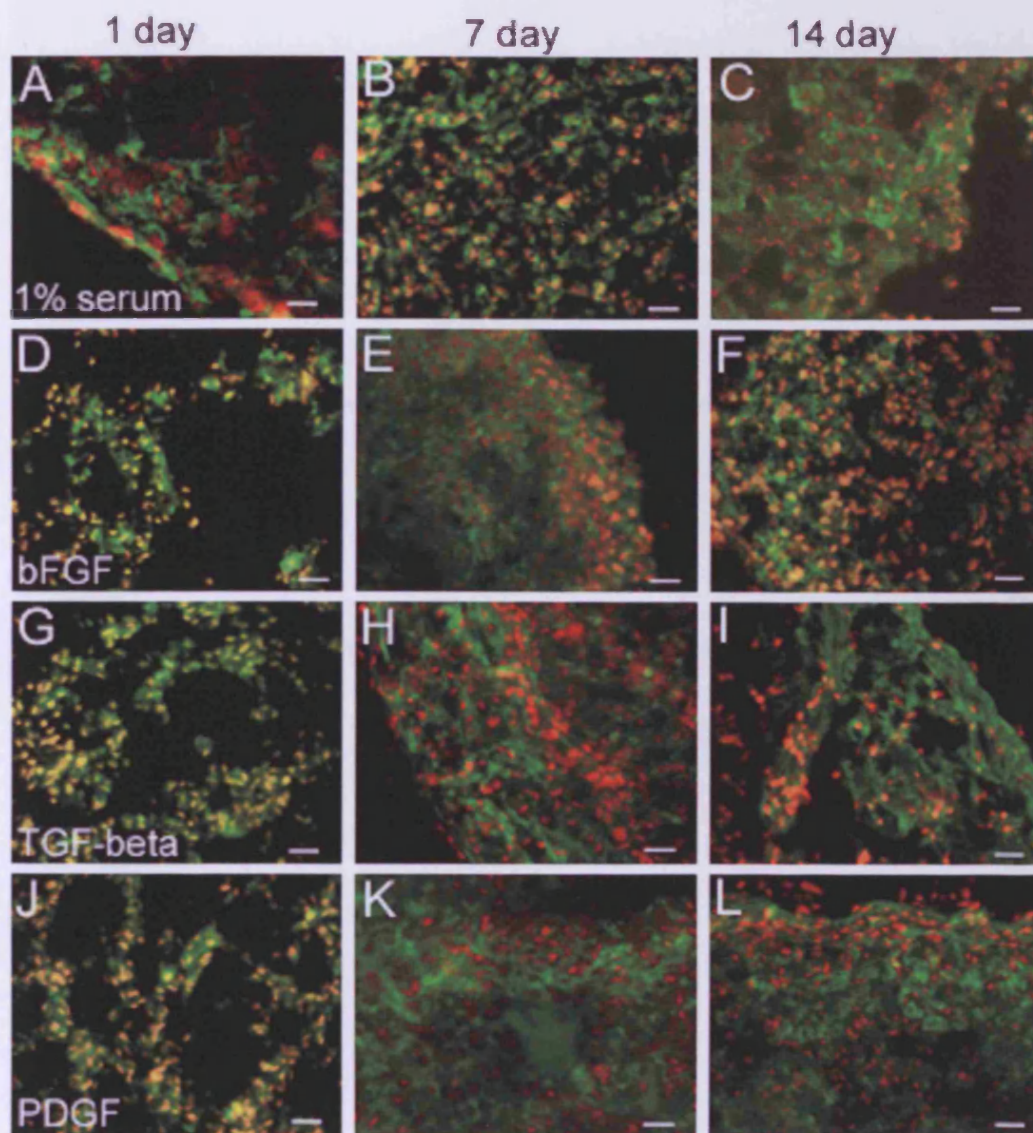


Figure 5.3 Pellet cultures treated with growth factors, x40 objective, Scale bar= 40µm. Type I pro-collagen.

1% sera treated controls

A- 1day.

B- 7day.

C- 14day.

bFGF treatment

D- 1day. Type I pro-collagen is present in isolated cells throughout the pellet.

E- 7day. Positively labelled type I pro-collagen can be observed clearly at this time period.

F- 14day. Type I pro-collagen remains sporadic in the pellets.

TGFβ treatment

G- 1day. At all stages the type I pro-collagen present is similar to the bFGF treated pellets.

H- 7day.

I- 14day.

PDGF treatment

J- 1day. At all stages the type I pro-collagen present is similar to the bFGF and TGFβ treated pellets.

K- 7day.

L- 14day.

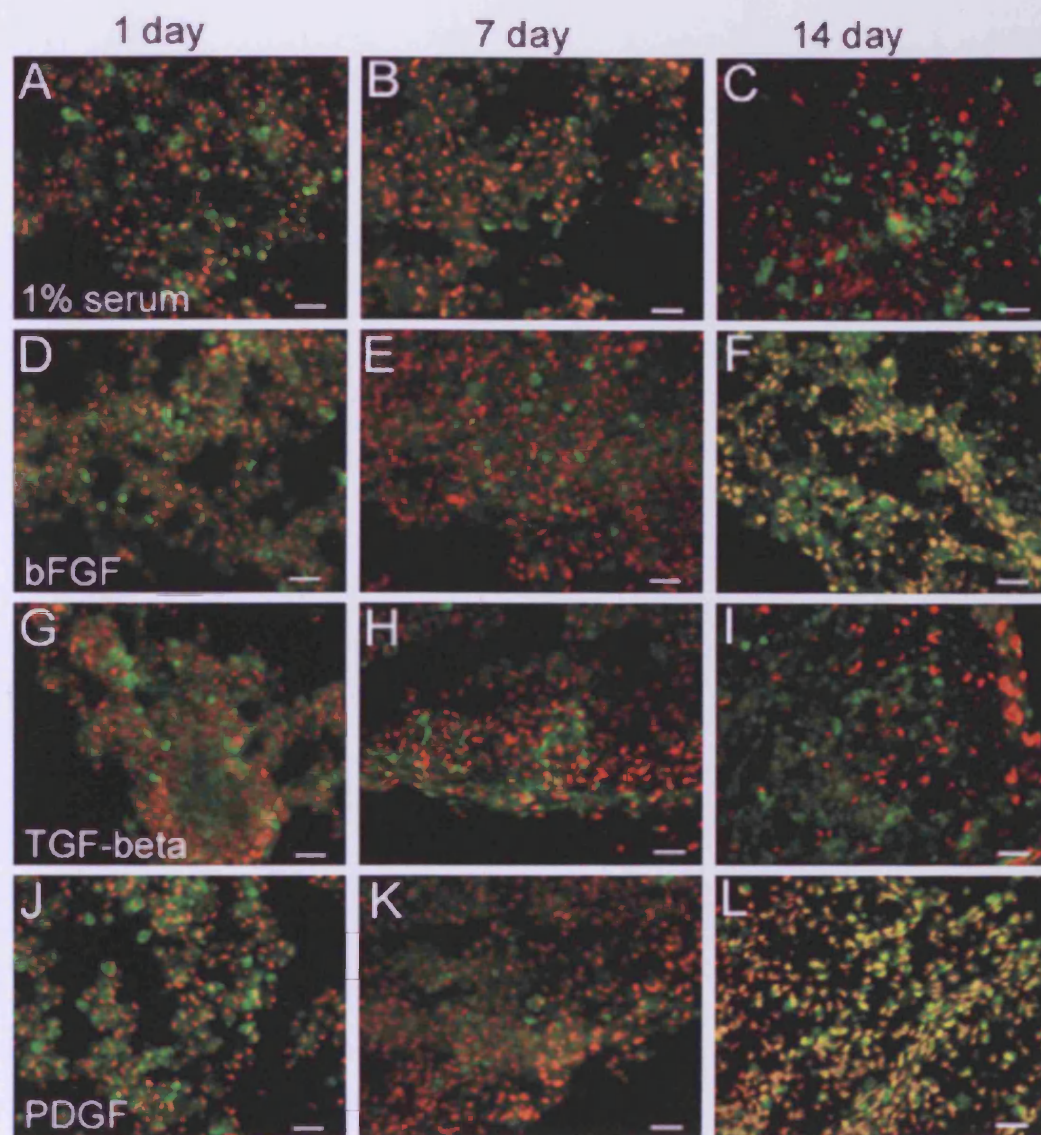


Figure 5.4 Pellet cultures treated with growth factors, x40 objective, Scale bar= 40µm. **Tenascin.**

1% sera treated controls

A- 1day.

B- 7day pellet.

C- 14day pellet.

bFGF treatment

D- 1day. Tenascin is positive and in areas appeared to be extracellular.

E- 7day. Label for tenascin remained positive and there is a possible increase in labelling intensity.

F- 14day. Tenascin label is positively concentrated at the edge of the cell pellet.

TGFβ treatment

G- 1day. Tenascin is present.

H- 7day. Tenascin is present after 7 days in culture, and is increasingly extracellular.

I- 14day. After 14 days in culture tenascin is focused in the periphery of the cell pellet.

PDGF treatment

J- 1day. Tenascin is present after 1 day in pellet culture.

K- 7day. An increase in tenascin labelling intensity is apparent and extracellular tenascin is evident.

L- 14day. Tenascin label is positive and extracellular.

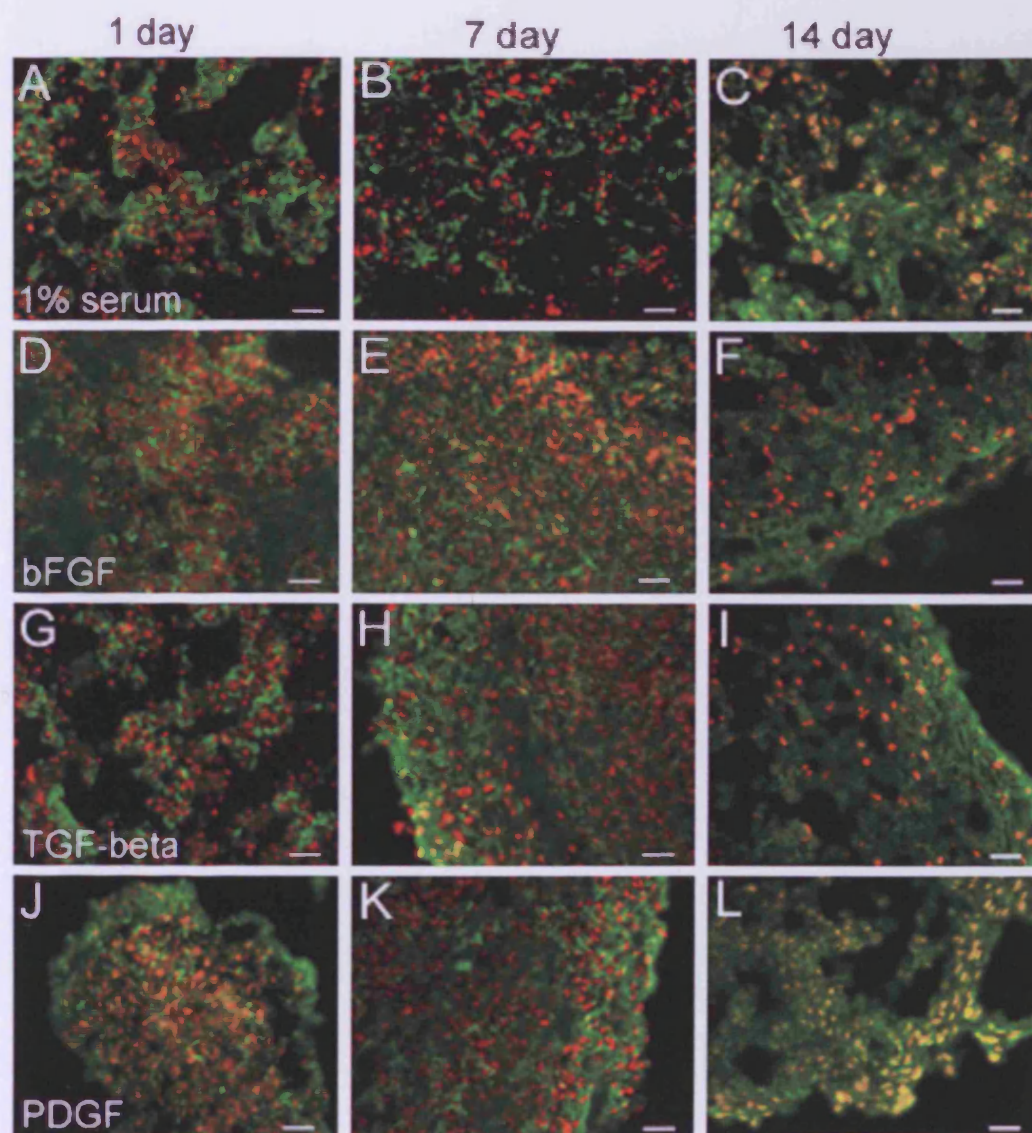


Figure 5.5 Pellet cultures treated with growth factors, x40 objective, Scale bar= 40µm. **Fibronectin.**

1% sera treated controls

A- 1day.

B- 7day.

C- 14day.

bFGF treatment

D- 1day. Positive label for fibronectin is observed in areas of the cell pellet.

E- 7day. Distinct areas of extracellular fibronectin are present.

F- 14day. Fibronectin remained present, although a possible decrease in intensity is seen.

TGFβ treatment

G- 1day. Fibronectin is present.

H- 7day. Positively labelled fibronectin is clear at the periphery of the pellet.

I- 14day. Fibronectin has a similar distribution pattern after 24 days in culture.

PDGF treatment

J- 1day. Little or no fibronectin is present.

K- 7day. The fibronectin identified is extracellular.

L- 14day. Fibronectin remains in areas nearing the periphery of the cell pellet.

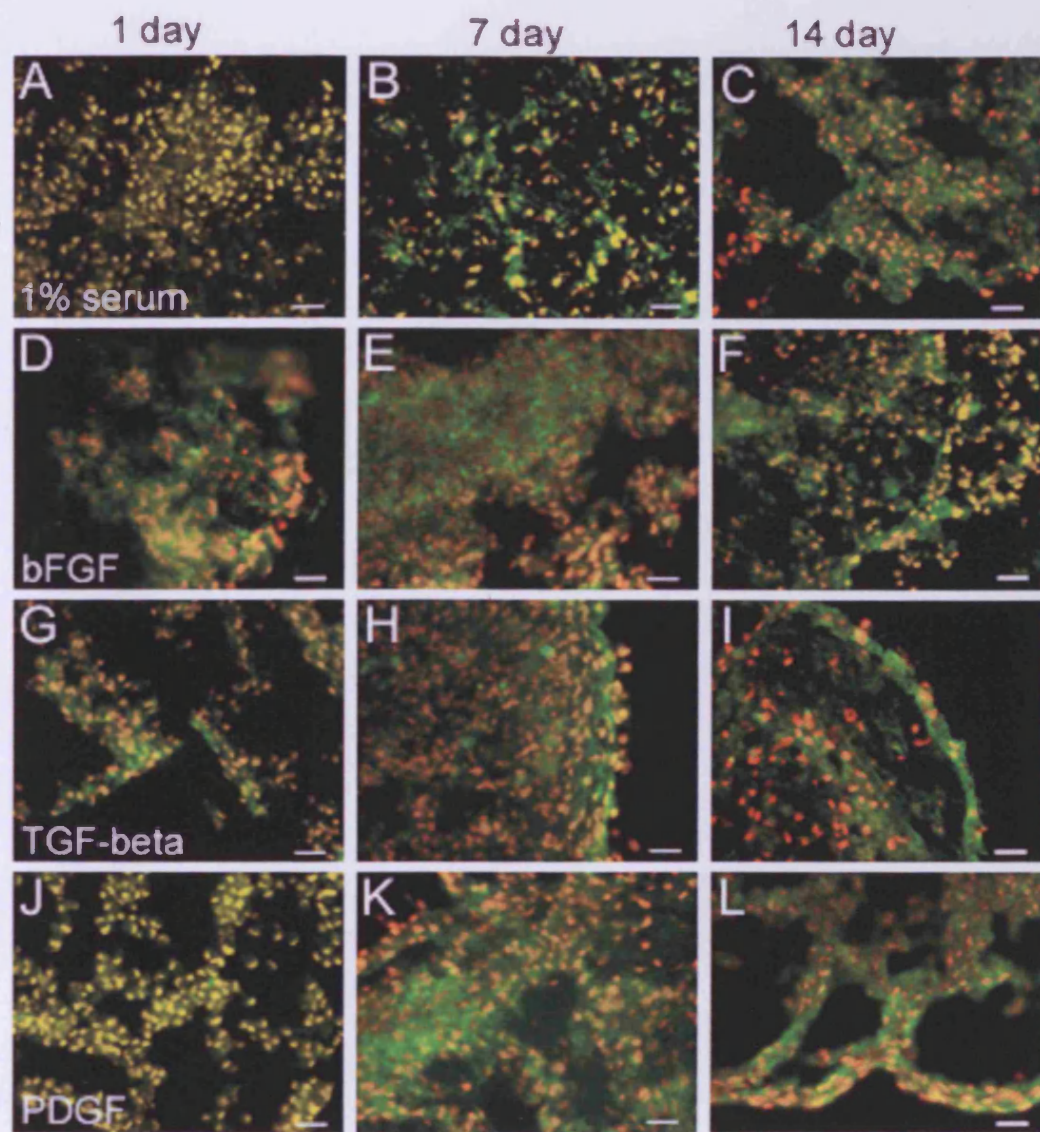


Figure 5.6 Pellet cultures treated with growth factors, x40 objective, Scale bar= 40µm. Actin.

1% sera treated controls

A- 1day.

B- 7day.

C- 14day.

bFGF treatment

D- 1day. Actin filaments are present, although have a hazy style of labelling.

E- 7day. Areas of actin label clearly show an increase in intensity on some cells. Rounded cells indicate cellular polarity in the labelling, although no stress fibres are observed.

F- 14day. Positively labelled actin is observed throughout the cell pellet.

TGFβ treatment

G- 1day. Strong actin filamentous networks are observed.

H- 7day. Centrally the more rounded cells labelled positively for actin, but no stress fibres can be identified. Cells that are flattened at the periphery of the pellet show the first signs of distinct actin stress fibres.

I- 14day. Actin is present in central areas of the pellets.

PDGF treatment

J- 1day. Actin is present in the cells within the pellet.

K- 7day. There was an increase in positively labelled actin and a decrease in the hazy style of labelling. Short actin stress fibres can be identified.

L- 14day. Areas of actin remain present and short actin stress fibres are clearly visible.

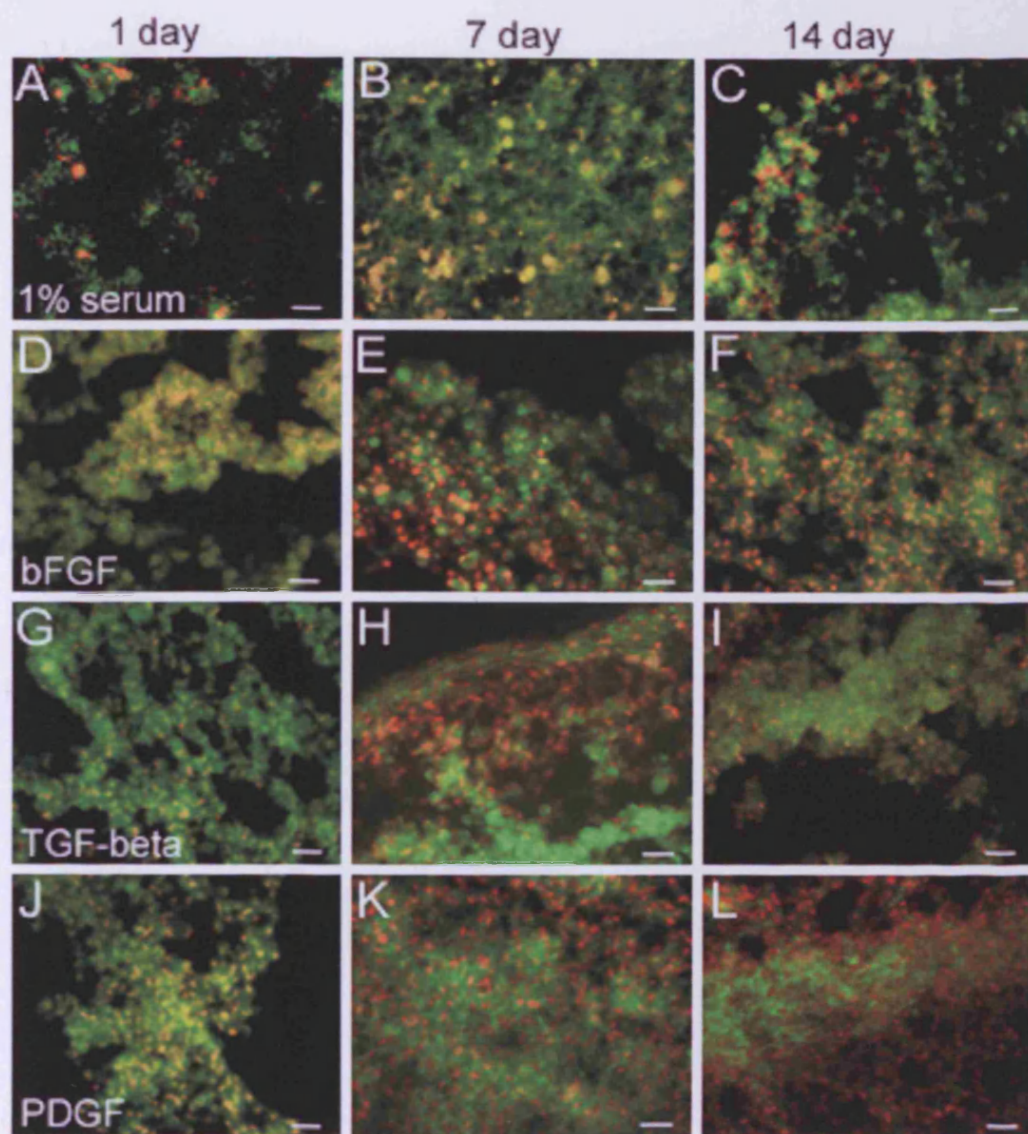


Figure 5.7 Pellet cultures treated with growth factors, x40 objective, Scale bar= 40µm. **Vimentin**.

1% sera treated controls

A- 1day.

B- 7day.

C- 14day.

bFGF treatment

D- 1day. Vimentin is present and can be observed in all cells.

E- 7day. An increase in positively labelled vimentin was apparent and in areas distinct filaments can be distinguished.

F- 14day. There is a similar distribution in 14 days as in 7 days and filaments can be identified.

TGFβ treatment

G- 1day. Vimentin is apparent, although more abundant at the pellet periphery.

H- 7day. Vimentin is positive and seen throughout the cell pellet.

I- 14day. The intensity and distribution remains in 14day pellets, and in areas distinct filaments can be detected.

PDGF treatment

J- 1day. Vimentin can be identified throughout the cell pellet.

K- 7day. Individual vimentin filaments are observed, with an increase in label at the pellet periphery.

L- 14day. Vimentin is present.

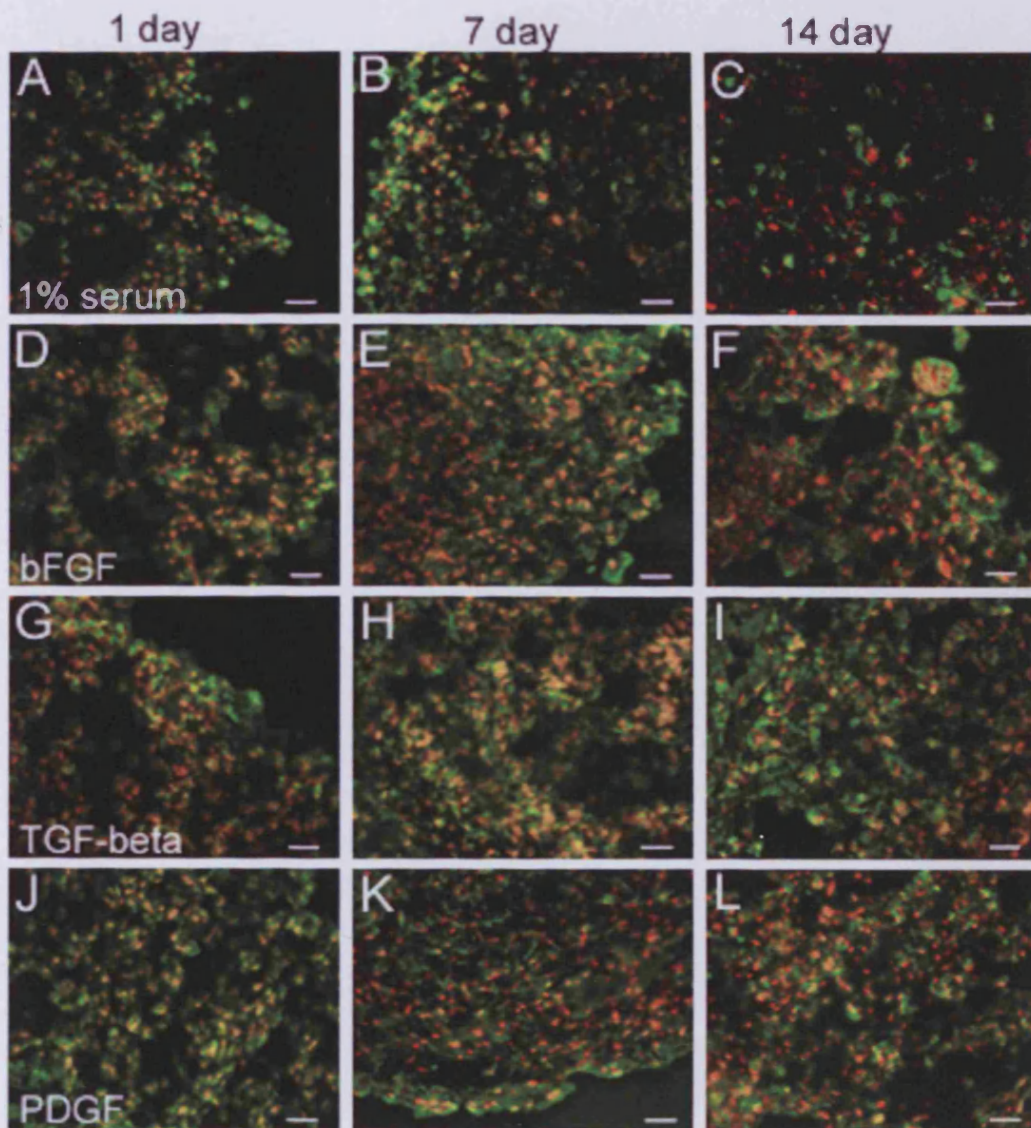


Figure 5.8 Pellet cultures treated with growth factors, x40 objective, Scale bar= 40µm. Pan-cadherin.

1% sera treated controls

A- 1day.

B- 7day.

C- 14day.

bFGF treatment

D- 1day. Pan-cadherin is present.

E- 7day. Pan-cadherin increased in distribution and is focused in the periphery of the cells.

F- 14day. Pan-cadherin is present and continues to be concentrated at the cell borders.

TGFβ treatment

G- 1day. Pan-cadherin shows a similar distribution as n-cadherin, but with an increase in intensity.

H- 7day. An apparent increase in pan-cadherin is observed together with labelling remaining strongest at the cell periphery.

I- 14day. Pan-cadherin remains present.

PDGF treatment

J- 1day. Positively labelled pan-cadherin can be observed throughout the cell pellet.

K- 7day. Pan-cadherin distribution and intensity is clear.

L- 14day. Pan-cadherin remains in areas of the pellet after 14 days in pellet culture.

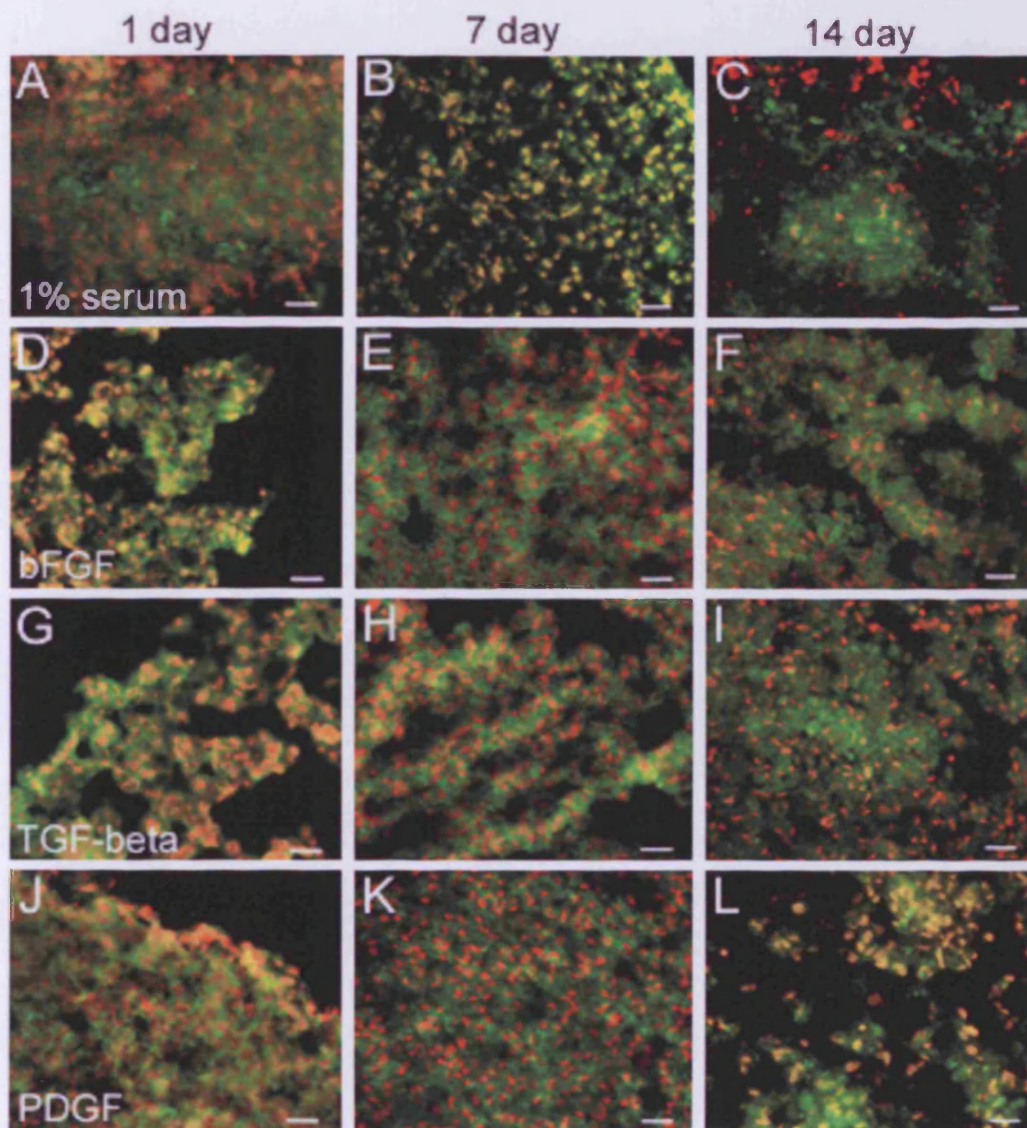


Figure 5.9 Pellet cultures treated with growth factors, x40 objective, Scale bar= 40µm. n-cadherin.

1% sera treated controls

A- 1day.

B- 7day.

C- 14day.

bFGF treatment

D- 1day. N-cadherin is positive and generally localised at the periphery.

E- 7day. There is an increase in labelling for n-cadherin throughout the pellet, compared to that in 1 day pellets.

F- 14day. The n-cadherin present is concentrated at distinct areas of the pellet, but remains widespread.

TGFβ treatment

G- 1day. n-cadherin is present and situated at the pellet periphery.

H- 7day. The n-cadherin shows a possible increase in n-cadherin labelling intensity and distribution.

I- 14day. N-cadherin remains present, but slightly diminished compared to 7 days in culture.

PDGF treatment

J- 1day. Positively labelled n-cadherin is present and localised to the pellet periphery.

K- 7day. There is a significant increase in label intensity and distribution for n-cadherin.

L- 14day. A decrease in n-cadherin distribution is observed, although the labelling remained positive.

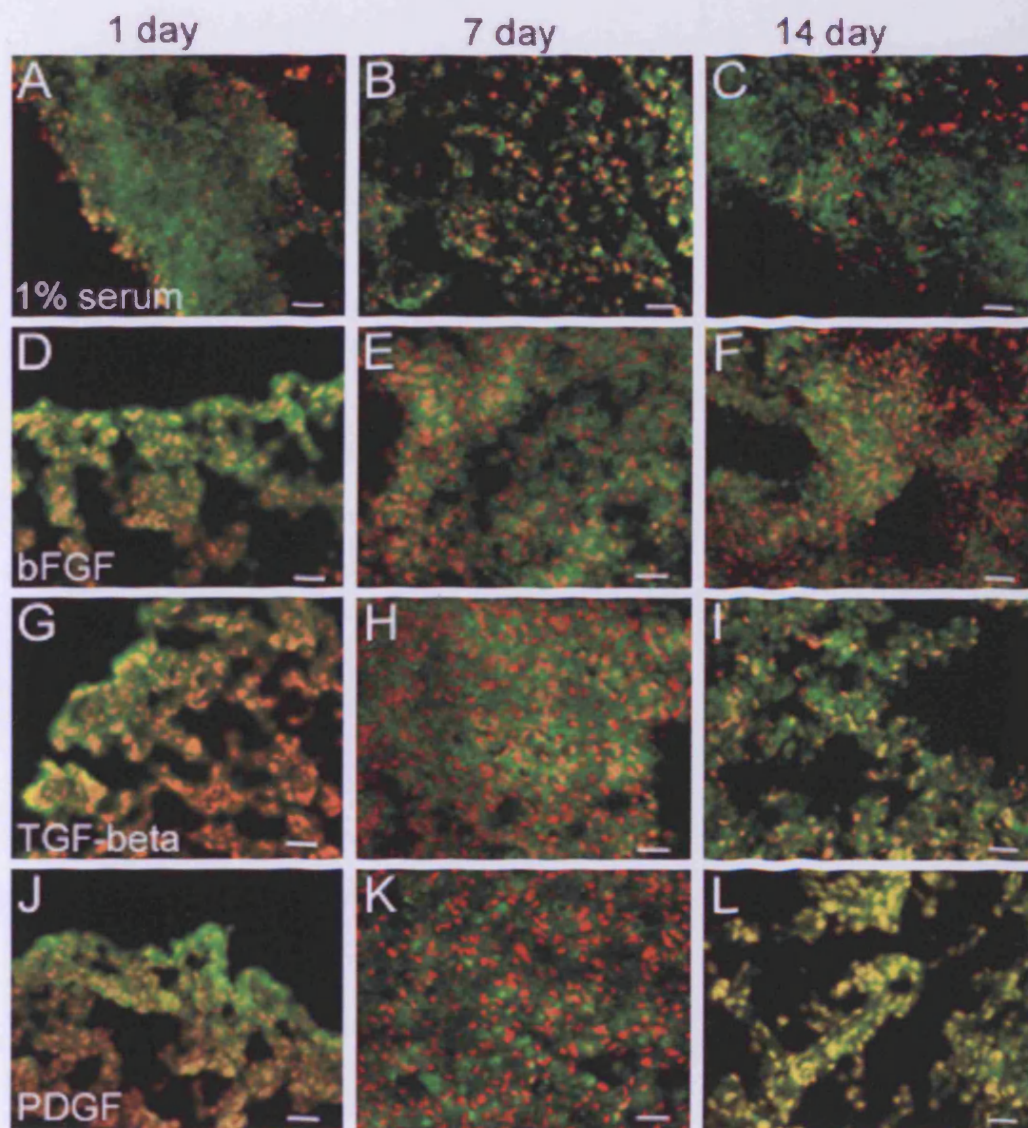


Figure 5.10 Pellet cultures treated with growth factors, x40 objective, Scale bar= 40µm. Vinculin.

1% sera treated controls

A- 1day.

B- 7day.

C- 14day.

bFGF treatment

D- 1day. Vinculin is present and clearly seen at the cell borders.

E- 7day. Vinculin remains present.

F- 14day. Positive vinculin label can be seen throughout the cell pellet.

TGFβ treatment

G- 1day. Vinculin label can be observed in 1day cell pellets.

H- 7day. Vinculin is present throughout the cell pellet, but there is a higher intensity at the periphery of the pellet.

I- 14day. The labelling pattern of vinculin remains at the periphery of the cells.

PDGF treatment

J- 1day. Positively labelled vinculin can be observed throughout the pellet with the cells retaining peripheral labelling.

K- 7day. Vinculin remains evident.

L- 14day. Positively labelled vinculin is maintained at the cell pellet periphery.

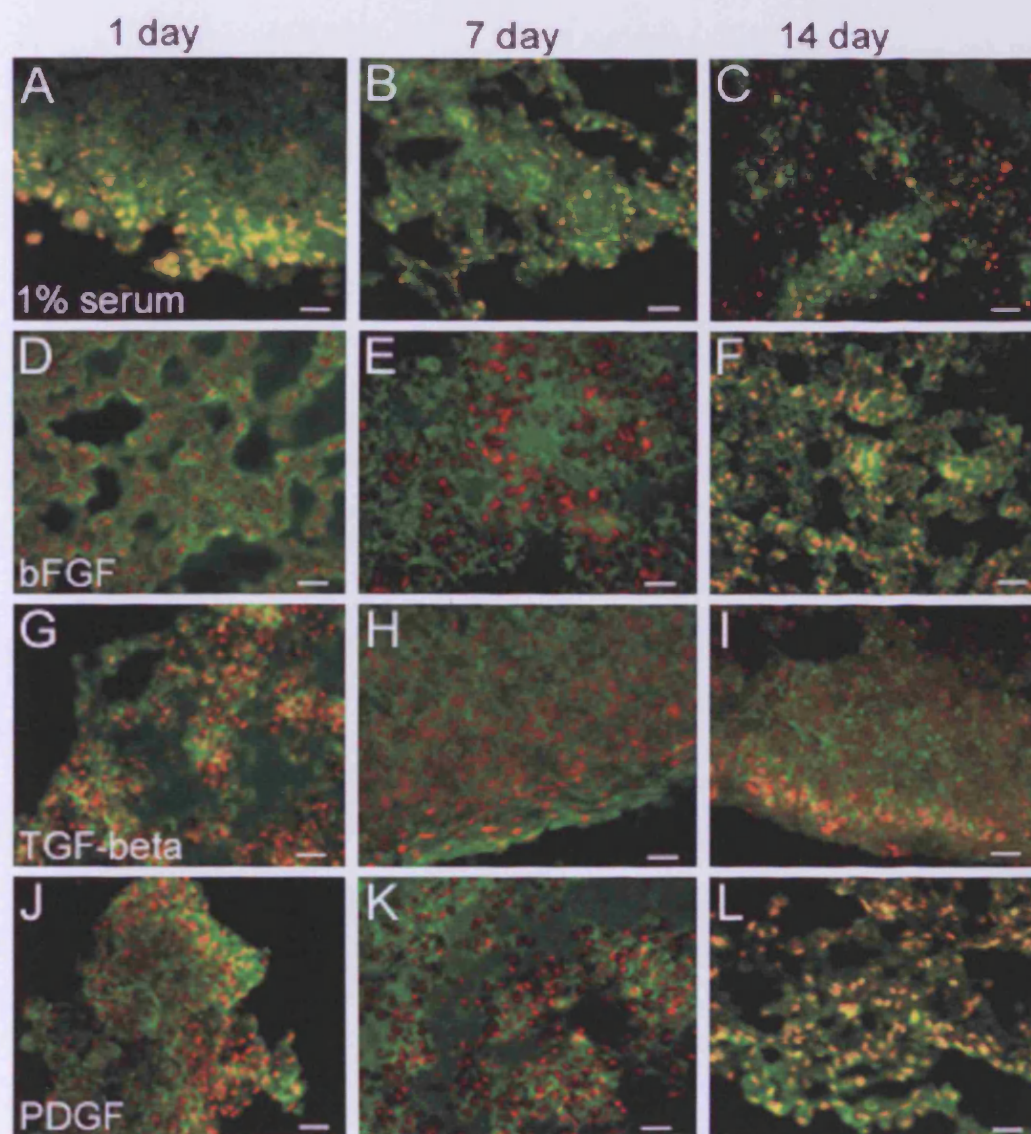


Figure 5.11 Pellet cultures treated with growth factors, x40 objective, Scale bar= 40µm. Connexin 32.

1% sera treated controls

A- 1day.

B- 7day.

C- 14day.

bFGF treatment

D- 1day. Cells label positively for connexin 32.

E- 7day. Cells continue to label positively for connexin 32 and an increased 'wisp-like' labelling pattern can be observed.

F- 14day. Areas of connexin 32 are strong at the pellet periphery.

TGFβ treatment

G- 1day. Connexin 32 label is present and labelling is increasingly intense at the periphery.

H- 7day. Connexin 32 has distributions similar to bFGF-treated pellets.

I- 14day. The strongest areas of connexin 32 remain at the pellet periphery.

PDGF treatment

J- 1day. Connexin 32 can be observed in 1 day pellet cultures.

K- 7day. The labelling intensity of connexin 32 increases after 7 days.

L- 14day. Connexin 32 is abundant after 14 days of culture.

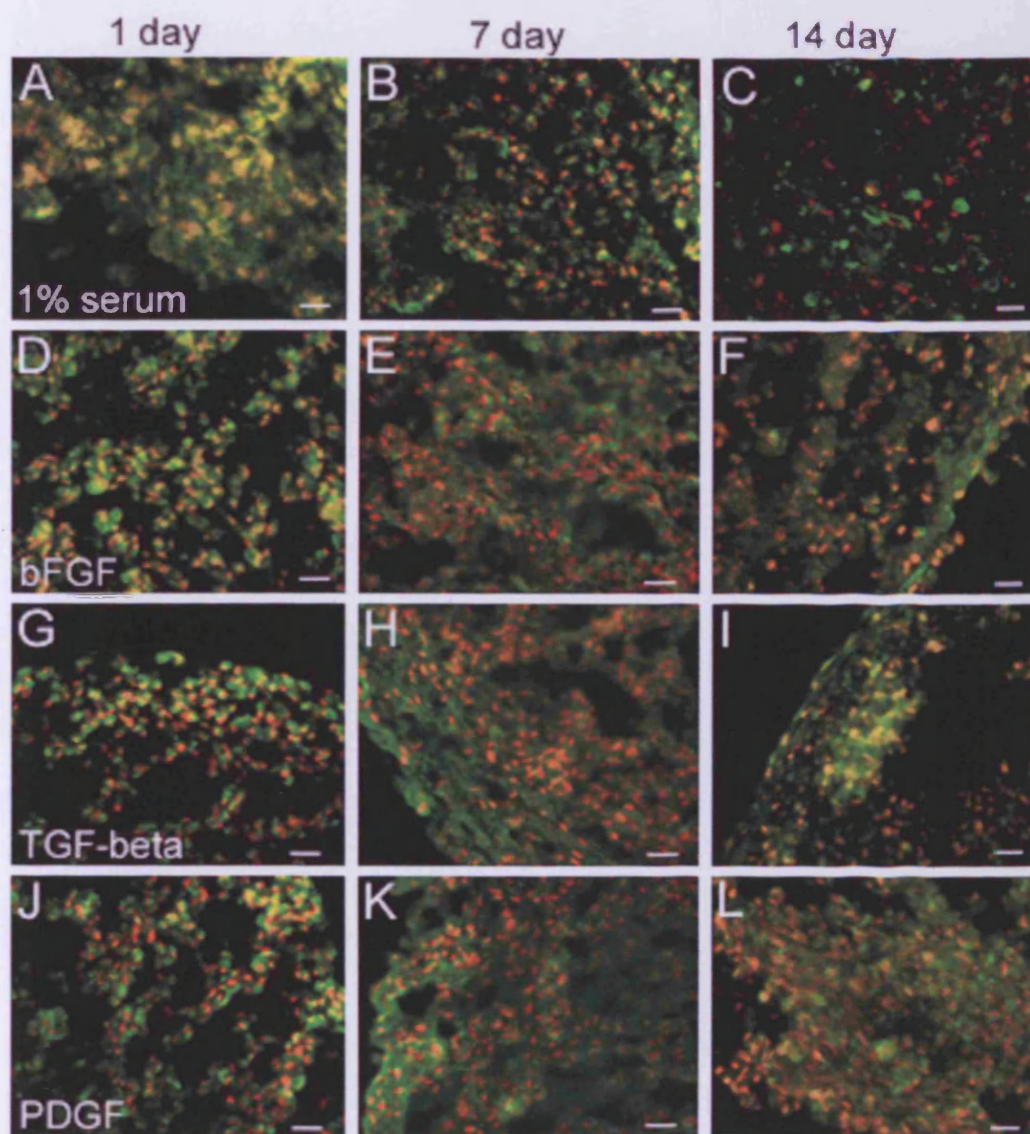


Figure 5.12 Pellet cultures treated with growth factors, x40 objective, Scale bar= 40µm. **Connexin 43.**

1% sera treated controls

A- 1day.

B- 7day.

C- 14day.

bFGF treatment

D- 1day. Though there is a green tinge to the image, connexin 43 is absent from these pellets.

E- 7day. Small areas of connexin 43 can be seen, but the presence of connexin 43 is sparse.

F- 14day. Connexin 43 distribution is similar to that at 7 days.

TGFβ treatment

G- 1day. Areas of connexin 43 can be observed under close scrutiny, although there remains an amount of auto-fluorescence.

H- 7day. Connexin 43 is clearly be identified.

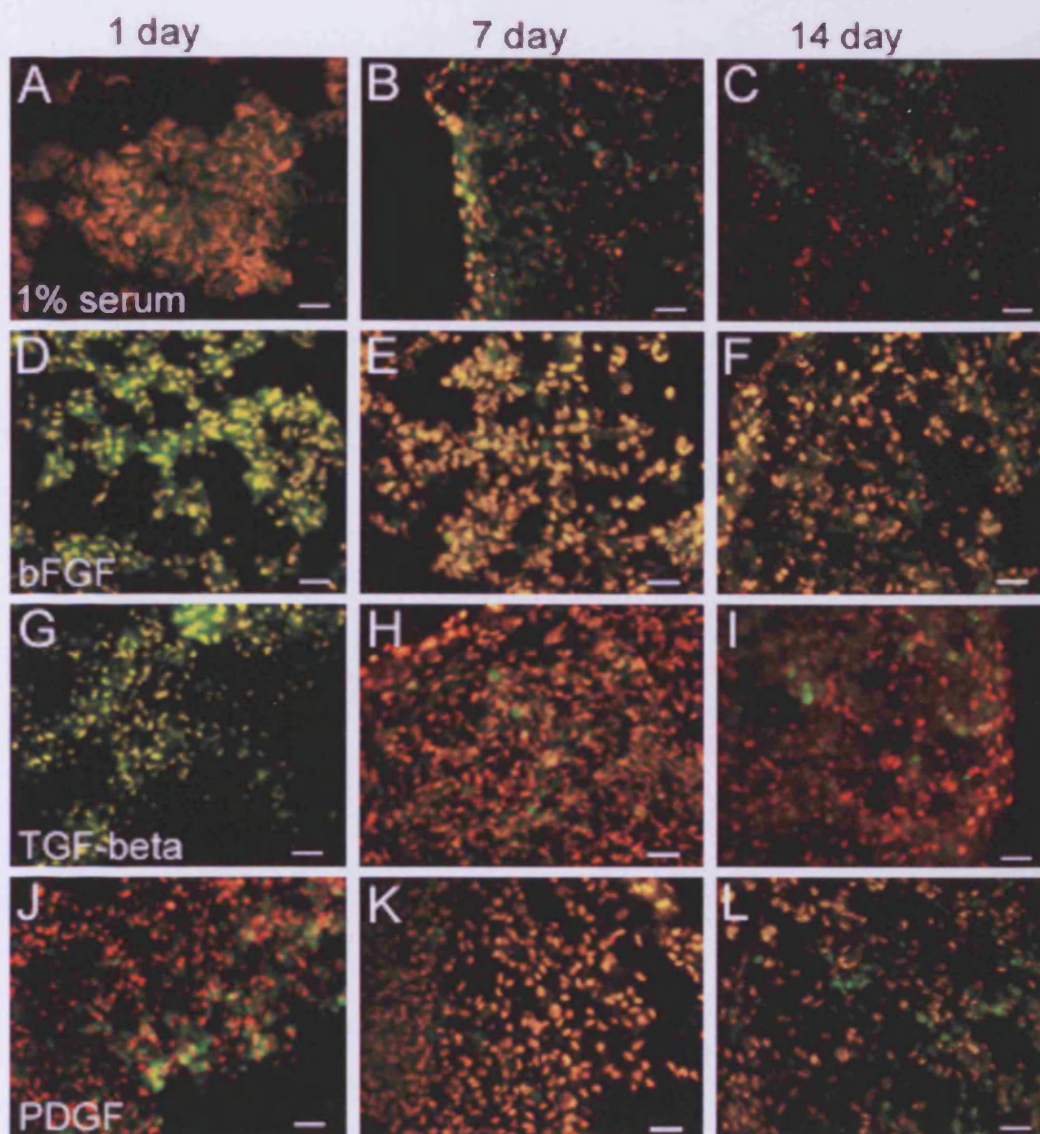
I- 14day. Individual cells label positively for connexin 43.

PDGF treatment

J- 1day. Positively labelled connexin 43 is found within the pellet.

K- 7day. There is little or no connexin 43 present.

L- 14day. Areas of positively labelled connexin 43 cells are observed.



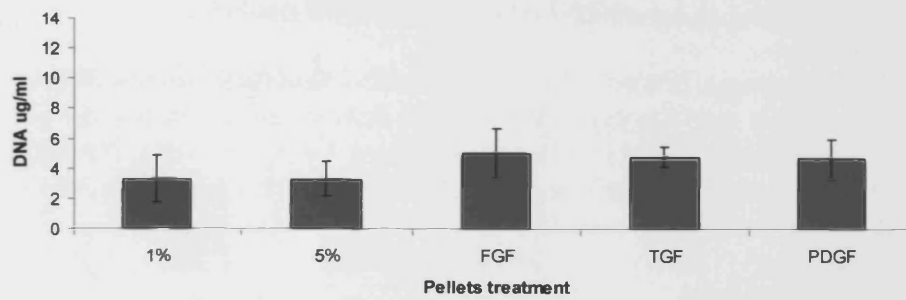
Extracellular matrix molecules						Cytoskeletal and cell junctional components						
	Type I	Type III	Type I pro	Tenascin	Fibronectin	Actin	Vimentin	Pan-cad	n-cad	vinculin	Cxn 32	Cxn 43
bFGF+PDGF 1day	*	**	**	***	—	**	**	**	*	*	*	*
bFGF+PDGF 7day	***	***	**	***	**	***	**	**	**	***	***	**
bFGF+PDGF 14day	**	***	**	***	**	***	**	*	**	**	**	*
TGFβ+bFGF 1 day	**	**	**	***	*	**	**	**	*	**	**	*
TGFβ+bFGF 7 day	***	***	**	***	**	***	**	**	**	**	**	*
TGFβ+bFGF 14 day	**	***	**	***	*	**	**	*	**	**	**	*
PDGF+TGFβ 1 day	**	**	**	***	—	**	**	**	*	**	**	**
PDGF+TGFβ 7 day	***	***	**	***	**	**	**	**	**	***	**	*
PDGF+TGFβ 14 day	**	***	**	***	*	**	**	*	**	**	**	*

Table 5.1 Summary to show immunolabelling patterns of pellets treated with growth factor combinations, from 1 to 14 days.

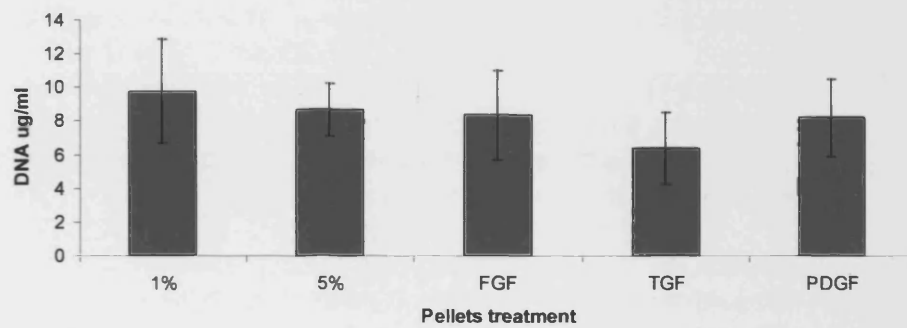
Key- - negative labelling
*-weak labelling present

** - labelling present
*** - strong labelling present

DNA in 1 day pellets



DNA in 7 day pellets



DNA in 14 day pellets

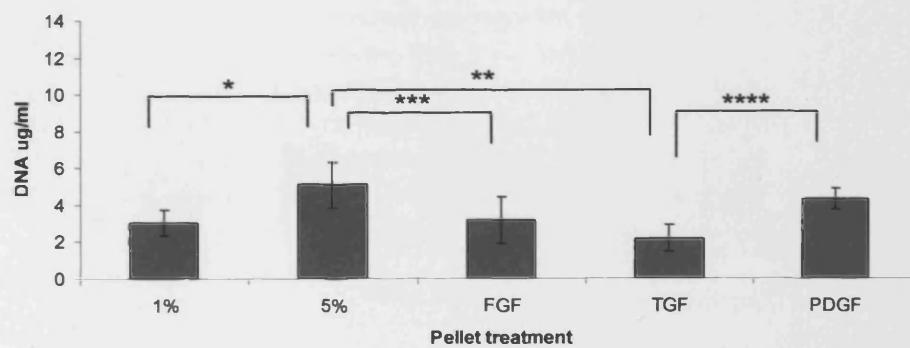


Figure 5.13. Effects of time and growth factors on DNA content of pellet cultures. Error bars = Standard deviation.

i) Graph of DNA (ug/ml) in tendon fibroblast pellets cultured for 1 day and treated with single growth factors. There was no significant difference between the pellets treated with growth factors and the controls.

ii) Graph of DNA (ug/ml) in tendon fibroblast pellets cultured for 7 days and treated with single growth factors. There was a significant increase in DNA in all pellets cultured compared to 1 day ($p < 0.05$) but no significant difference between the pellets treated with growth factors and the controls.

iii) Graph of DNA (ug/ml) in tendon fibroblast pellets cultured for 14 days and treated with single growth factors. There was a significant decrease in DNA compared with pellets cultured for 7 days ($p < 0.05$). Between treatments at 14 days, 5% serum pellets had significantly elevated DNA content compared to 1% serum, bFGF and TGF β ($p < 0.05$). Also, PDGF treated pellets had a greater effect on DNA content than TGF β ($p < 0.05$).

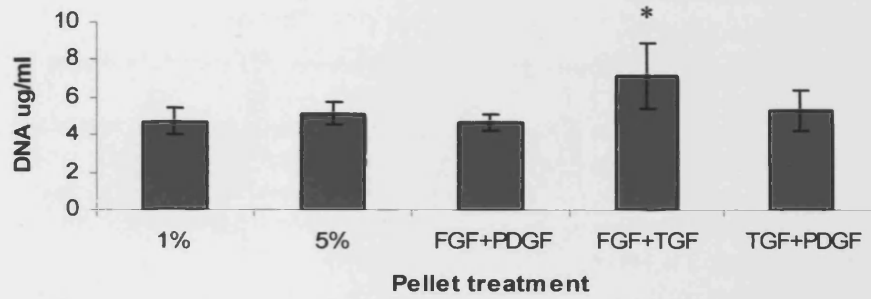
*= $p < 0.05$

**= $p < 0.05$

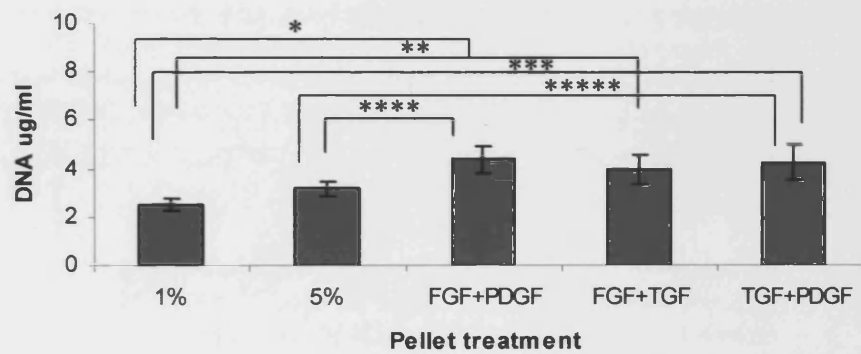
***= $p < 0.05$

****= $p < 0.05$

DNA in 1 day pellets



DNA in 7 day pellets



DNA in 14 day pellets

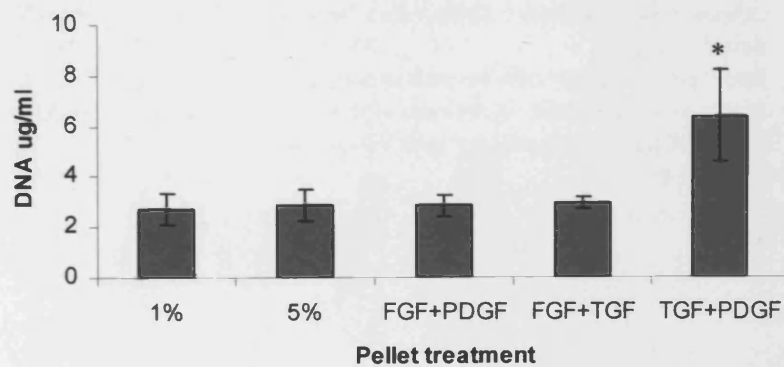


Figure 5.14. Effects of time and growth factors on DNA content of pellet cultures. Error bars = Standard deviation.

i) Graph of DNA (ug/ml) in tendon fibroblast pellets cultured for 1 day and treated with growth factor combinations. bFGF+TGF β treated pellets showed elevated DNA ($p<0.05$). There was no significant difference between the other pellets treated with growth factors and the controls ($p>0.05$).

*= $p<0.05$

ii) Graph of DNA (ug/ml) in tendon fibroblast pellets cultured for 1 week and treated with growth factor combinations. There was a significant decrease in DNA in pellets cultured for 7 days compared to 1 day pellets ($p<0.05$). The DNA content is significantly reduced in 1% controls, 5% serum and bFGF+TGF β treated cultures ($p<0.05$). Significant differences between treatments at 7 days include all growth factor treated pellets have increased DNA contents compared to the 1% control ($p<0.05$). Also, 5% serum was significantly lower than both bFGF+PDGF and TGF β +PDGF treated pellets ($p<0.05$).

*= $p<0.05$

**= $p<0.05$

***= $p<0.05$

****= $p<0.05$

*****= $p<0.05$

iii) Graph of DNA (ug/ml) in tendon fibroblast pellets cultured for 14 days and treated with growth factor combinations. There was no significant difference between the pellets after 14 days in culture compared to 7 day pellets ($p>0.05$), except bFGF+PDGF treated pellets showed a decrease in DNA content ($p<0.05$) and there was significant increase in DNA in PDGF + TGF β treated pellets ($p<0.05$). Between treated pellets and controls cultured for 14 days, only TGF β +PDGF treated pellets showed significantly elevated levels of DNA ($p<0.05$).

*= $p<0.05$

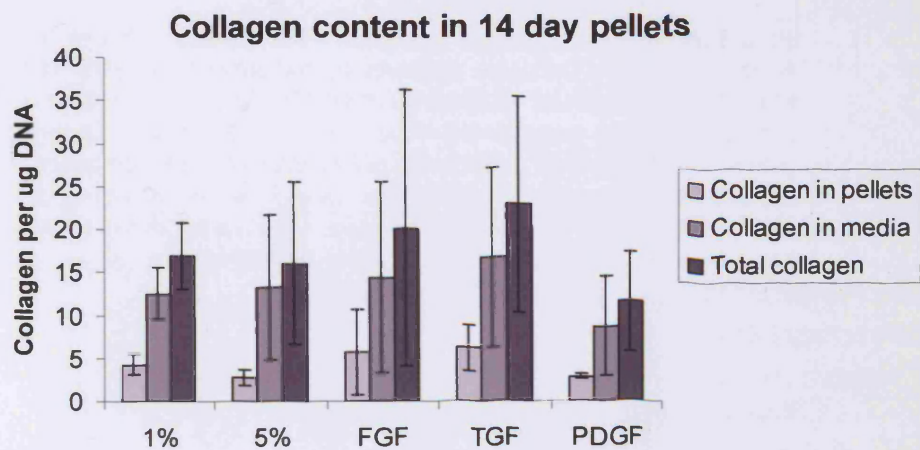
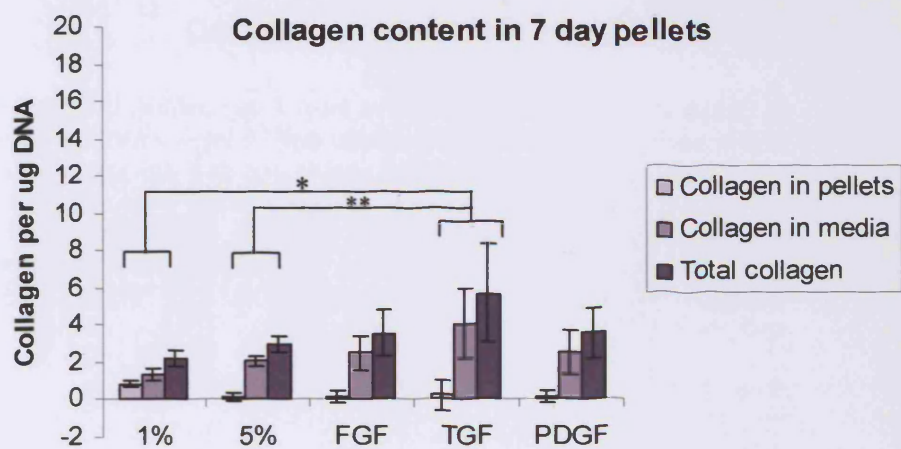
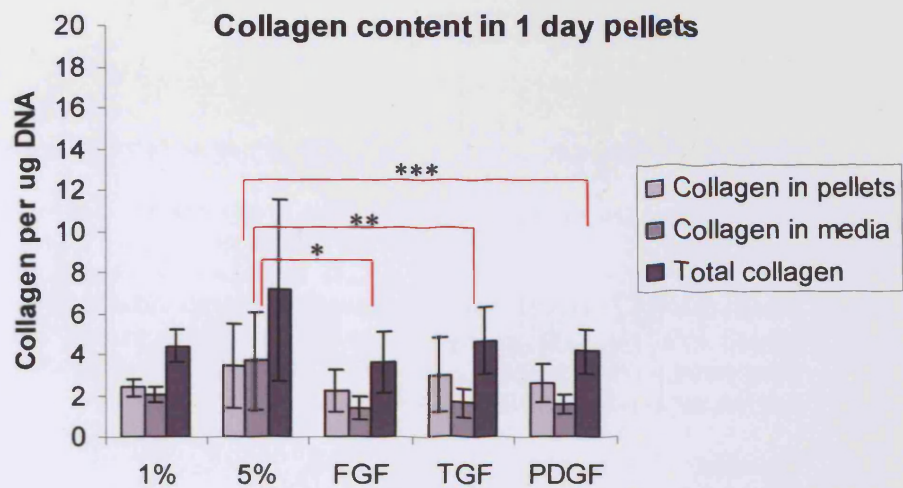


Figure 5.15 . Effects of growth factors on collagen production

i) Graph to show collagen amounts retained in pellets, released into the media and in total after 1 day of incubation. The collagen released into the media from the 5% serum treated pellets was significantly greater than that released by each growth factor treated pellet ($p < 0.05$). $n=7$, except 1% serum and TGF where $n=6$.

*= $p < 0.05$

**= $p < 0.05$

***= $p < 0.05$

ii) Graph to show collagen amounts retained in pellets and released into the media and in total after 7 days of incubation. There are significant differences in the collagen content of each pellet, media and in total between both the 1% and 5% serum and TGF treated pellets. $n=7$.

*= $p < 0.05$

**= $p < 0.05$

iii) Graph to show collagen amounts retained in pellets and released into the media and total after 14 days. There were no significant differences between the sera and growth factor treated pellets at this time, however the error bars show the variance in the data. $n=7$.

Blue lines indicate differences between total collagen amounts.

Green lines indicate differences between collagen retained within the pellet

Red lines indicate differences in the collagen released into the media

Black lines indicate significant differences between all pellet, media and total collagen amounts for that treatment

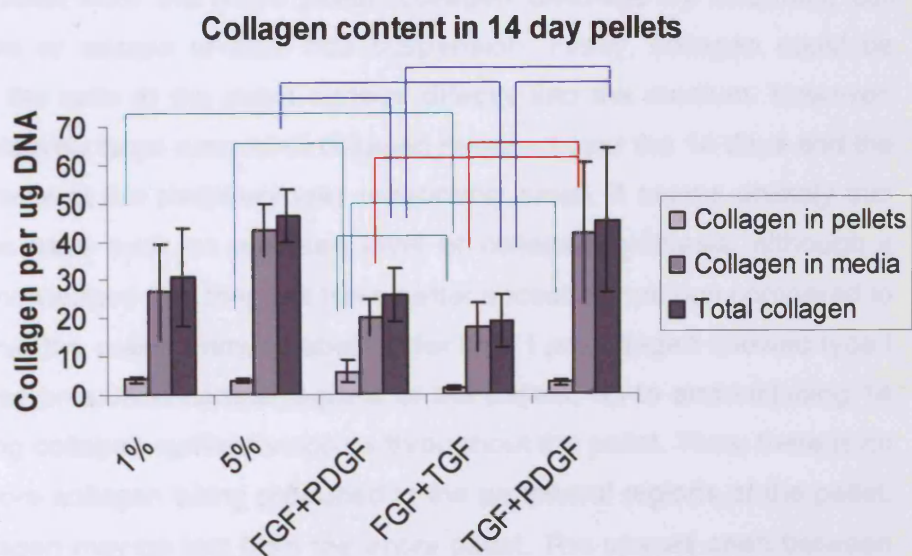
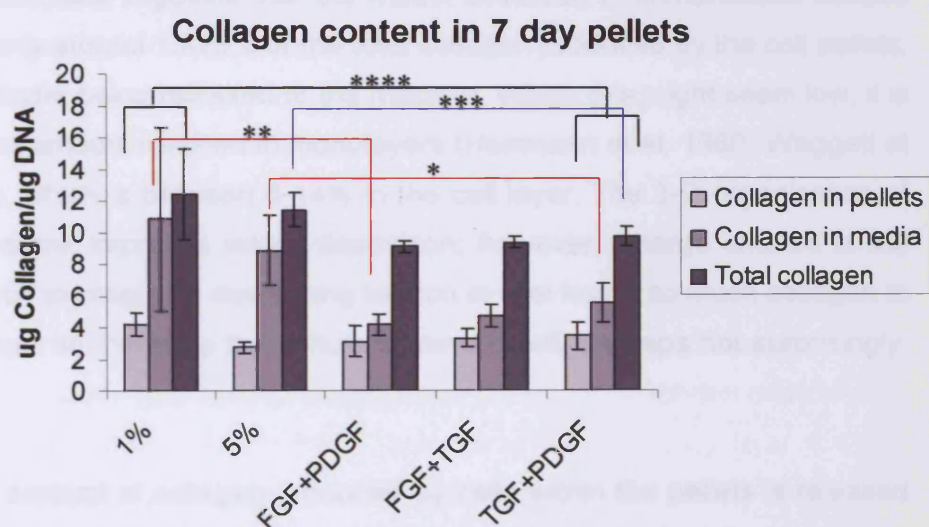
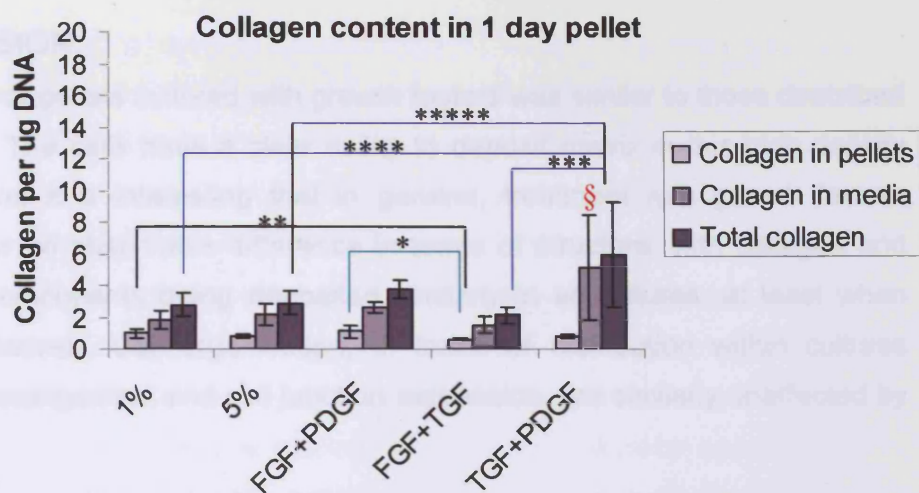


Figure 5.16 Effects of growth factor combinations on collagen production

i) Graph to show collagen amounts from 1 day pellets, retained in the pellet, released into the media and total. Collagen retained in the pellets of 1% and bFGF+PDGF treated pellets is greater compared to that of TGFβ+bFGF. Collagen released from the media from TGFβ+PDGF treated pellets is significantly greater than the other treated pellets. Total collagen is significantly greater in TGFβ +PDGF compared to 1% and 5% serum treated pellets.. n=7.

*=p<0.05

**=p<0.05

***=p<0.05

****=p<0.05

*****=p<0.05

ii) Graph to show collagen amounts from 7 day pellets, collagen retained in the pellet, released into the media and in total. There is significantly more collagen between the pellet, media and total collagen of 1% serum control compared with the TGFβ +PDGF treated pellet. The 1% serum control also has greater levels of collagen released into the media compared with bFGF+PDGF, and 5% serum cultures have more collagen released into the media compared to TGFβ +PDGF. n=7, except TGF+PDGF where n=5.

*=p<0.05

**=p<0.05

***=p<0.05

****=p<0.05

iii) Graph to show collagen amounts from 14 day cultures. 1% sera control has more collagen retained in the pellet than bFGF+FGF, and bFGF+PDGF treated pellets had more collagen within the pellet compared to 5% sera, bFGF+FGF and TGFβ +PDGF treated pellets. TGFβ +PDGF treated pellets had more collagen released into the media compared to the other growth factor combinations, and both 5% serum and TGFβ +PDGF treated pellets had significantly more total collagen compared to the other growth factor combinations. n=7 and each significant difference shown was at p<0.05.

Blue lines indicate differences between total collagen amounts.

Green lines indicate differences between collagen retained within the pellet

Red lines indicate differences in the collagen released into the media

Black lines indicate significant differences between all pellet, media and total collagen amounts for that treatment

5.4 DISCUSSION

The structure of pellets cultured with growth factors was similar to those described in chapter 4. The cells have a clear ability to deposit matrix in this high density culture system. It is interesting that in general, treatment with growth factors makes little or no observable difference in terms of structure, with collagen and other ECM components being deposited similarly in all cultures, at least when viewed qualitatively. Cell organisation, in terms of distribution within cultures cytoskeletal arrangement and cell junction expression, are similarly unaffected by treatments.

The quantitative data suggests that the matrix observed in immunolabel studies represented only around 15-25% of the total collagen produced by the cell pellets, with the remainder being released to the medium. Whilst this might seem low, it is higher than the amount retained in monolayers (Herrmann et al, 1980; Waggett et al, submitted), which is between 6-14% in the cell layer. The 3-D organisation of the cells therefore, improves matrix deposition; however, a large amount is still lost. It is difficult to imagine a developing tendon *in vivo* losing so much collagen to its surroundings, so therefore the culture system is still, perhaps not surprisingly, inadequate.

A substantial amount of collagen produced by cells within the pellets is released into the media: but how does this occur? Possible explanations involve direct secretion, diffusion from the entire pellet, collagen cleavage by enzymes, cell death and lysis or escape of cells into suspension. Firstly, collagen could be secreted from the cells at the pellet surface directly into the medium. However, there was a relatively large amount of collagen released over the 14 days and the population of cells at the periphery was reasonably small. It seems unlikely that peripheral cells have such an elevated level of collagen synthesis, although it should be acknowledged that they will have better access to nutrition compared to other cells within the pellet. Immunolabelling for type I procollagen showed type I procollagen was present in central regions of the pellets, up to and including 14 days suggesting collagen synthesis occurs throughout the pellet. Thus, there is no evidence of more collagen being produced in the peripheral regions of the pellet. Secondly, collagen may be lost from the entire pellet. The spaces seen between cells within the pellet (shown in electron microscopy images in chapter 4) may be continuous with the outside environment, rather than bounded by endotenon in fascicles or an epitenon at the periphery as *in vivo* (Kannus, 2000) and thus

collagen could be lost through these channels. Thirdly, it is possible that there is collagen cleavage within the pellet. It may be that MMPs, including collagen degrading enzymes MMP-1, 8 and 13 (Sodek and Overall, 1992; Krane, 1995; Leeman et al, 2002; review Overall, 2002; Stamenkovic, 2003), are produced and that secreted collagen is broken down and lost as fragments. MMPs can modulate the activity of TGF β and bFGF (Levi et al, 1996; Yu and Stamenkovic, 2000) and TGF β can inhibit MMPs (Ma and Chegini, 1999a). Since the hydroxyproline assay used can assay these fragments as well as intact collagen we cannot rule out this possibility. A further possibility is that cell death within the pellet causes cell and tissue lysis. Although not directly assayed, there was no particular evidence of extensive cell death at any stage within the pellets; indeed DNA contents tended to be maintained during the culture period. An additional possibility is that cells are lost from the pellet into the supernatant and secrete collagen directly into the media, although cells were generally not observed here.

The hydroxyproline assay allows measurement of collagen, but clearly this assay does not distinguish between types of collagen. Immunolabelling showed that both types I and III collagen were present. There are other collagens present in tendon *in vivo* (Kannus, 2000), and therefore likely to be produced by tendon cells *in vitro*. These include types V, VI, IX, XII and XIV (Duance et al, 1977; Lapiere et al, 1977; von der Mark, 1981; Linsenmayer et al, 1990; Birk et al, 1990; Shaw et al, 1991; Waggett et al, 1998). In monolayer it is known that 86% of type I collagen secreted is lost to the medium (Waggett et al submitted; Waggett et al, 2001), which is around the same proportion as total collagen (Herrmann et al, 1980). It seems likely that the majority of the collagen from pellets is type I, but there is likely to be a contribution from other types. Interestingly, type III collagen production increases with the number of cell passages (Herrmann et al, 1980). By keeping all cells used in experiments at the same passage number, P3, it was possible to reduce this variation.

Before discussing the effects of growth factors on collagen production, it is important to address their effects on DNA content and cell proliferation. The key problem is that when assessing the effects of individual growth factors, results show a large DNA increase at day 7. However, the results from the experiments assessing combined growth factors do not. Since collagen results were normalised

to pellet DNA content, to try and eliminate effects of cell proliferation on collagen content, it is necessary to assess these changes.

There are various possibilities for the DNA increase observed at day 7 of the individually treated growth factor pellets. There may have been some experimental error. Although all cultures were set up from the same sample suspension, all DNA measurements performed at the same time and calculated from the same standard curve. Unless a systemic pipetting error was made for the day 7 cultures, this does not seem a likely source of the variation. Comparing the single growth factors and combined growth factor experiments, and in particular considering control 1% serum and 5% serum treated cultures, it is clear that this DNA increase is not related solely to the growth factor treatments. One possibility is that a different serum batch was used and stimulated the 7 day cultures in the first experiment, although it is not clear as to why this should occur just at this time. Another possibility is that there may have been microbial contamination of the 7 day pellet cultures. These types of infection are relatively easy to identify in monolayer cultures, through a dramatic change in media colour and clarity, as well as visible bacteria under the microscope. In pellet cultures, however, this type of infection might have been missed, particularly in the early stages as such infection cannot be observed live on an inverted microscope. The presence of bacteria within the cultures would cause an increased DNA value measured by the fluorometer. Although this type of infection is possible, it is odd that it would also appear at just one time point. If the infection was caused through feeding the cultures, a similar infection might be expected in the 14 day cultures as well. It is not possible to decide what has caused this change, whether it is real or caused by other influences and so it has to be accepted as genuine.

In the single treatments of pellet with growth factors, the effects of bFGF, TGF β and PDGF were examined. bFGF is known to promote matrix synthesis (Chan et al, 2000; Okada-Ban et al, 2000) and cell proliferation (Chan et al, 1997, 2000; Nugent and Iozzo, 2000), however, within these pellet culturing systems there were no differences in the DNA and collagen contents compared to the 1% serum control. TGF β is also known to stimulate ECM synthesis in fibroblasts (Ignatz and Massague, 1986; Roberts et al, 1986; Grotendorst, 1997). Its role in cell proliferation is complex, sometimes stimulating it, at other times inhibiting it, dependant on cell type or environment (Assoian et al, 1983; Tucker et al, 1984;

Roberts et al, 1985a; Nilsen-Hamilton, 1990; Moses et al, 1994; Grotendorst, 1997; Klein, 2002; Yanai et al, 2002; Haber et al, 2003; Lu et al, 2004; Tomlinson et al, 2004). In the pellet cultures, however, it increases collagen secretion. The explanations for this may involve the ability of TGF β to affect the differentiated state of cells (Nilsen-Hamilton, 1990). In this situation, it appears that treated with TGF β the cells favour their differentiated function of matrix synthesis, rather than cell proliferation (Klein, 2002). Experiments on cultured fibroblasts showed bFGF and PDGF do not stimulate such significant amounts of ECM as TGF β (Frazier et al, 1996). Treatment with PDGF does not affect cell behaviour with regards to proliferation or collagen synthesis, although it is known to fulfil both these roles *in vitro* and *in vivo* (Ross et al, 1974; Ross and Vogel, 1978; Pierce et al, 1991; Yoshikawa and Abrahamson, 2001; Molloy, 2003). It is known for its involvement in fibroblast proliferation in tendon cells when they are under load (Banes et al, 1995). In the pellet cultures the cells are not under load and this may explain why PDGF fails to induce any effect on the treated cells. Importantly none of the three growth factors tested are able to increase the amount of collagen retained by the cells within the pellet. In fact, where collagen secretion is enhanced, the proportion incorporated in the pellet is unchanged. It is possible that matrix turnover is enhanced by the growth factors (Ma et al, 1999; Coletta et al, 1999; Hall et al, 2003; Nahm et al, 2004) and not just matrix synthesis.

The use of growth factors *in vitro* to induce a range of changes in cell behaviour has been taking place for decades. In the past, pellet cultures have been used primarily to induce chondrogenesis (Solursh et al, 1991), as discussed previously. Cells from tissues other than hyaline cartilage can also undergo chondrogenesis with growth factor treatment in high density culture. Cells from the fibrous part of the foetal annulus fibrosus undergo this response if treated with TGF β (Hayes, thesis, 1999), as do cells of the adult intervertebral disc (Yung Lee et al, 2001). Tendon fibroblasts showed no evidence of this, in this study. The intervertebral disc cells are from fibrocartilaginous tissue (Yung Lee et al, 2001) or in the case of the foetal cells (Hayes, thesis, 1999), cells that will form fibrocartilage. Whilst tendon cells can form fibrocartilage *in vivo* in response to compression (Evanko and Vogel, 1990) and dermal fibroblasts in micromass culture can take a chondrogenic phenotype when treated with lactic acid (Nicoll et al, 2001), it appears that chicken flexor tendon fibroblasts do not respond in this way.

Whilst single growth factors had few effects on DNA content, combined treatments did demonstrate some significant outcomes. Early on, bFGF+TGF β enhanced DNA content, both growth factors are well known to promote cell proliferation individually (Shibley et al, 1995; Nilsen-Hamilton, 1990; Moses et al, 1994; Chan et al, 1997; Bikfalvi et al, 1998; Verrecchia and Mauviel, 2002), but here needed to be used together to generate significant effects. At 7 days all factor treatments were higher than the controls, and at 14 days TGF+PDGF was highest of all. All appeared to act synergistically as when treated individually there was little effect on proliferation (see above). The pronounced TGF β +PDGF effect at 14 days may reflect the stimulatory effects of both. The mitogenic effects of TGF β involve the activation of PDGF receptors (Ishikawa et al, 1990) and can be stimulated via an autocrine production of PDGF related peptides (Soma and Grotendorst, 1989; Battegay et al, 1990). Hence, these factors together will enhance cell proliferation. Cells also appear to change their responsiveness to the growth factor combinations with time in culture, this may reflect the differentiated state of cells. One day cultures have recently been formed from monolayer, where they were rapidly proliferating and therefore could have had different factor responsiveness to cultures in the 3-D for the long term. Older cultures (14 day) have a different environment, they have more deposited matrix in direct contact with cells and this may have altered their sensitivity to different growth factors.

In early cultures TGF β +PDGF appear marginally more effective at promoting collagen synthesis, bFGF+TGF β was the least effective. Notably, bFGF+TGF β was the most effective at increasing DNA content at this stage and the TGF β +PDGF contribution may be enhancing differentiated function, and the bFGF+TGF β a proliferative function. By 7 days the situation has changed and the serum treated cultures generally secrete more collagen, the growth factor treated ones are significantly lower than the 1% control culture. These growth factor cultures did have higher DNA, indicating their addition to the 1% serum-containing medium again favoured a proliferative phenotype. By 14 days, there are further changes, and the bFGF containing combinations remain low, whereas the 5% serum and TGF β +PDGF are elevated. Strikingly, the TGF β +PDGF are the only cultures that have an enhanced DNA content at this time, indicating that both proliferation and matrix production are enhanced.

No growth factor combination appeared to enhance matrix deposition in pellets, with the possible exception of bFGF+PDGF at 14 day, which does have a greater

proportion, of a fairly low collagen production, in its pellets. The TGF β containing cultures were poor at matrix incorporation, even in the presence of bFGF. TGF β has been shown to reduce MMP activity by increasing TIMPs and reducing MMP expression (Ma et al, 1999; Coletta et al, 1999; Hall et al, 2003; Nahm et al, 2004), and so might be expected to enhance incorporation into matrix; however, there is no evidence of these here, either singly or in combinations of growth factors. It has been shown that the combination of TGF β +PDGF has a negative effect on ligament healing *in vivo* (Hildebrand et al, 1998), and that PDGF can also increase MMP activity (Matrisian and Hogan, 1990; Laurent et al, 2003). Here we show good matrix synthesis, but poor matrix accumulation in association with these factors, suggesting that much of it is lost. If this happened *in vivo*, it might explain the poor healing response.

Another possible explanation for the differences observed within the cultures is that the cells are producing endogenous growth factors. These could either affect the exogenous growth factors applied to the culture system, or the exogenous growth factors could affect the cell metabolism and consequently the endogenous growth factor production (Banes et al, 1995; Tsuzaki et al, 2000; Dalhgren et al, 2005). PDGF can stimulate the synthesis of other growth factors (Lynch et al, 1989) and TGF β -1 can induce TGF β -2 and bFGF expression in the cornea (Song et al, 2002). Alternatively these factors can increase growth factor receptor expression, also affecting cell behaviour (Tsuzaki et al, 2000; Rubini et al, 1994; Ngo et al, 2001). Growth factors and their control in promoting matrix synthesis is such that the deposited matrix can sequester other endogenous growth factors. TGF β and matrix synthesis have been investigated with the ECM molecules especially affected including fibronectin, type I collagen, laminin and GAGs (Ignatz and Massague, 1986; Roberts et al, 1986; Verrecchia et al, 2004). TGF β acts to stimulate ECM accumulation, which in turn supports the actions of other growth factors, namely PDGF, bFGF, EGF (also known as α FGF) and TGF α , each of which directly stimulate cell proliferation (Nilsen-Hamilton, 1990). Each of these roles must be evaluated when using exogenous growth factors in culture systems. There is evidence that endogenous growth factors are present as the 1% serum treated growth factors show little difference in DNA content compared to 5% serum treated, or indeed other growth factor treated pellets, and continue to synthesise considerable amounts of collagen. In addition, growth factors are signalling systems used by these cells, for example in gap junctional communication and mechanical attachment. Therefore the addition of one or two different exogenous

factors will not necessarily be predictable given the complexity of the culture system.

In bFGF+TGF β treated pellets it is important to note that different concentrations of TGF β can have varying effects on bFGF (Pepper et al, 1993). It was discovered that low concentrations of TGF β enhance bFGF activity and high concentrations arrest it. Although this experiment was associated with the angiogenic roles of both growth factors, this combination at these concentrations may hinder effects to the cells within the pellet cultures. From this experiment, the growth factor concentration used in our study was towards the higher end of their scale, indicating a possible counteraction of effects. The effects of TGF β , however, on endothelial cells and fibroblasts are known to differ and even the effect of TGF β can vary dependant on the proliferation state of the cells or the surrounding ECM (Sutton et al, 1991). Therefore, it is only possible to speculate, that rather than acting together, the TGF β and bFGF treated fibroblasts may, in fact, cancel out some of the growth factors effects. In chondrogenic experiments, using bFGF and TGF β in media, while expanding the chondrocytes, displayed the low doubling times, suggesting their mitogenic effect is apparent in monolayer. When these cells were re-differentiated in pellet cultures this specific combination of growth factors expressed the largest mRNA amounts for type II collagen and aggrecan (Jacob et al, 2001). While this combination of growth factors may be counter productive in some cell types, in others it can encourage matrix synthesis.

In summary, single growth factor treatments showed no significant differences between the controls, and there is little difference between the 1% and 5% serum treated controls. Growth factor combinations do increase matrix synthesis significantly compared to single growth factor treatments, but the majority is still discharged into the media. It is impossible to say that the single growth factors encouraged cellular proliferation within the first week in culture as the controls followed the same pattern, and this is not observed in the controls or the growth factor combination treated pellets. The 5% serum treatments still appear the most beneficial treatment for pellets and collagen synthesis. This is likely to be caused from the mixture of growth factors present in the serum or endogenous growth factor production. Although in these experiments, the levels of protein secretion have been measured there are other ways these measurements could be confirmed and expanded upon. These methods include western blots and radio immuno assays. Both of these procedures make it possible to assess differences

in the types of collagen secreted. Quantitative PCR has been used in many experiments to quantify the mRNA, using this method together with the techniques described above could increase the accuracy of the collagen measurements. This could, in turn, provide increased significances between pellet treatments, allowing a greater understanding of the whole culture system involved in collagen synthesis and secretion from the pellets. These experiments have shown that treating tendon fibroblasts in a high cell density pellet culture with growth factors does aid the retention of collagenous matrices by the fibroblasts, but a significant level of collagen is released into the media. Consequently, the pellet, although easy to duplicate and manipulate and better than monolayers at promoting collagen deposition, does not possess certain factors required for efficient matrix deposition.

6.1 INTRODUCTION

The process of cell condensation is central to the early development of many connective tissues, including tendon and cartilage, whose development has already been discussed (see chapter 1). Cell-cell interactions become prominent in the condensation process, and in cartilage have been shown to be important in early differentiation (Zimmerman et al, 1982). The cadherins, notably n-cadherin, have been shown to be important in this process, along with NCAM and gap junctions comprised of connexin 43 (Dealy et al, 1994; Oberlender and Tuan, 1994; Hall and Miyake, 1995; Packer et al, 1997; Chimal-Monroly et al, 1999; Delise and Tuan, 2002; Yajima et al, 1999 and 2002). Equivalent studies have not been performed on tendon condensations, but n-cadherin and gap junctions are of particular interest. They are prominent in regions of cell-cell contact in adult tendons (McNeilly et al, 1996; Ralphs et al, 2002), and therefore might be expected to be present in developing tendon.

In this chapter, the importance of cell-cell interactions in establishing 3-D tissue structures is examined *in vitro*. These studies were performed firstly, to establish the extent, timing and factors controlling of cell aggregation in suspension cultures in the short term, and secondly to examine the role of cell-cell interaction and 3-D organisation in establishing large scale cultures, of potential value in tissue engineering approaches to tendon repair. The behaviour of tendon cells is examined in detail, and compared with chondrocytes. An important difference between the two tissues is, while they both start out as cell condensations, chondrocytes differentiate and lose contact with one another, whereas tendon cells retain extensive cell-cell interactions and form networks throughout the tissues (McNeilly et al, 1996).

In vitro, a variety of tissue culture systems have been used to culture fibroblasts and chondrocytes. These range from simple monolayers to complex bioreactors involving incorporation of cells onto scaffold structures and growing them in mechanically and/or chemically active environments (Jasmond and Bader, 2002; Sodian et al, 2002; Garvin et al, 2003; Radisic et al, 2003). Monolayers, although useful for expansion of cell number, were not considered suitable for studies of early cell-cell interaction. The purpose of these studies was to investigate the site of cell-cell interactions in cell aggregation and tissue differentiation. Cells in monolayers have a relatively large area of interaction with the culture substrate, which is a cell-matrix interaction (Freshney, 1983). A relatively small area of any

given cell is in contact with surrounding cells around the periphery. Similarly, the use of scaffold type structures for the incorporation of cells into 3-D arrays was not attempted, as the cells would be likely to have a greater interaction with the scaffold substrate than with one another. Accordingly, cells were grown in suspension culture, so that cells did not encounter a solid substrate, and could only interact with one another.

In the first part of the work described in this chapter, aggregation behaviour of tendon cells and chondrocytes is investigated in short term cultures, with particular reference to cadherin, gap junction and extracellular matrix expression. The second part of this chapter examines the feasibility of using suspension culture systems to generate large scale *in vitro* structures from isolated tendon cells. Many attempts have been made to create large scale connective tissue structures, potentially, for use in tissue engineering. Most of them require the incorporation of isolated cells into 3-D scaffold structures. These scaffolds can be made from a variety of materials, natural or synthetic, e.g. collagen, hyaluronan, polylactic acid, polyethylene glycol (reviewed in DeFranco et al, 2004).

Using cell seeded scaffolds in tissue repair (see chapter 1) can have advantages, for example the production of a construct with intrinsic mechanical strength that when implanted, enables the construct to perform its function. It is then hoped that the cells seeded within the scaffold will synthesise a matrix and take over the function of the original scaffold components (Yamamoto et al, 1993; Dunn et al, 1994). The disadvantages however, include the scaffold materials having adverse effects *in vivo*, in terms of breakdown products or triggering a host response. Using degradable scaffolds can cause problems if the rate of degradation is not compatible with matrix synthesis from the host, or if the load the construct is subjected to during integration is not suitable, too much or too little may cause a failure of the implant (Qin et al, 2004). Experiments into the suitability of the scaffolds in long-term implantation are few, scaffolds are often implanted for a few months and the results harvested. Longer experiments could evaluate the longevity and effects of the scaffolds *in vivo*.

The experiment described here represents an attempt to take advantage of connective tissue cells potential ability to create their own scaffold, their natural ECM. A system has been developed to allow large numbers of tendon cells to interact with one another in suspension culture to form orientated structures. The

basis of the design is that cells must interact with, and deposit matrix between one another without disturbance from regular medium changes. However, nutrition of large-scale cultures is potentially problematic, as diffusion distances are relatively small (typically 1mm). The system works by growing cells within a dialysis tube containing a small volume of medium that remains undisturbed, contained within a large volume of medium that can be regularly changed. Cell suspensions are placed in the inner tube, which is placed in an outer tube and cultured on a roller. These cultured cells show a degree of orientation and matrix deposition hitherto undescribed and could provide the basis of a system for forming fairly large-scale materials for tissue engineering graft production.

Tendon consists mainly of extracellular type I and type III collagens, and tendon cells are known to continue the synthesis of these *in vitro* (Herrmann et al, 1980). The synthesis of these ECM components by cells in suspension cultures will be investigated using immunolabelling techniques. As the behaviour of tendon cells will be compared with chondrocytes, type II collagen will also be examined. Type II collagen is the major component of cartilaginous matrix, but is absent from tendon ECM, except in regions of fibrocartilage (Benjamin and Ralphs, 2004). The synthesis of type II collagen decreases in chondrocytes in culture as they de-differentiate (Benya and Shaffer, 1982; Schnabel et al, 2002). The cytoskeleton is known to be involved in cell interactions and in the control of connective tissue phenotype (Benya et al, 1978; Grundmann et al, 1980). The distribution of cytoskeletal fibres and proteins associated with cell-cell adhesion and linkage of cytoskeletal networks will be investigated together with proteins involved in cell-cell adhesion joining the actin networks of adjacent cells, namely cadherins. Cadherins and vinculin are known to be present in adult tendon and regulated through mechanical loading (Ralphs et al, 2002). The orientation of the actin cytoskeleton has been implicated in the control the cell orientation and therefore the organisation of matrix deposition (Hayes et al, 1999). To ascertain whether this is the case for tendon fibroblasts within this culturing system, the presence of filamentous actin will be investigated.

In short term and long term cultures, features of tendon structure and composition that are important in final tissue structure and behaviour are mapped in the context of early cell aggregation and large scale tissue differentiation. The results are described in terms of remodelling of *in vitro* cell condensation and matrix deposition, in relation to possible tissue engineering applications.

6.2 MATERIALS AND METHODS

Two types of suspension culture system were used. First, cells were suspended and placed in 1.5ml cryotubes on a roller for experiments of duration 0-6 hours and secondly, cells were incorporated into a more complex large-scale system for experiments lasting up to 3 weeks.

Aggregation of tendon cells in short term suspension culture

For tissue source, cell isolation and maintenance see general material and methods (chapter 2). Tendon fibroblasts at P 3, were suspended at $20\text{-}30 \times 10^6$ cells/ml DMEM (5%FBS), placed into sterile screw-top 1.5ml cryotubes (Greiner Ltd.), sealed and rotated at 35 rpm at 37°C for 0 hours, 0.5, 1, 1.5, 2, 3, 4, 5 and 6 hours. At these times, 20 μl samples were taken and spotted directly onto a microscope slide, covered with a coverslip and examined live under phase contrast. 80 μl samples taken at the same time were fixed immediately in 500 μl 90% alcohol at 4°C , and aliquots dropped onto slides and allowed to dry at room temperature ready for immunolabelling (see general materials and methods, chapter 2).

To test the hypothesis that cadherins are important in cell aggregation, suspension cultures were treated with blockers of cadherin function. HAV peptide (amino acid sequence AHAVSE), which competes for binding on the extracellular loop of the cadherin molecule (Ilversaro et al, 1998), and control peptide sequence (VASHEA) were used at 20 $\mu\text{g}/\text{ml}$ and 200 $\mu\text{g}/\text{ml}$ (Alta Bioscience), respectively. Sterile PBS was used as a carrier control. 20 μl of peptide or PBS were added to a tendon fibroblast cell suspension at the beginning of the experiment and run as above. In addition, experiments were repeated using n-cadherin blocking antibody (Sigma, Clone CH-19) (Wanner and Wood, 2002) and anti-vinculin (Sigma, Clone-hVIN-1), both at 5 $\mu\text{g}/\text{ml}$. Vinculin acts as a control because it is a component of the intracellular complex formed at adherens junctions and focal contacts. Sterile PBS was used as a carrier control.

6.2.1 Long term, large scale cell suspension culture of tendon cells

To investigate cell aggregation in long term culture systems we developed a large scale disposable 'bioreactor' from parts readily available from tissue culture equipment and specialised dialysis tubing (Medicell Ltd. Int) (fig.6.1). Essentially, the culture system involves placing cell suspension of $20\text{-}30 \times 10^6$ cells into a

dialysis tube of volume 1ml. The dialysis tube is placed within a standard 50ml tissue culture centrifuge tube. Cultures can then be fed by changing the medium within the centrifuge tube, leaving the contents undisturbed. The cultures are incubated on a roller; the second lid at the lower part of the tube has its central part removed and is pushed over the tube so the whole thing runs evenly on the roller.

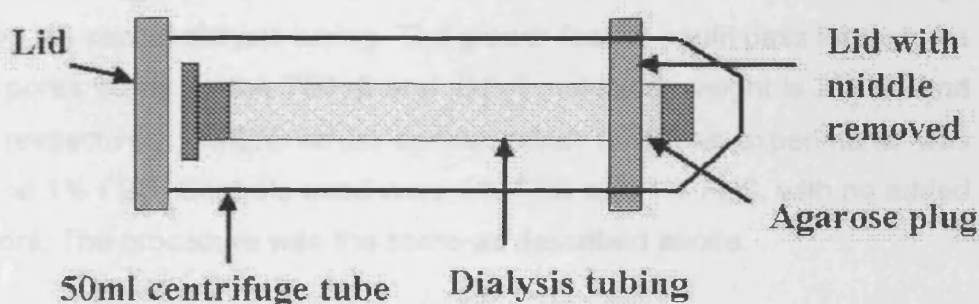


Fig 6.1 Schematic diagram of DispoDialyzer tube within centrifuge tube.

Description of culture system and procedure

Cells isolated above and in general methods were adjusted to $20\text{-}30 \times 10^6$ cells/ml, and 1ml of cell suspension was pipetted into each sterile Spectra/Por® CE (Cellulose Ester) Irradiated DispoDialyzer tube (Medicell Int.Ltd). The dialysis tube is 5cm in length with a 5mm diameter, a plastic base and a plastic screw-top lid assembly. Initially experiments were performed using tubes with 300KDa molecular weight cut off (MWCO) membranes. Unfortunately during the experiments SpectraPor ceased manufacturing these, and so they were replaced with 100KDa MWCO and 50KDa MWCO membranes. The key modification to the tubing was to place a small volume (200µl) of 2% agarose (Sigma) into the bottom of the tube and allowing it to set at 4°C before adding the cells. This method was used because in preliminary experiments cells tended to become trapped in the complex shape of the plastic moulding of the base; the agarose plug prevented this adhesion. After the addition of cell suspension, the dialysis tubes were sealed and placed in a standard 50ml polypropylene sterile centrifuge tube (Corning). The addition of 40ml DMEM (5% FBS and 1% antibiotics/antimitotic, 12.5mM HEPES buffer (Invitrogen/GIBCO) and ascorbate (1mg/ml) created the complete culturing system. Experimentation with chondrocytes required nutrition with DMEM/10%

FBS. The concentric tubes were incubated at 37°C and rotated in a rock and rolling motion at 35rpm. Media was changed every three days and experiments were terminated at 24 hours, 7, 14 and 21 days.

To investigate the differences between adult and embryonic cells within this system, embryonic cells isolated from E17 chick embryos (see general materials and methods, chapter 2) were used, the procedure was the same as above. To investigate the effect of growth factors in this system TGF β and IGF-1 were used at 10ng/ml and 50ng/ml, respectively added to the media in the 50ml centrifuge tube housing the sealed dialysis tubing. The growth factors could pass through the membrane pores easily as the TGF- β and IGF-1 molecular weight is 27KDa and 21.8 KDa, respectively. DMEM serum concentration for these experiments was maintained at 1% FBS. Controls used were 5% FBS and 1% FBS, with no added growth factors. The procedure was the same as described above.

Cell aggregates were separated from the dialysis tubing by cutting a section of tubing with scissors and removing the aggregate with tweezers. Cell aggregates were immediately fixed in 4°C and frozen onto a chuck using Cryo-m-bed ready for cryosectioning. Sections were taken at 8-10 μ m on histobond slides.

Immunohistochemical Analysis

Fixed cell aggregates and cell aggregations from short-term experiments were labelled for extracellular proteins (types I, II and III collagen, tenascin, some with the proteoglycan decorin), cytoskeletal components (direct labelling of actin with phalloidin and vimentin) and cell junctional associated proteins (pan-cadherin, n-cadherin, vinculin and the connexins 32 and 43). (See general materials and methods, chapter 2 (table 2.1)).

Biochemical Analysis

To measure the collagen amounts produced by the cell aggregates, media from the total experiment, media held within the dialysis tubes and collagen contained within the cell aggregates samples were prepared for the hydroxyproline assay (See biochemical analysis, hydroxyproline assay, materials and methods chapter 2). To measure the DNA content of cell aggregates, aggregates were digested in papain and the Hoechst assay was used to assess DNA quantities (See biochemical analysis, Hoechst assay, materials and methods chapter 2).

Due to the large number of cells used and the relatively lengthy incubation periods of these cultures, it was not always possible to collect complete data for each aggregate. In early experiments the cell aggregates were required for immunolabelling and therefore, accurately replication for quantitative analysis was difficult.

6.3 RESULTS

6.3.1 Initial cell aggregations in short term suspension culture

Live cells examined under phase contrast showed that cell suspension of both tendon cells and chondrocytes formed aggregates within the first hour (fig. 6.2 and fig. 6.3). Aggregates enlarged presumably recruiting new cells from the cell suspension (fig. 6.2 and 6.3). After 4 hours most cells are incorporated into cell aggregates, although some individual cells remain (fig 6.2 G, H, I).

Tendon fibroblasts treated with HAV peptides or n-cadherin blocking antibody, and corresponding controls showed no change in aggregation. Cell aggregates were seen within the first hour of the cell suspension and cultures progressed as described above (fig 6.4). Neither concentration of HAV peptide (1 and 10 mg/ml) appeared to hinder cell aggregation.

Immunolabelling of collagens and cell adhesion associated proteins

Fibroblasts

Collagens

Positive label for type I collagen was present at all time intervals (fig. 6.5). Initially it appeared intracellular but in 3hr cultures it appeared that some aggregates had extracellular label (fig. 6.5 F). The type I collagen label appears to increase in intensity after 1 hour in culture (fig 6.5 D). Type III collagen label was sparse and not associated with all cells, it appeared to be principally intracellular (fig. 6.7). There was no type II collagen at any stage (fig.6.6), it was used as a negative control but also indicated no fibroblasts can develop a chondrogenic phenotype.

Cytoskeletal

Filamentous actin is positive in all cells, as shown by a faint green hazy label (fig. 6.8). However, there are no large scale actin assemblies such as actin stress fibres. Vimentin label showed positive at all stages (fig 6.9).

Cell junctional proteins

Label for cadherins was present in all cells at all time points, generally located at the cell periphery (fig. 6.10 and fig. 6.11). The Pan-cadherin and n-cadherin antibodies showed similar distribution suggesting the predominant cadherin to be n-cadherin. Vinculin label was similarly distributed (fig.6.12). Label for connexin 32 was also present throughout the experiment. In some areas it appeared intracellular in a dense, bright regions, possibly the Golgi apparatus. In others it

was at the cell surface and in aggregates gave the appearance of a regular network where many cells were pressed together (fig. 6.13). Connexin 43 label was much less prominent being sparse, with the odd bright spot of labeling occurring between cells (fig. 6.14).

Chondrocytes

Collagens

For comparative purposes immunolabelling of chondrocytes showed some differences compared to the fibroblasts. Label for type I collagen was present, but weak compared to tendon cells (fig 6.15 A). Type II collagen was present up to and including 3 hours in culture (fig. 6.15 B). Type III collagen label was present, but weak at the 0 hour time interval. No other type III collagen label was present for the remainder of the experiment (fig. 6.15 C).

Cytoskeleton

Actin labeling was positive at all time intervals, the filamentous nature seen in cells incorporated into aggregates (fig. 6.15 D). Vimentin label was negative in isolated cells and cell clusters (fig. 6.15 E). This negative result should be thoroughly investigated for confirmation; it is possibly due to poor immunolabelling.

Cell junctional proteins

Vinculin label is positive at all stages, showing labelling concentrated at the cell periphery (fig. 6.15 F). Connexin 32 label is abundant throughout the experiment until the 6 hour time point, where it is diminished. There is no conclusive connexin 43 label present at any time point (fig. 6.15 G). At this time no cadherin antibodies were available for labelling.

6.3.2 Long term, large scale suspension cultures: Adult cells

Cells within this large scale system aggregated within 24 hours in medium containing 5%FBS. They formed structures that were clearly visible to the naked eye, and which became larger, increased in strength and became easy to handle over the following 7-21 days of culture.

The older (7-21 days) structures took a variety of forms (fig 6.16). In most cultures there were elongated, thin, rod shaped aggregates, although funnel shaped sheets or a solid sphere of cells were sometimes formed. All, or just one of these types could occur within the culture. The elongated structures could reach large sizes,

some were over 3cm in length (fig 6.16 A). These structures formed in all of the dialysis tubes tested with 300kDa, 100kDa or 50kDa MWCO membranes. Cultures grown in DMEM/1% FBS, formed no aggregates. Loose cellular material was visible in the tube but with no structure. When chondrocytes were substituted for tendon cells and maintained with DMEM/10% FBS similarly, no structures were formed, even up to 28 days of culture fine, particulate material was present but no comparable structures to the fibroblast aggregates were made.

Immunolabelling of ECM and cellular components

Cell distribution within aggregates could be observed in all of the immunolabelled material because propidium iodide was used to counterstain the nuclei. In 24 hour and 7 day aggregates nuclei appeared evenly distributed (fig 6.18 A, B) although in some central regions of larger aggregates at 14 days onwards there appeared to be a lower cell density. These regions tend to crack and break up during cryosectioning, suggesting different material properties compared to the outer parts. However, in thinner aggregates the cell densities appeared consistent. Twenty one day aggregates were similar in structure and labelling characteristic to 14 day cultures.

Nuclear distribution showed a degree of orientation in the thinner rod-shaped or sheet and funnel shaped aggregates. Cells here were arranged in parallel rows separated by ECM, reminiscent of the *in vivo* organisation of tendon cells. This was particularly clear at 14days onwards (fig 6.18 C), but was seen in regions of 7 day cultures. There were no effects of the different MWCO pore sizes of dialysis tube membranes.

Extracellular matrix

Types I and III collagen were detected in cell cultures at all stages, and type II collagen was absent (figs 6.19, 6.20, 6.21). Type I collagen was observed as extracellular, with label being strongest in 14 day cultures (fig 6.19 C). There was no obvious orientation to type I collagen label although in 7 day (fig 6.19 B) and older cultures it was positioned between orientated rows of tendon cells in rod-like and thin sheet cell aggregates. In the more rounded cell aggregates areas of cell and matrix orientation were restricted to the periphery of the aggregate. There were no visible differences in cultures grown in 300kDa, 100kDa or 50kDa MWCO tubes.

Compared to the type I collagen, the type III collagen labelled very brightly and formed long, relatively widely spaced interwoven fibres after 24 hours in culture (fig 6.21 A). In 7 day aggregates type III collagen labelled strongly, with an interwoven pattern as above. There was a zone of cells, 3-5 cell layers, at the periphery of the aggregate that did not label (fig 6.21 B). Orientated type III collagen was present in 14 day aggregates (fig 6.21 C) with bright longitudinal fibres positioned between orientated nuclei. This pattern of type III collagen is consistent in cell aggregates formed in with DispoDialyzer tubes of 50 KDa, 100 KDa and 300KDa MWCO (fig 6.28). Quantitative data for collagen produced by these cultures is presented in the final part of these results.

The presence of tenascin was examined because of its role in tendon development and its known adhesive qualities (Hurle et al, 1990; Sriramarao et al, 1993). Tenascin was evident in 14 day cell aggregates (fig 6.29 A) with an extracellular distribution of longitudinal fibres.

Cytoskeletal labelling

There were no prominent actin fibres in 24 and 7 day cultures. The characteristic green haze was visible in the cells, indicating the presence of actin filaments (fig 6.22 A, B) but clearly there were no larger scale structures. Obvious filamentous actin was only identified in elongated cell aggregates at 14 days (fig 6.22 C), where cells and matrix had the longitudinal tendon-like organisation indicated above. Vimentin was present in cell aggregates particularly at the periphery of the aggregate (fig 6.29 C) and showed a degree of longitudinal orientation in longitudinal aggregate structures.

Cell junction labelling

N-cadherin and pan-cadherin were present in cell aggregates cultured for 7 to 14 days (fig 6.30). Both cadherin antibodies labelled predominantly at the periphery of the cells, outlining their membranes, but with no clear focal spots. At 7 days in cultures pan-cadherin and n-cadherin distribution was similar (fig 6.30 A, B), whereas at 14 days pan-cadherin labelled more strongly (fig 6.30 C), suggesting expression of additional cadherins. Vinculin was present throughout the cell aggregates, at 24 hours an intense vinculin distribution was identified in cells at the periphery (fig 6.23 A). The positive labelling is indifferent as to whether the cell aggregates are formed in 50, 100 or 300KDa MWCO tubes.

Connexin 32 was present in all cultures at all times (fig 6.24 A, B, C). There was an increase in labelling intensity at the aggregate periphery in some areas (fig 6.24 A). Highly aligned cells within a 7 day cultured aggregate showed thin streaks of connexin 32 (fig 6.24 B), the 14 day culture had a less organised structure but connexin 32 remained evident (fig 6.24 C). Positive labelling for connexin 43 showed small and occasional foci of labelling throughout the aggregates, but did appear to increase after 14 days in culture (fig. 6.25 C). Both connexin patterns are consistent with aggregates from 50, 100 or 300KDa MWCO Disposable tubes.

6.3.3 Long term, large scale suspension cultures: Embryonic cells

To examine whether embryonic tendon fibroblasts behave differently to the adult cells described above, fibroblasts from 17day chick embryos were cultured for 14 days. Essentially labelling patterns for ECM components and cell junctional components were the same as adult cells.

Immunolabelling of ECM and cellular components

Cell aggregates formed from embryonic fibroblasts showed no differences in labelling patterns compared to the adult tendon fibroblasts. Types I and III collagen and tenascin were present extracellularly, and type II collagen was absent (fig 6.26). An antibody for decorin was available at the time of this study, therefore decorin was immunolabelled for because of its involvement in collagen fibrillogenesis in development. Decorin was restricted to the peripheral zone of the aggregates (fig 6.26 E) with small and infrequent areas of label visible centrally.

Cell junction labelling

Pan and n-cadherin showed similar patterns of labelling (fig 6.27 C, D) at the cell periphery, but showed more punctate arrangement than cultures derived from adult cells. Connexin 32 and connexin 43 were identified in the cell aggregates (fig 6.27 E) and the connexin 43 spotted labelling was difficult to identify (fig 6.27).

6.3.4 Long term, large scale suspension cultures: Adult cells treated with growth factors

To examine the effects of TGF β and IGF-1 in large scale suspension cultures, cultures were maintained in DMEM/1%FBS, which as indicated above does not support cell/matrix aggregate formation in the presence or absence of the growth factors. Both growth factor treatments promoted the formation of aggregates,

which showed the same range of morphology as cultures described above (fig 6.33 and 6.34).

Biochemical analysis

The hydroxyproline assay was used to quantify the amount of collagen produced by the cells cultured in suspension cultures. Collagen content was measured in three separate components of the cultures: growth media from the outer centrifuge tube, media from within the dialysis tubing at time of termination and collagen retained by the cells within the formed aggregates. Since most cell aggregates were required for immunohistochemistry these numbers are low. No n values are more than 3 and consequently, no statistical analysis was possible. The data is presented below, however, it must be stated that this has no statistical validity. Some interesting observations can nevertheless, be made.

A full set of data was obtained from 3 replicates of cultures grown with 100kDa MWCO tubes, cultured for 7 days. The total amount of collagen made by each culture was on average 304ug collagen. This was divided, with 69% in the cell aggregate, 7% in the dialysis tube and 25% released from the dialysis tube to the growth medium. By comparison, 50kDa MWCO (albeit n=1) produced considerably more collagen in total 498 ug, but there appeared to be a similar proportion of collagen in the cell aggregates (78%, 4%, 18% respectively), although clearly more replicates would have to be performed to demonstrate this. DNA values analysed with the Hoechst assay (described in chapter 2, general material and methods) indicate there was possibly increased cell death in aggregates cultured in tubes with the smallest pore size (50kDa). This may be due to an increase in blockage of these pores by collagen fragments or other waste products, and a resulting decrease in nutrient availability into the dialysis tube.

By comparison cultured cell aggregate data for the 300kDa MWCO DispoDialyzer tubes was unavailable because all were used for immunolabelling studies, and the manufacturers ceased production of this size before further cultures could be grown. The only comparison that can be made therefore is between the amount of collagen in the dialysis tube media and main tube growth media, without DNA normalisation. These results show that much more collagen appears in the main tube than with the other dialysis tube MWCO. This suggests that the larger pore size enables collagen fragments produced by the cells to pass freely through the membrane. A similar pattern is evident in dialysis tubes where the pore sizes are

smaller, 50-100kDa MWCO. It is important to note that the total collagen released into the media from these experiments is significantly lower than in 300kDa MWCO tubes. This result would have to be thoroughly investigated. Experiments with the smaller pore sized membranes show a decrease in collagen released into the media after 14 days. This is possibly due to a build up of matrix metalloproteases, discussed later.

The effects of growth factors added to cells culturing within these suspension cultures showed aggregates forming with reduced serum levels (1%FBS/DMEM). Growth factor experiments used dialysis tubes with 300kDa MWCO, as with the above quantification analysis the aggregates from these cultures were used to obtain qualitative data. Collagen amounts released into the media showed similar levels of collagen compared to the aggregates treated with 5% FBS/DMEM. Cells with added IGF-I showed increased levels of collagen when cultured for 7 days compared to those treated with TGF- β . After 14 days in culture total collagen released into the media had increased in both growth factor treated cultures, raising contradictory indications to the 14 days 50kDa and 100kDa MWCO cultures.

Figure 6.2. Cell-cell interactions in short term suspension cultures: tendon cells

Phase contrast images, x40 objective lens, Scale bars= 50 μm

A- 0 hour time point. Freshly isolated cells with no dense aggregates.

B- 0.5hour time point. Similar to A.

C- 1hour time point. Cell aggregations can be observed.

D- 1.5hour time point. Larger cell aggregates are consistently present.

E-I- 2- 6 hour time points. Cell aggregates present at all time points; cells tend to enlarge with time, although individual cells are always present.

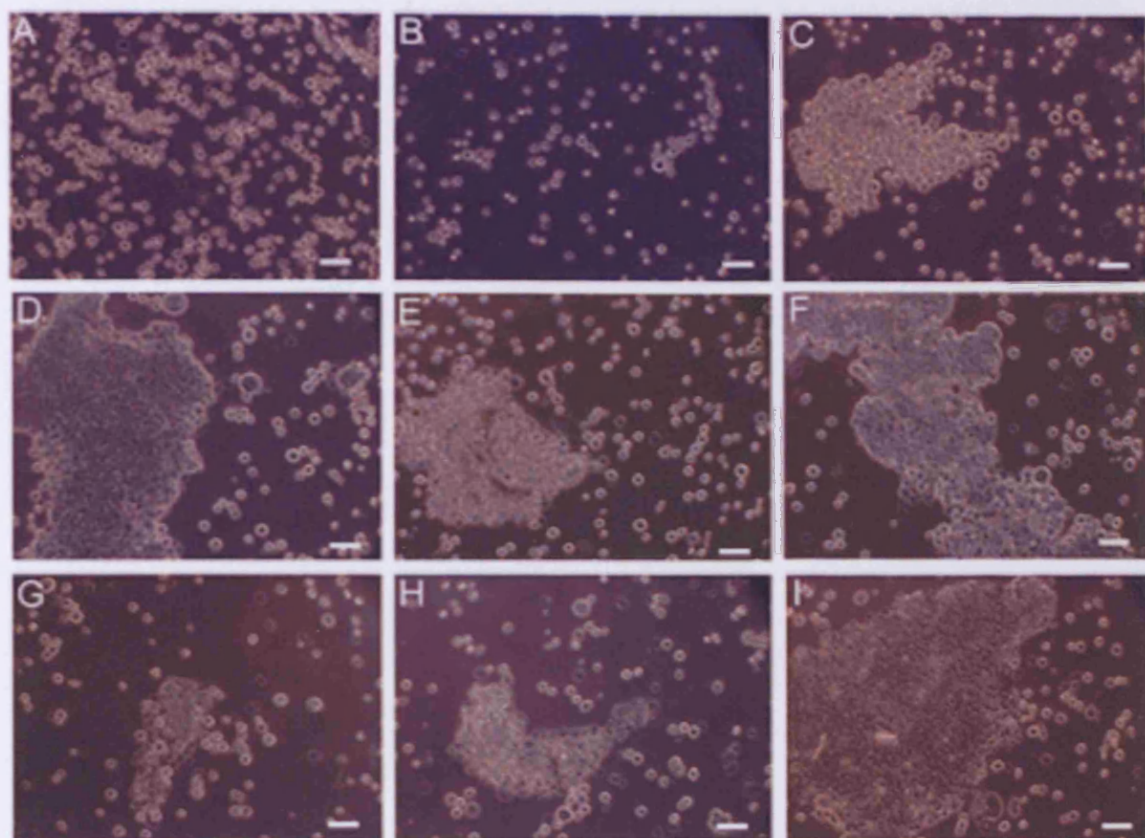


Figure 6.3 Cell-cell interactions in short term culture; chondrocytes

Phase contrast images, x40 objective lens, Scale bars= 50 μ m

A- 0 hour time point. Cells are isolated within the cell suspension.

B- 0.5hour time point. Cells begin to adhere to one another.

C- 1hour time point. Cells form loose aggregates.

D- 2hour time point. Aggregates formed are increasingly tightly packed.

E-H- 3-6 hour time point. Cell aggregates increase in size, there are a few isolated cells left in suspension.

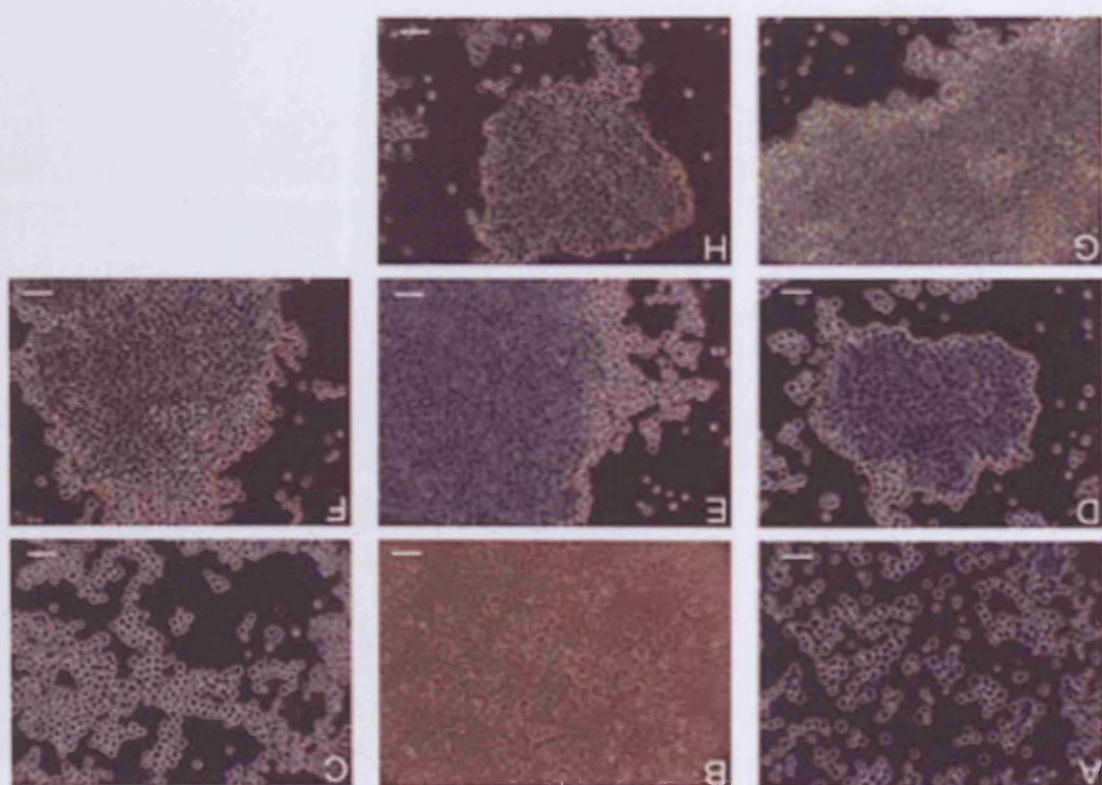


Figure 6.4 Cell-cell interactions in short term cultures; fibroblasts treated with cadherin function blockers.

Phase contrast images, x40 objective lens, Scale bars= 50 μ m

A- Fibroblasts treated with HAV peptide, 0 hour time point

Cells are isolated in suspension.

B- Fibroblasts treated with HAV peptide, 1 hour time point

Cells are beginning to aggregate

C- Fibroblasts treated with HAV peptide, 4 hour time point

Tight cell aggregates form and fewer cells are in suspension.

D- Fibroblasts treated with HAV (10xconc), 0 hour time point

Cells accumulate loosely.

E- Fibroblasts treated with HAV (10xconc), 1 hour time point

Large cell aggregates are identified.

F- Fibroblasts treated with HAV (10xconc), 4 hour time point

Large amounts of cells are still isolated in the cell suspension.

G- Fibroblasts treated with N-cadherin blocking antibody, 0 hour time point. Cells are isolated in the cell suspension.

H- Fibroblasts treated with N-cadherin blocking antibody, 1 hour time point. Aggregates forming show isolated cells attaching at the periphery.

I- Fibroblasts treated with N-cadherin blocking antibody, 4 hour time point. Isolated cells are present in the cell suspension.

FIG. 10. Same as in Fig. 9, but for the 1000-hPa level.

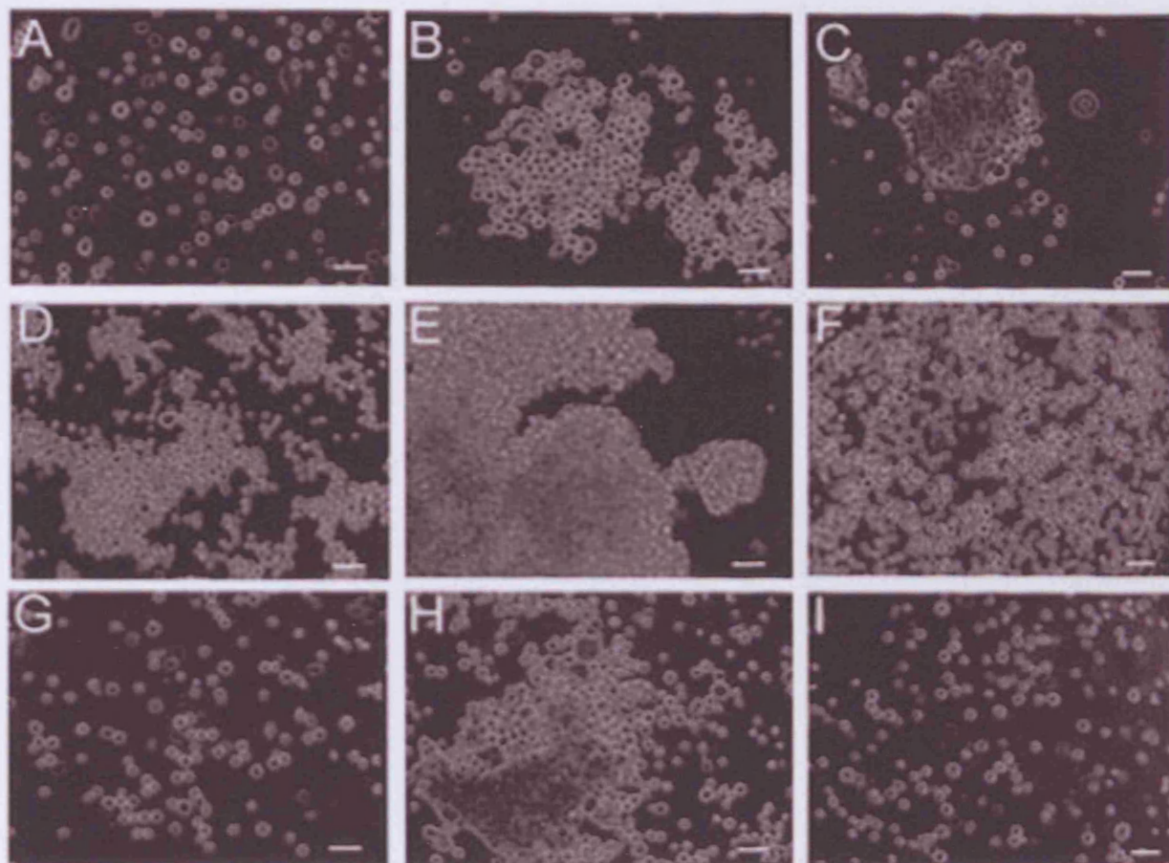


Figure 6.5 Tendon fibroblasts in a 3-D suspension system

Scale bars at 50 μm .

Type I collagen

A- 0 hour time interval. Type I collagen label is positive intracellularly and in varying amounts in individual cells.

B- 0.5 hour time interval. There is no visible change between 0 and 0.5 hours of incubation.

C- 1 hour time interval. The type I collagen label remains present, though its expression is still intermittent.

D- 1.5 hour time interval. The positive label appears to have increased and most cells label positive for type I collagen.

E- 2 hour time interval. There is little difference between 1.5 and 2 hours of incubation.

F- 3 hour time interval. This cluster of cells shows extracellular type I collagen label.

G- 4 hour time interval. At this time interval the cells all label positive for type I collagen.

H- 5 hour time interval. There is little change between 4 and 5 hours of incubation.

I- 6 hour time interval. After 6 hours there may be a small decrease in type I collagen label, but it is still positive in most cells.

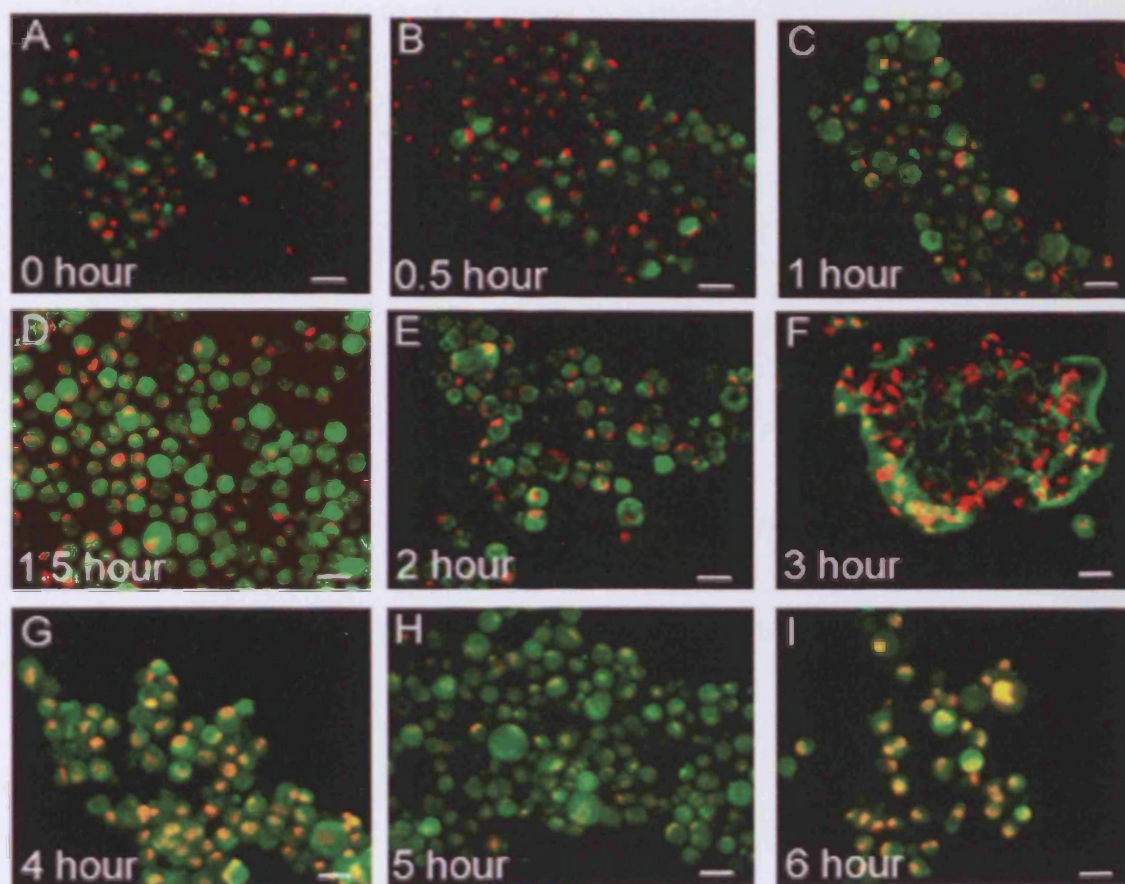


Figure 6.6 Tendon fibroblasts in a 3-D suspension system

Scale bars at 50 μm

Type II collagen

Type II collagen label is negative at all time intervals.

A- 0 hour time interval.

B- 0.5 hour time interval

C- 1 hour time interval

D- 1.5 hour time interval

E- 2 hour time interval

F- 3 hour time interval

G- 4 hour time interval

H- 5 hour time interval

I- 6 hour time interval

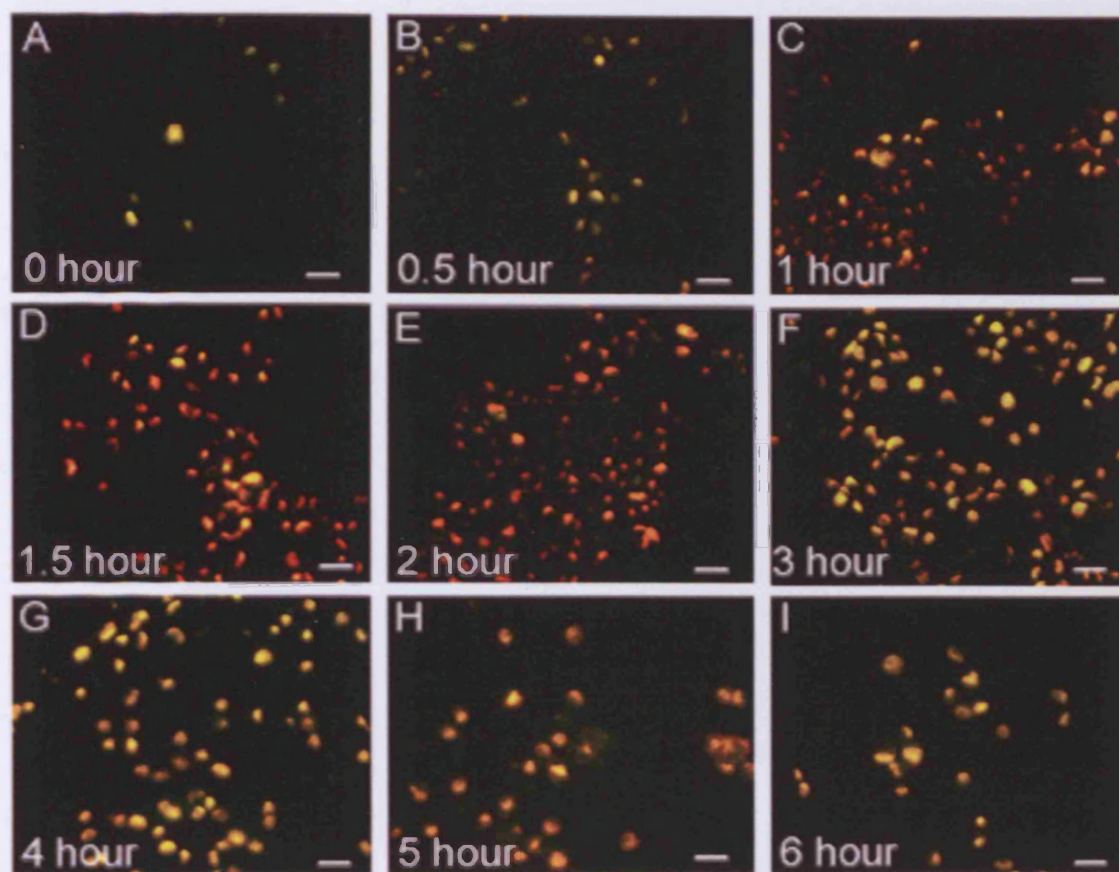


Figure 6.7 Tendon fibroblasts in a 3-D suspension system

Scale bars at 50 μm

Type III collagen

A- 0 hour time interval. Type III collagen label is positive intracellularly on intermittent cells.

B- 0.5 hour time interval. There is little difference between 0 and 0.5 hours of incubation.

C- 1 hour time interval. Type III collagen still displays a sporadic pattern of labelling.

D- 1.5 hour time interval. There is neither an increase nor a decrease in labelling.

E- 2 hour time interval. Type III collagen label remains intracellular.

F- 3 hour time interval. Individual cells still label positive for type III collagen.

G- 4 hour time interval. There is no change in type III collagen label between 4-6 hours of incubation time.

H- 5 hour time interval

I- 6 hour time interval

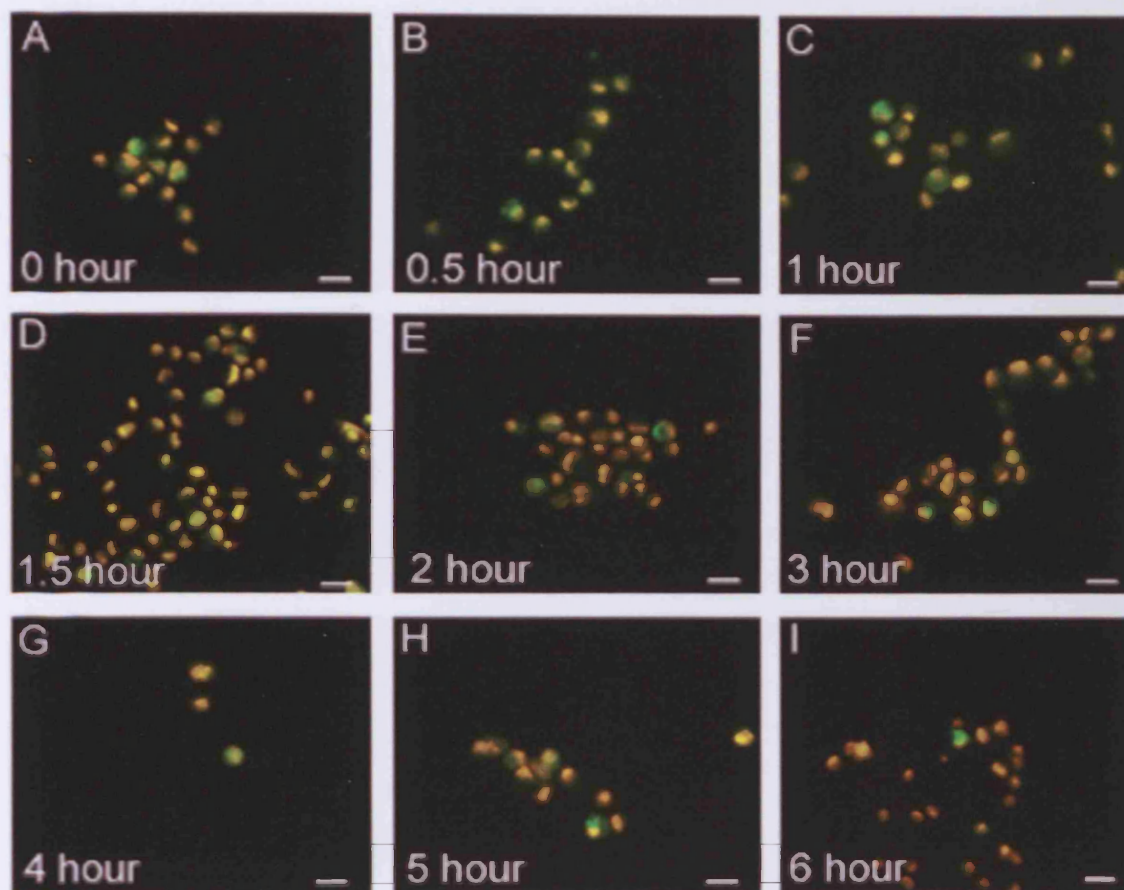


Figure 6.8 Tendon fibroblasts in a 3-D suspension system

Scale bars at 50 μm

Actin

A- 0 hour time interval. Actin label appears as a hazy green label covering the cytoplasm of each cell.

B- 0.5 hour time interval. There is no difference at any time interval and no actin stress fibres can be observed.

C- 1 hour time interval

D- 1.5 hour time interval

E- 2 hour time interval

F- 3 hour time interval

G- 4 hour time interval

H- 5 hour time interval

I- 6 hour time interval

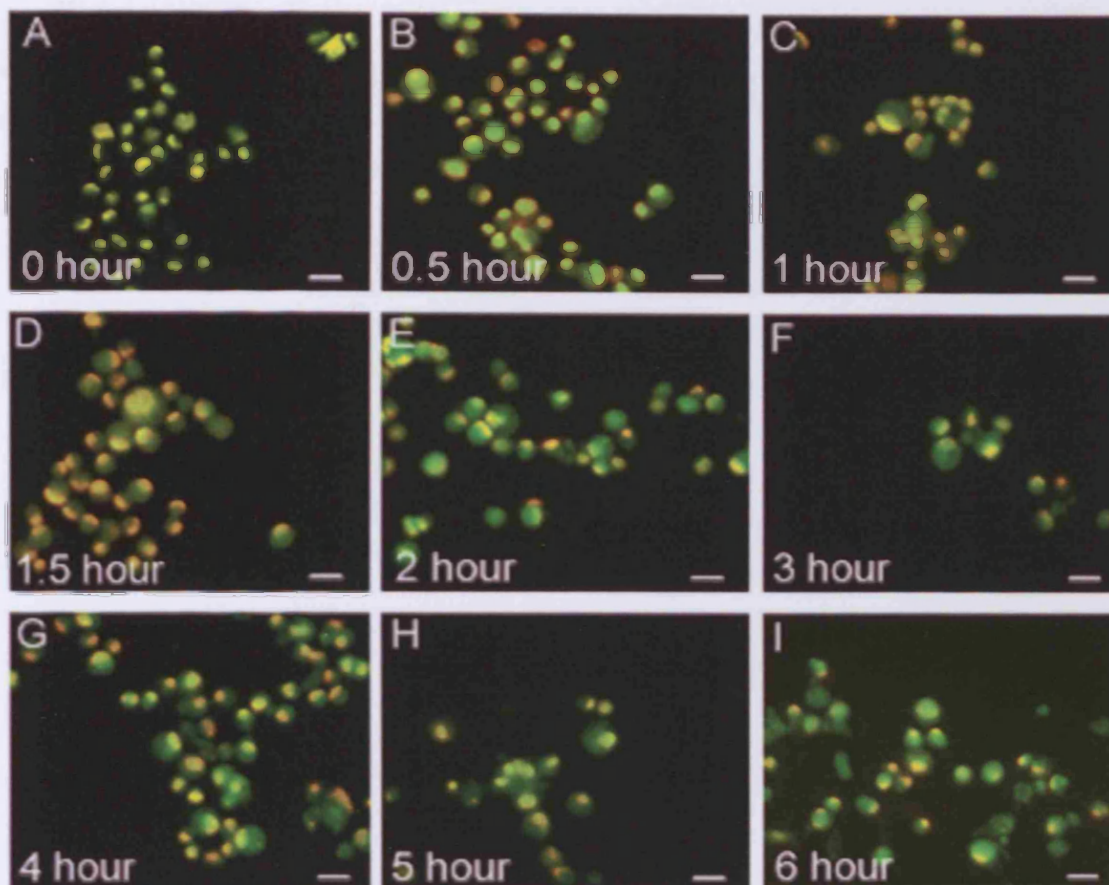


Figure 6.9 Tendon fibroblasts in a 3-D suspension system

Scale bars at 50 μm

Vimentin

A- 0 hour time interval. Labelling for vimentin closely follows labelling patterns for vinculin. With certain cells showing dominant label and labelling preference to the cell periphery.

B- 0.5 hour time interval

C- 1 hour time interval

D- 1.5 hour time interval

E- 2 hour time interval

F- 3 hour time interval

G- 4 hour time interval

H- 5 hour time interval

I- 6 hour time interval

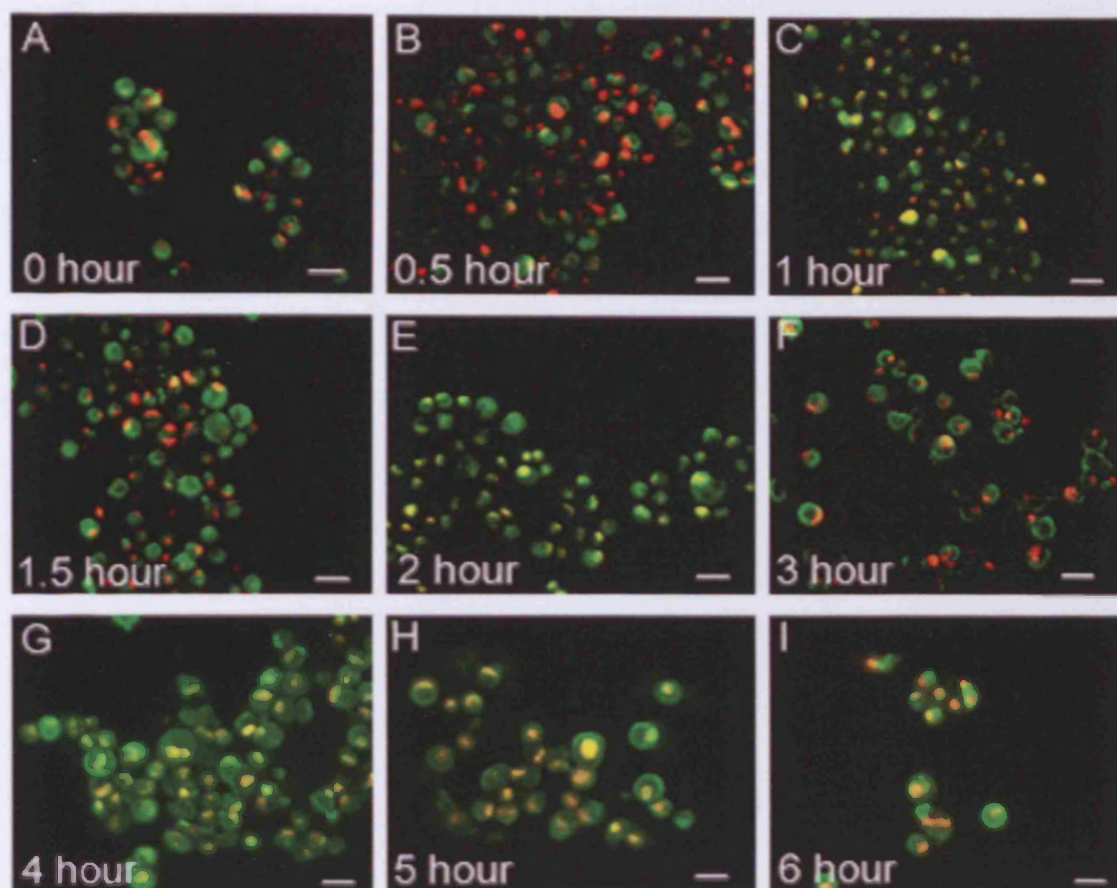


Figure 6.10 Tendon fibroblasts in a 3-D suspension system

Scale bars at 50 μm

Pan-cadherin

A- 0 hour time interval. Positive labelling for pan-cadherin shows a similar pattern to n-cadherin, however pan-cadherin appears to be more abundant.

B- 0.5 hour time interval. Most cells label positive for pan-cadherin.

C- 1 hour time interval. There is little change from 1-6 hours incubation.

D- 1.5 hour time interval

E- 2 hour time interval

F- 3 hour time interval

G- 4 hour time interval

H- 5 hour time interval

I- 6 hour time interval

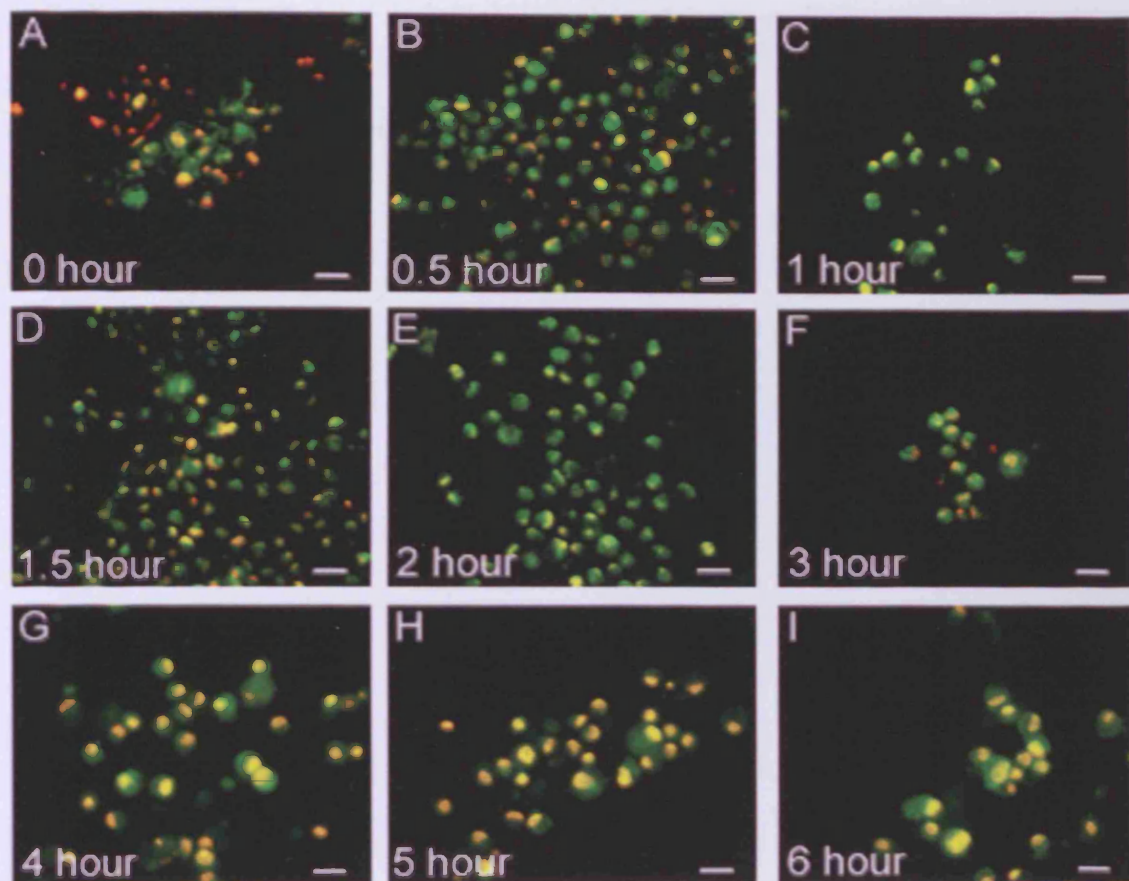


Figure 6.11 Tendon fibroblasts in a 3-D suspension system

Scale bars at 50 μm

n-cadherin

A- 0 hour time interval. Few cells label positive for n-cadherin, the positively labelled cells show label is concentrated at the cell edge.

B- 0.5 hour time interval. More cells label positive for n-cadherin.

C- 1 hour time interval. Small cell samples show labelling remains positive from 1-4 hours.

D- 1.5 hour time interval

E- 2 hour time interval

F- 3 hour time interval

G- 4 hour time interval. Positive label is once again strong at the cell borders.

H- 5 hour time interval. There is little difference in label between 4-6 hours incubation.

I- 6 hour time interval

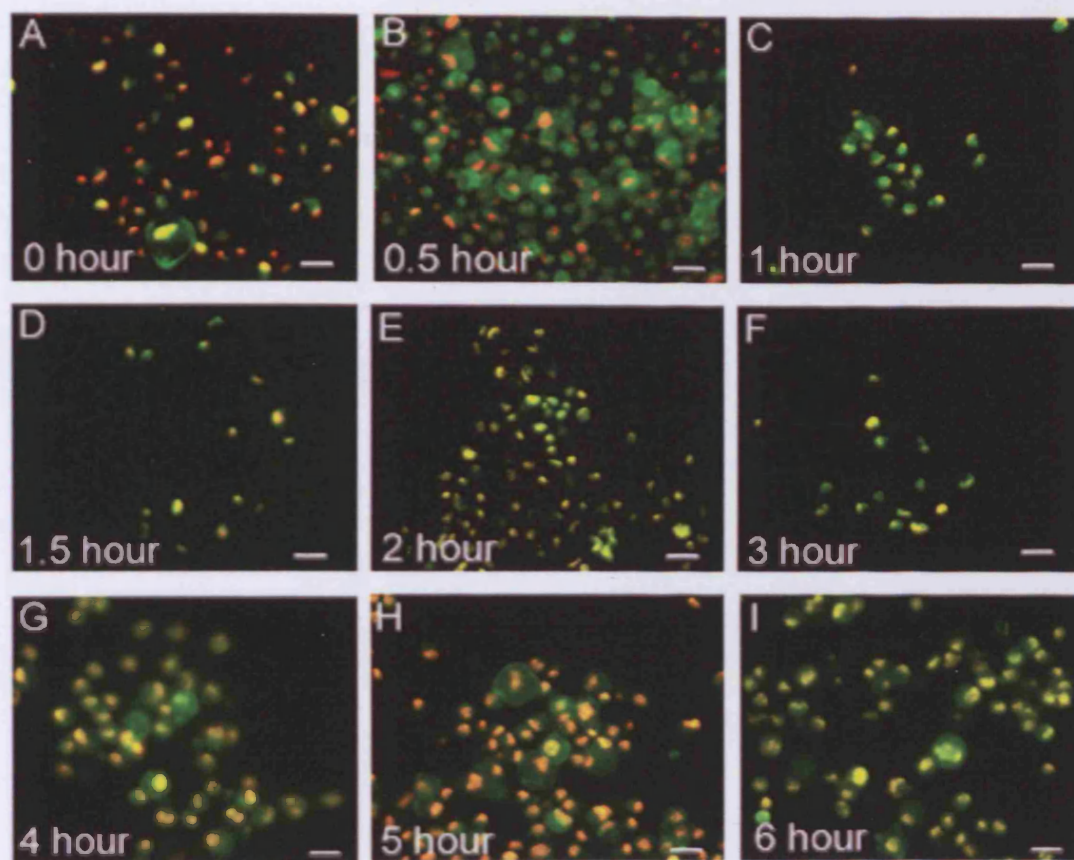


Figure 6.12 Tendon fibroblasts in a 3-D suspension system

Scale bars at 50 μm

Vinculin

A- 0 hour time interval. Vinculin label is positive at 0 hours incubation.

B- 0.5 hour time interval. Small clusters of vinculin can be observed and a preference to the periphery of the cell can be seen.

C- 1 hour time interval. Most cells label positive for vinculin.

D- 1.5 hour time interval. All cells label positive for vinculin.

E- 2 hour time interval. In cell clusters some cells show a dominant label for vinculin.

F- 3 hour time interval. There is little difference between 2 and 3 hours of incubation.

G- 4 hour time interval. Cells continue to label positive for vinculin.

H- 5 hour time interval. The concentration of vinculin label to the periphery of the cells can be seen.

I- 6 hour time interval. After 6 hours of incubation the cells are still labelling positive for vinculin.

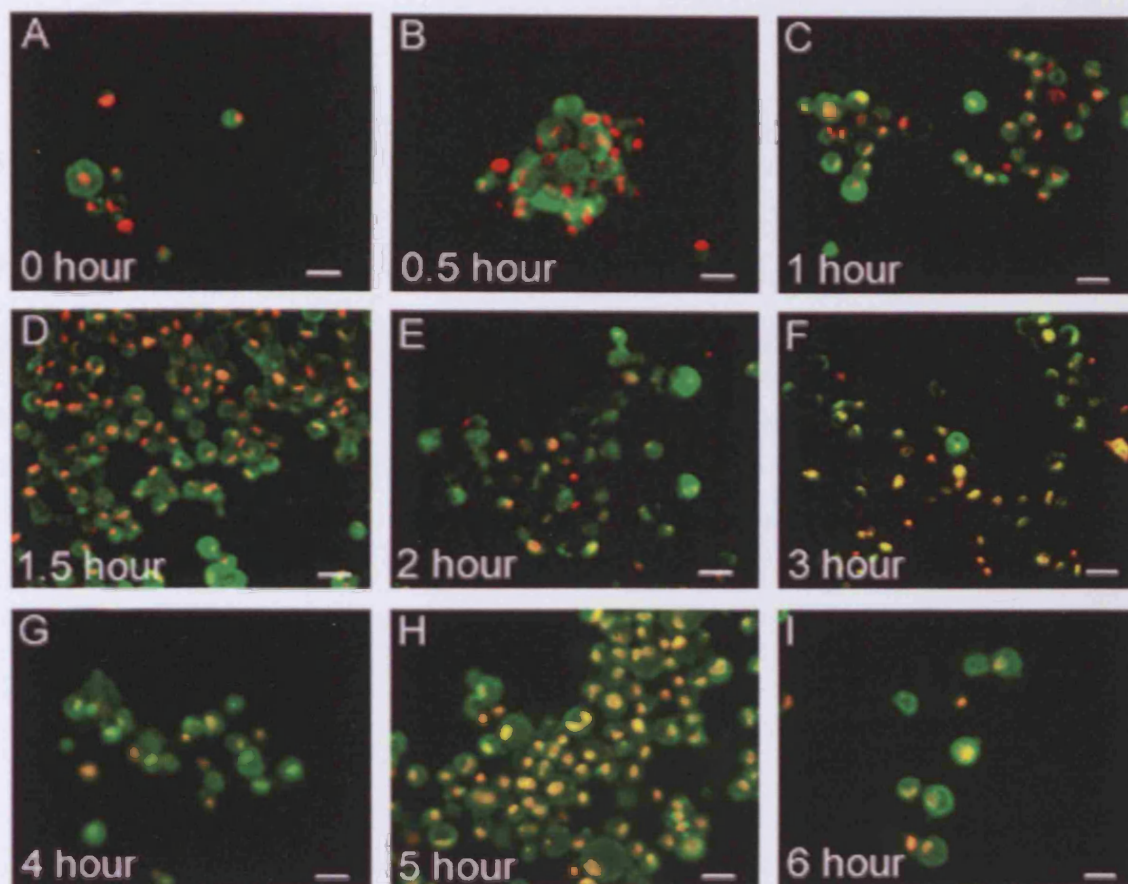


Figure 6.13 Tendon fibroblasts in a 3-D suspension system

Scale bars at 50 μm

Connexin 32

A- 0 hour time interval. Connexin 32 label is present from 0 hours.

B- 0.5 hour time interval. After 0.5 hours of incubation the label is clear and expressed by most of the cellular population.

C- 1 hour time interval. Some cells appear to show an increased label for connexin 32.

D- 1.5 hour time interval. Connexin 32 label is widely observed and often appears to show cell polarity, where the label is concentrated on one side of the cell at the periphery.

E- 2 hour time interval. Cells continue to label positive for connexin 32.

F- 3 hour time interval. A small cell sample at this stage revealed cells labelled positive for connexin 32.

G- 4 hour time interval. After 4 hours of incubation there was no difference in the labelling pattern for connexin 32.

H- 5 hour time interval. It appears that after 5 hours of incubation not all cells label positive for connexin32.

I- 6 hour time interval. After 6 hours these cells label positive for connexin 32, however some show a more dominant label.

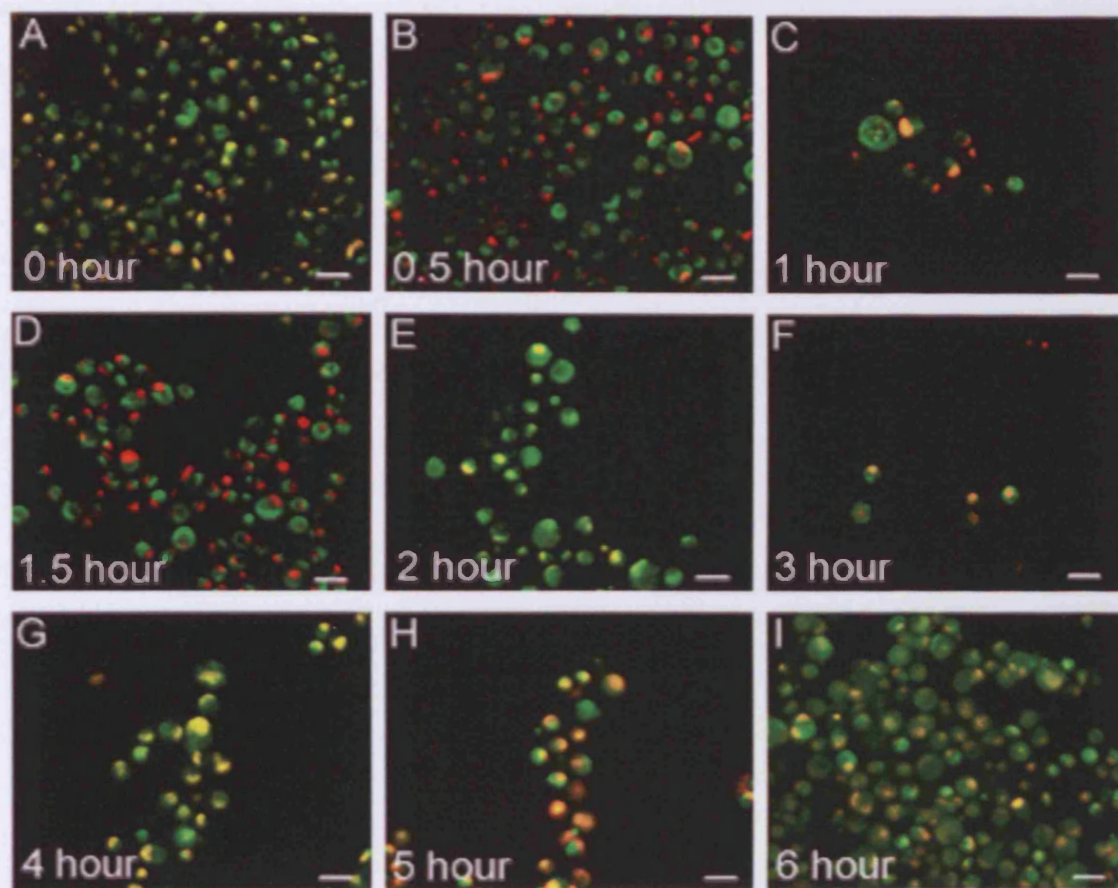


Figure 6.14 Tendon fibroblasts in a 3-D suspension system

Scale bars at 50 μm

Connexin 43

A- 0 hour time interval. There are individual cells that label positive for connexin 43.

B- 0.5 hour time interval. The spotted appearance of this label can be hard to identify. Few cells are positive for connexin 43 label throughout this experiment.

C- 1 hour time interval

D- 1.5 hour time interval

E- 2 hour time interval

F- 3 hour time interval

G- 4 hour time interval. Connexin 43 label is negative.

H- 5 hour time interval

I- 6 hour time interval

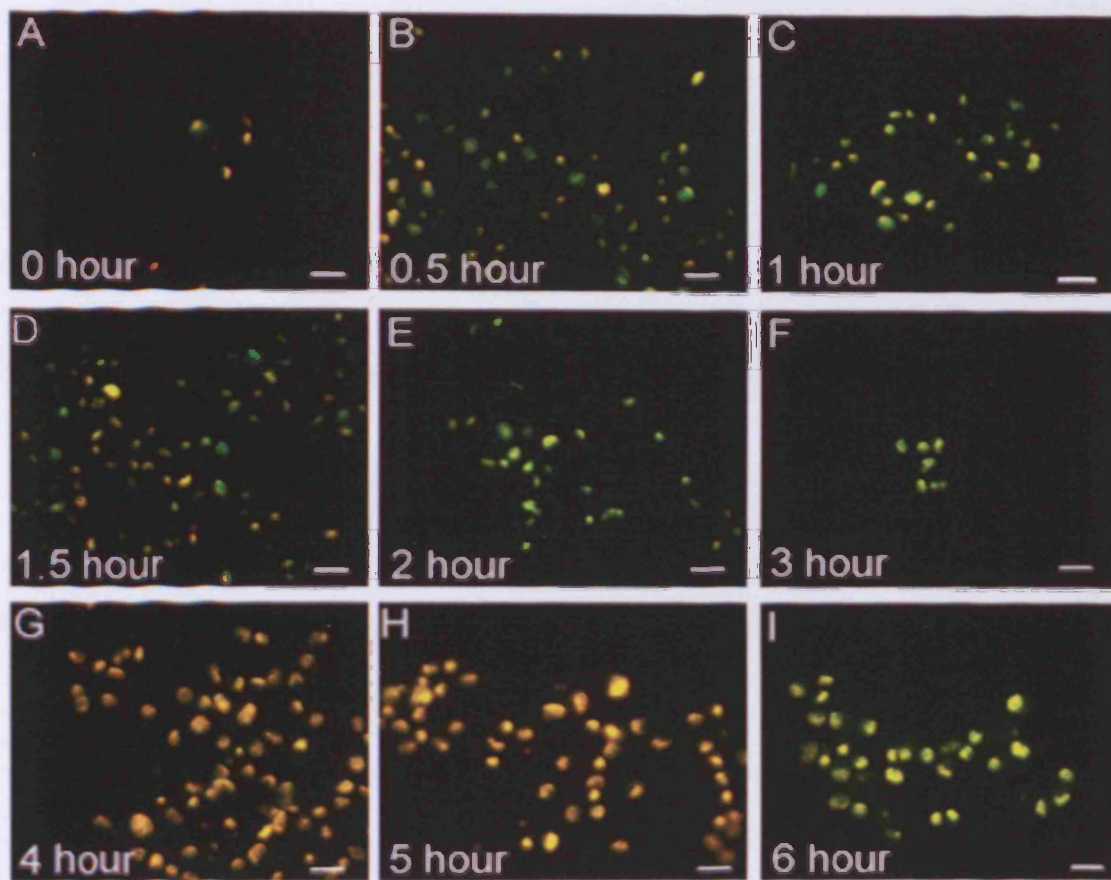


Figure 6.15 Initial cell-cell interactions investigations using articular chondrocytes in a 3-D suspension system, for 0-6hours. Scale bars at 50 μm .

A- 3 hour time interval. Type I collagen label. In small cell aggregates strong type I collagen label is present and possibly extracellular.

B- 0.5 hour time interval. Type II collagen label. Areas of positively labelled type II collagen are present in small cell aggregates.

C- 1 hour time interval. Type III collagen label is negative at all time intervals.

D- 2 hour time interval. Actin. The central regions of cell clusters label strongly for actin.

E- 2 hour time interval. Vimentin label is negative at all time intervals.

F- 2 hour time interval. Vinculin label is concentrated at the cell periphery throughout each time interval.

G- 2 hour time interval. Connexin 32 label is positive throughout the cell population.

H- 2 hour time interval. Connexin 43 label is negative continually in suspension cultures.

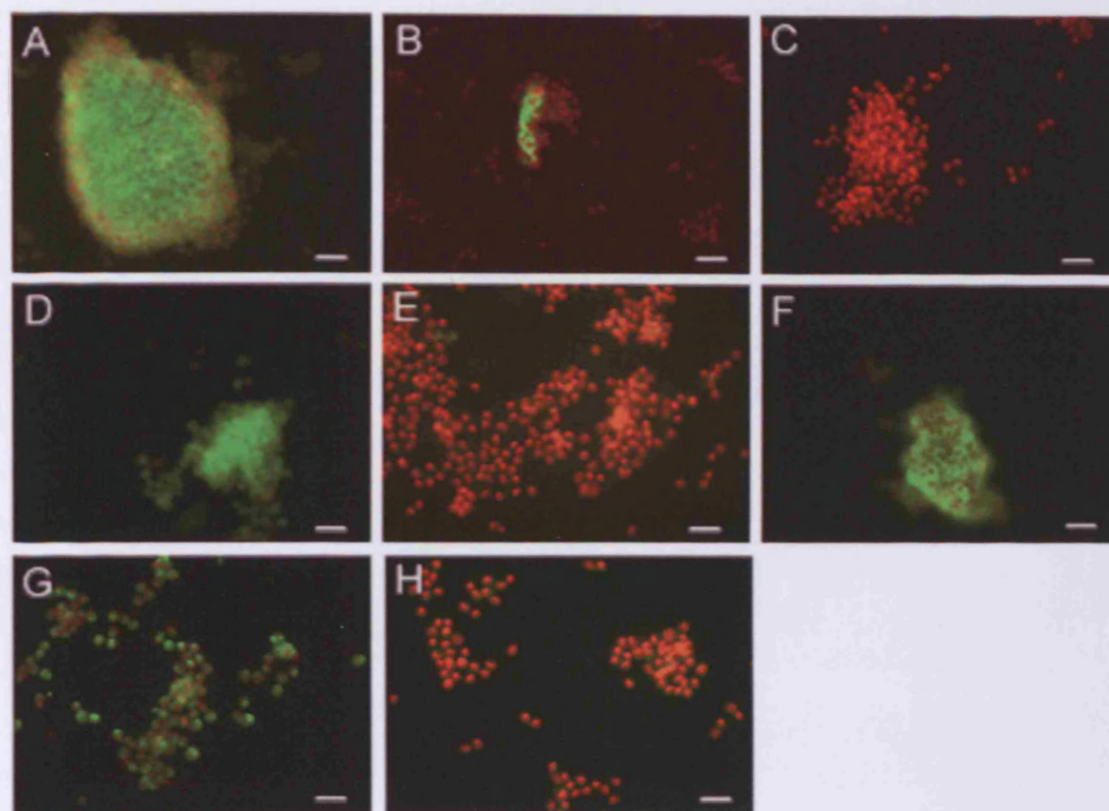


Figure 6.16. Photographs of tendon cell aggregates. Images were acquired on an Olympus SZH10.

A-Tendon cell aggregates after 14 days in 3-D suspension culture. Aggregate is approximately 3cm long in total. Cellular tail widens into a film-like funnel of cellular material and within the funnel is a ball of cells. Scale bar=1cm.

B-A higher magnification of the funnelled end of the aggregate shows the creases that have formed in the thin cellular layer. The two smaller cellular aggregates within the funnel are securely attached to the sides of the funnel. The larger ball of cells located at the end of the funnel were easily detached. Scale bar=0.25cm.

C-Tendon cell aggregates after 14 days in 3-D suspension culture. An overall similar shape as in A, though in this DispoDialyzer tube more than one cell aggregate was formed. There are a total of 4 cell aggregates that developed within this tube. However, only one shows an elongated aggregate with a thin sheet of cellular material at one end.

D- Another example of an elongated cell aggregate after 14 days in 3-D suspension culture. Again note the bulbous aggregation of cells at one end. In this aggregate the 'tail' has become coiled at one end. Scale bar=1cm.

E- Tendon cell aggregates after 14 days in 3-D suspension culture, as seen in C. Note the amount of sheet-like cellular material at the upper end. Scale bar=1cm.

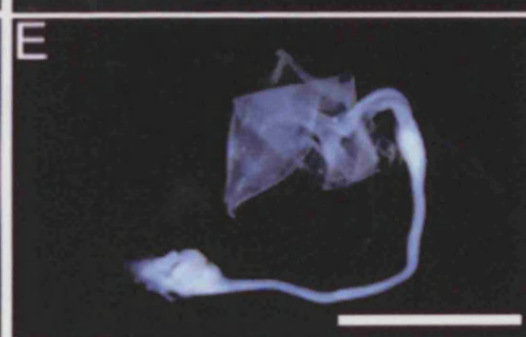
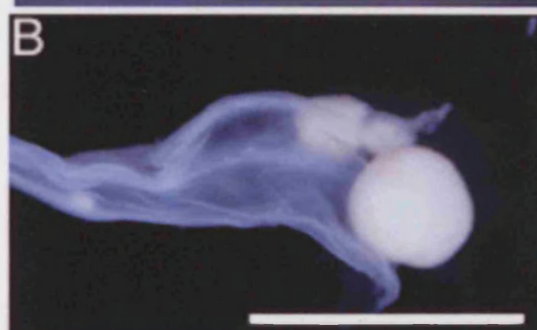
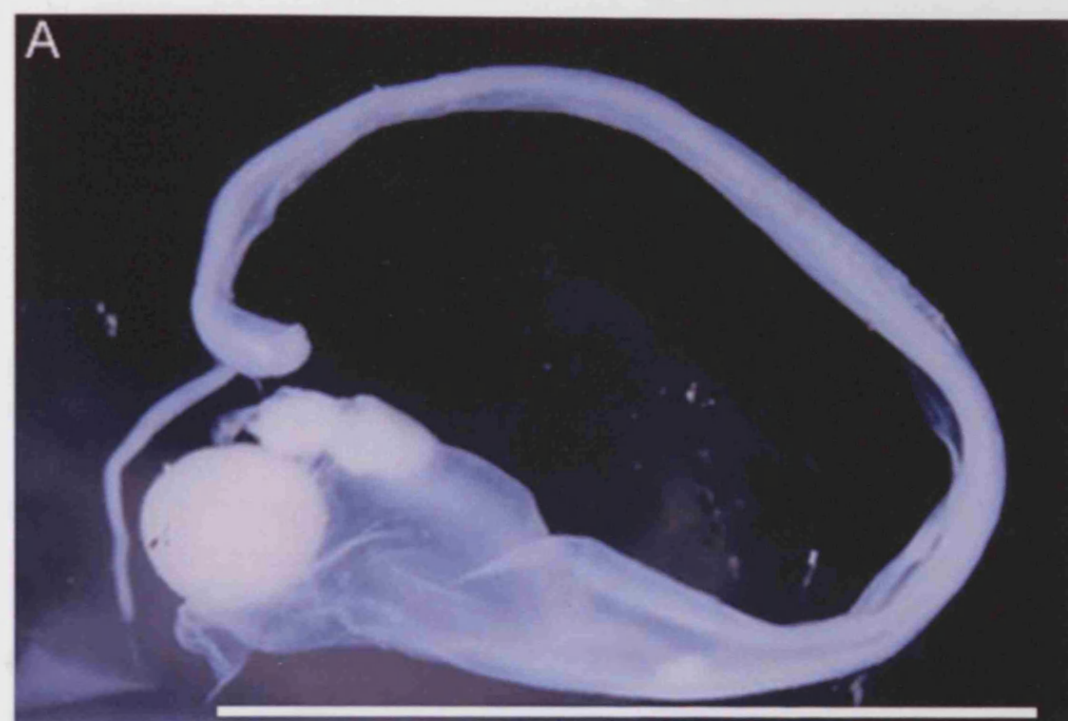


Figure 6.17. Photographs of articular cartilage cell aggregates.

In all images of the articular chondrocyte cell aggregates the difference compared to tendon fibroblasts is staggering. In none of the experiments did the chondrocytes form any large cell aggregates. The small clusters visible disintegrated through handling. Phase contrast images were taken on a CK 40 microscope.

A- Articular chondrocyte cell aggregates at 14 days in 3-D suspension culture.

Scale bar=1cm.

B- Articular chondrocyte cell aggregates at 14 days in 3-D suspension culture.

C- Articular chondrocyte cell aggregates at 14 days in 3-D suspension culture.

Scale bar=1cm.

D- Articular chondrocyte cell aggregates at 14 days in 3-D suspension culture.

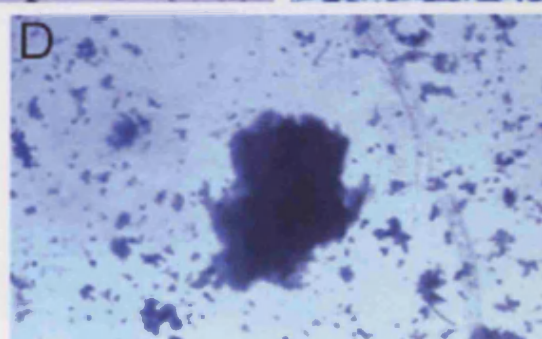
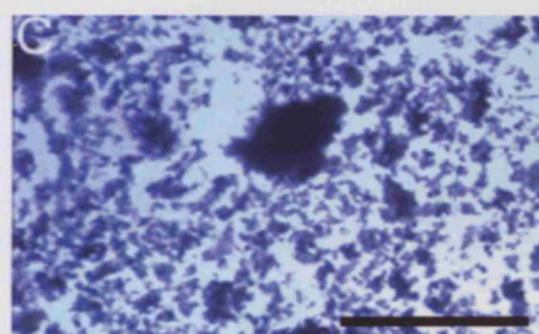
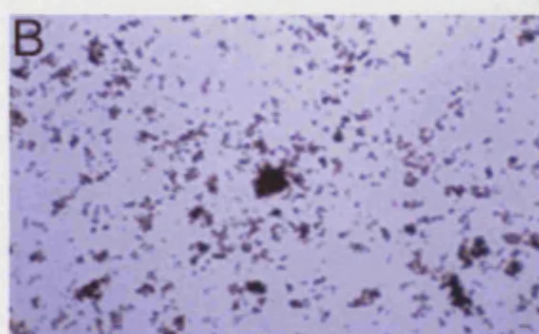
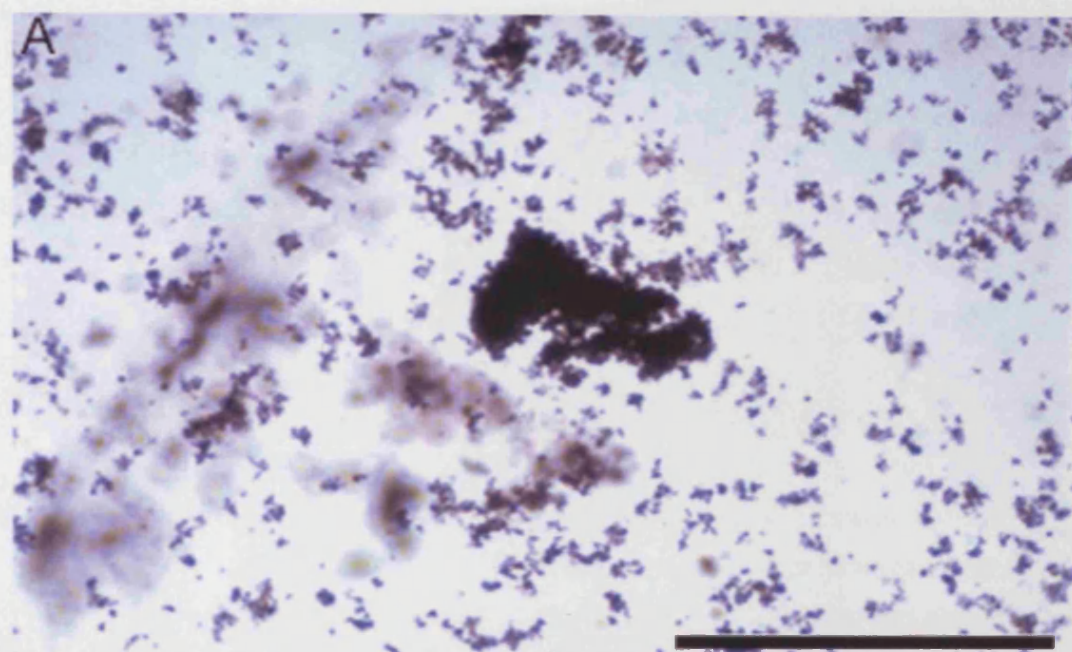


Figure 6.18 Long term large scale suspension culture experiments

Propidium iodide labelling of **cell nuclei** in tendon fibroblast aggregates in 3-D culture system

A- 24hour cell aggregate.

Nuclei labelled with propidium iodide show an even distribution throughout the cell aggregate. Even at this early stage there is evidence of flattened cells in peripheral regions.

Scale bar=20µm

B- 7 day cell aggregate.

Nuclei appear more condense, and in this section there is little evidence to suggest cell flattening at the aggregate surface.

Scale bar=50µm

C- 14 day aggregate.

An elongated section of the aggregate shows significantly elongated nuclei arranged in a longitudinal and parallel fashion. The spaces between labelled nuclei suggest deposited ECM.

Scale bar=20µm

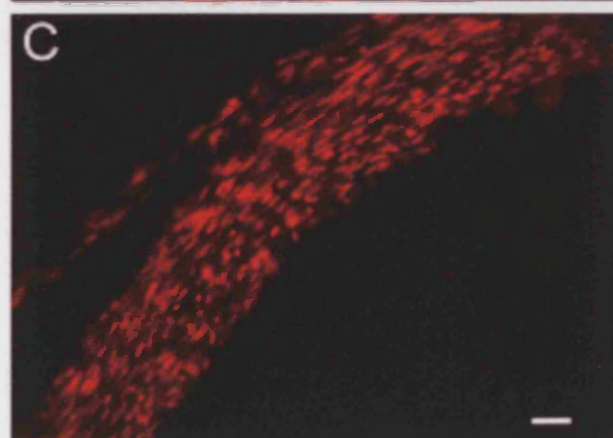
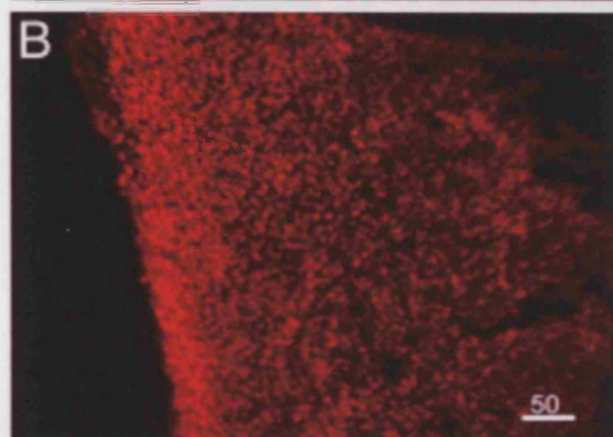
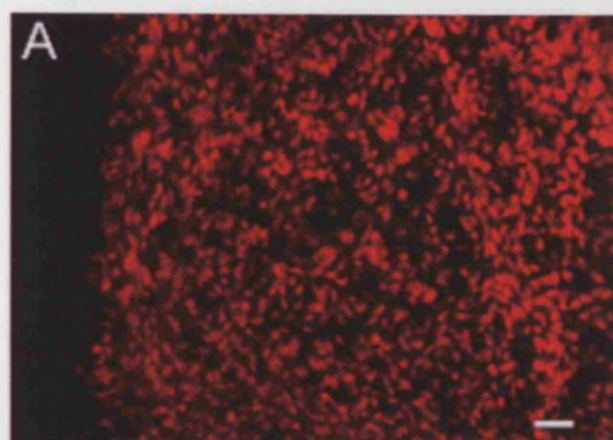


Figure 6.19 Long term large scale suspension culture experiments

Immunohistological labelling of **type I collagen** present in tendon fibroblast aggregates in 3-D culture system

A- 24 hour cell aggregate.

Type I collagen is present showing a fibrous network after 24 hours in culture. The aggregate appeared to be relatively open cellular structure and the type I collagen is present throughout the aggregate, with more intense staining at the periphery.

Scale bar= 20µm

B- 7day cell aggregate.

The increasingly dense aggregate continue to contain extracellular type I collagen. There is no significant difference between the intensity of labelling throughout the aggregate.

Scale bar= 20µm

C- 14 day cell aggregate.

After 14 days in culture, and in longitudinal areas of the cell aggregates, a type I collagen mesh can be observed. The distribution is ubiquitous in these regions and show no orientation with relation to the longitudinally orientated nuclei.

Scale bar= 50µm

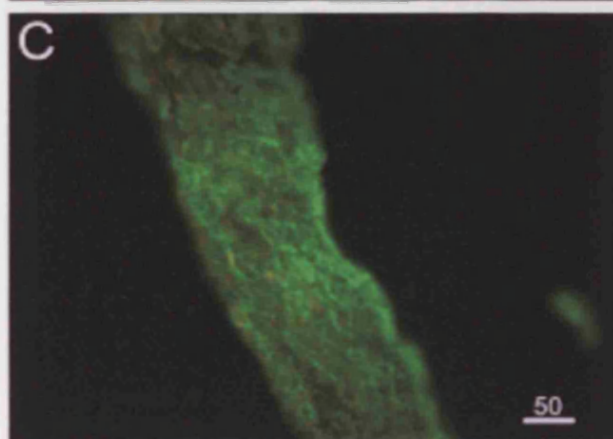
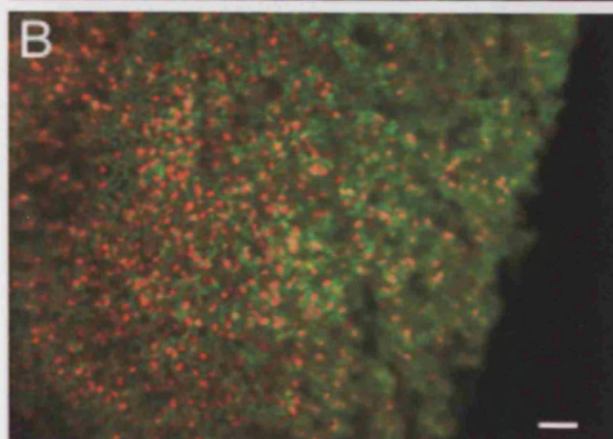
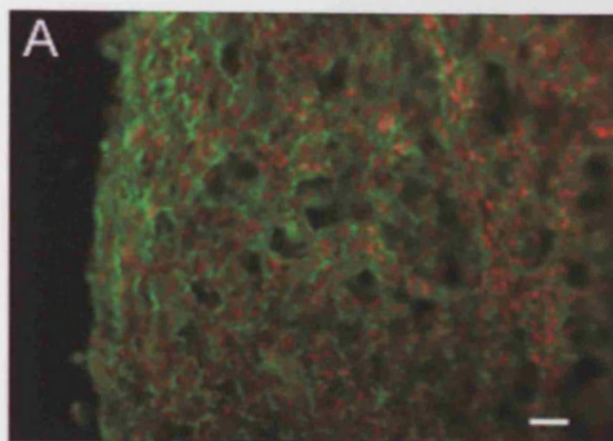


Figure 6.20 Long term large scale suspension cultures

Immunohistological labelling of **type II collagen** present in tendon fibroblast aggregates in 3-D culture system

A- 24 hour cell aggregate.

There is no type II collagen present at any stage throughout the aggregate. The open cellular structure of the aggregates after this time in culture is apparent.

Scale bar=20µm

B- 7 day cell aggregate.

Type II collagen is negative after 7 days in culture and the longitudinal orientation of the cells can begin to be identified.

Scale bar=20µm

C- 14day cell aggregate.

There is no type II collagen present in the cultures after 14 days in culture. This section, from a rounded region of the aggregate, shows no cellular organisation.

Scale bar=20µm

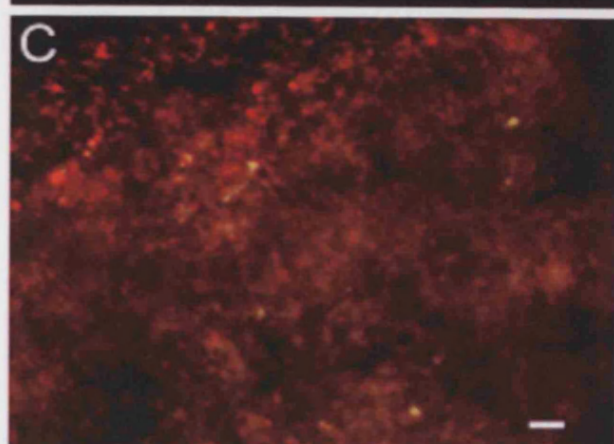
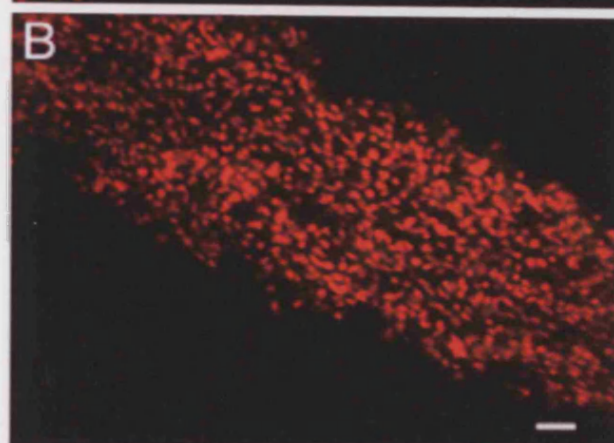
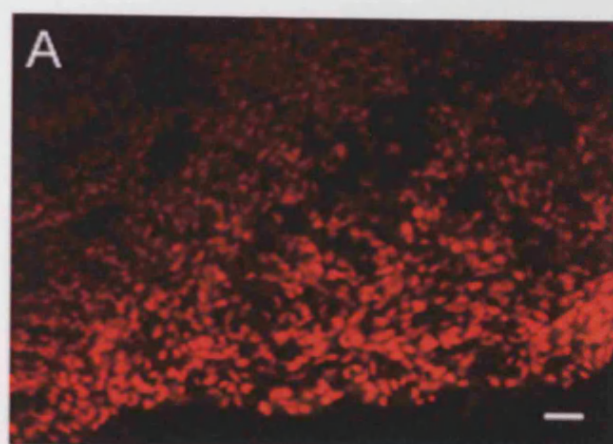


Figure 6.21 Long term large scale suspension cultures

Immunohistological labelling of **type III collagen** present in tendon fibroblast aggregates in 3-D culture system

A- 24hour cell aggregate

Extracellular and fibrous type III collagen is present throughout the cell aggregate.

Scale bar=20µm

B- 7 day cell aggregate

Positively labelled type III collagen can be observed in central parts of the aggregate. On either side there are 3-5 rows of cells showing no type III collagen label. Type III collagen remains fibrous and extracellular but continues to show no significant orientation, similarly to the nuclei labelled with propidium iodide.

Scale bar=20µm

C- 14 day aggregate

Type III collagen is significantly increased in this section compared to 7 day aggregates. The fibrous type III collagen have a longitudinal orientation and there is a possible collagen crimp detectable. Due to the type III collagen being particularly bright the nuclei labelled with propidium iodide are difficult to see, although they are elongate and orientated longitudinally. The label is evenly distributed and there are no signs of surface cells labelling negatively as in 7 day aggregates.

Scale bar=50µm

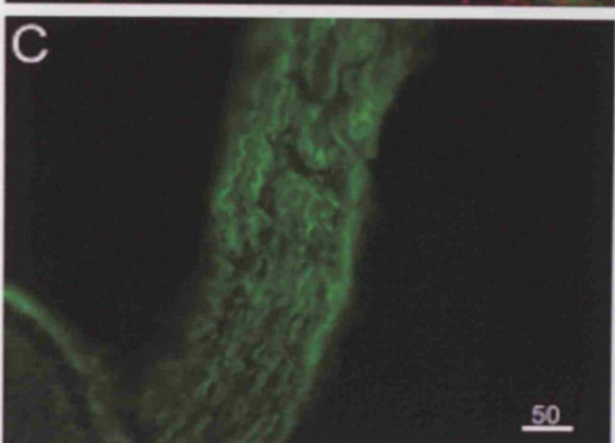
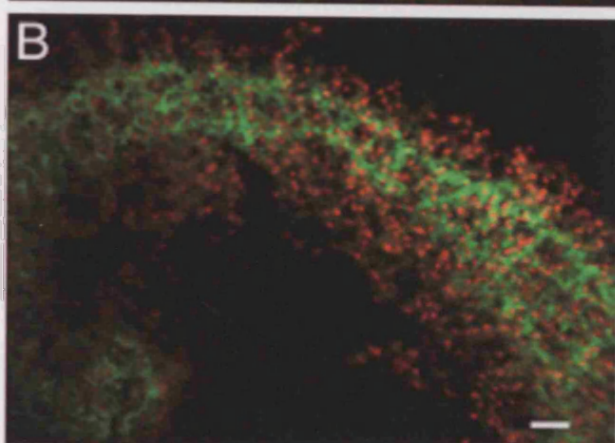
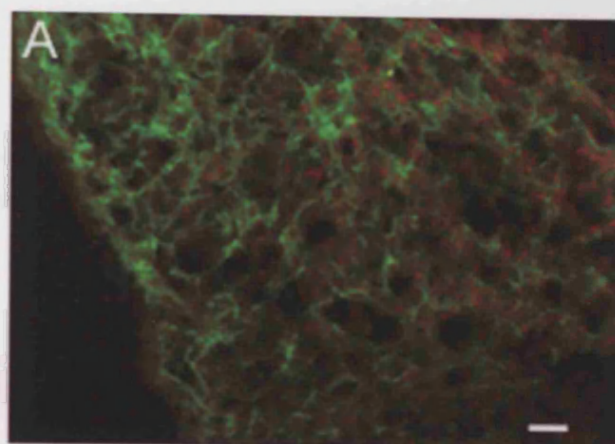


Figure 6.22 Long term large scale suspension cultures

Histological labelling of actin present in tendon fibroblast aggregates in 3-D culture system

A- 24 hour cell aggregate.

Actin labelling is positive, although consistently show as a green hazy labelling in the cells. There is no difference in the intensity of labelling throughout the aggregate.

Scale bar=20µm

B- 7day cell aggregate

Actin can be seen in all cells indicating the presence of a filamentous network, the cloudy label suggests no actin stress fibres are present in the cell aggregate.

Scale bar=20µm

C- 14day cell aggregate

In areas of the 14 day aggregates it was possible to identify some actin stress fibres, although only in areas of cells with a longitudinal orientation. In the majority of areas the characteristic hazy green label is predominant.

Scale bar=50µm

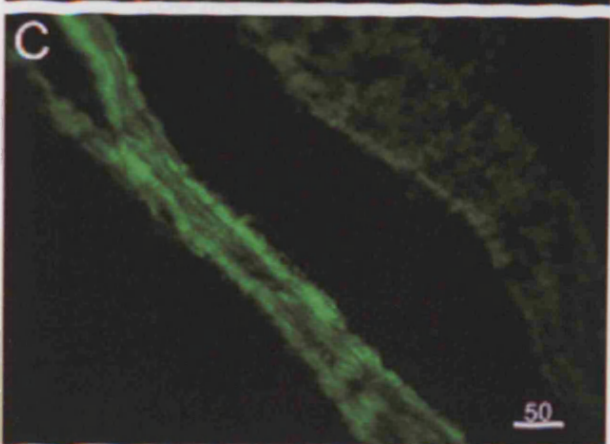
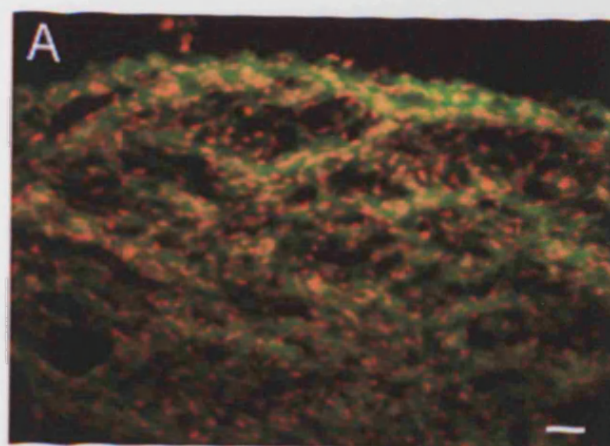


Figure 6.23 Long term large scale suspension cultures

Immunohistological labelling of **vinculin** present in tendon fibroblast aggregates in 3-D culture system

A- 24hour cell aggregate

Clearly labelled vinculin is observed throughout the cell aggregate and is increasingly intense at the periphery.

Scale bar=20µm

B- 7day cell aggregate

Vinculin was present in all areas of the aggregate. The characteristic peripheral patterning can be seen in each cell.

Scale bar=20µm

C- 14day cell aggregate

Ubiquitous vinculin is present, with an emphasis on peripheral labelling of the cells. There are areas of orientation, although not longitudinal.

Scale bar=20µm

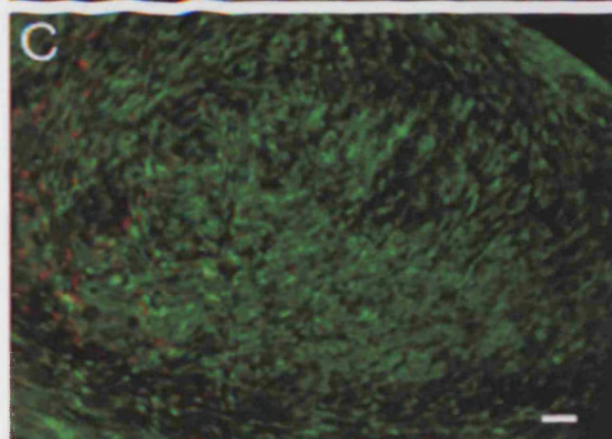
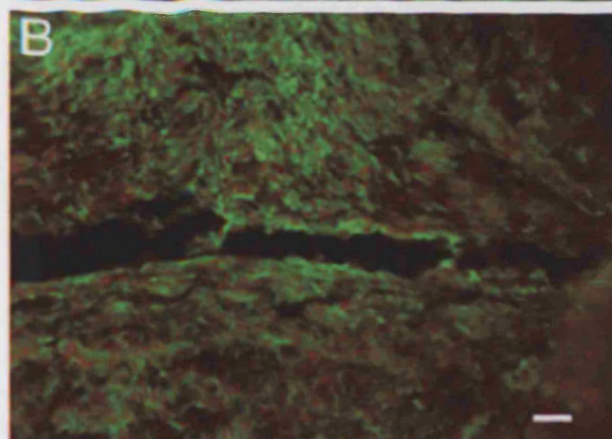
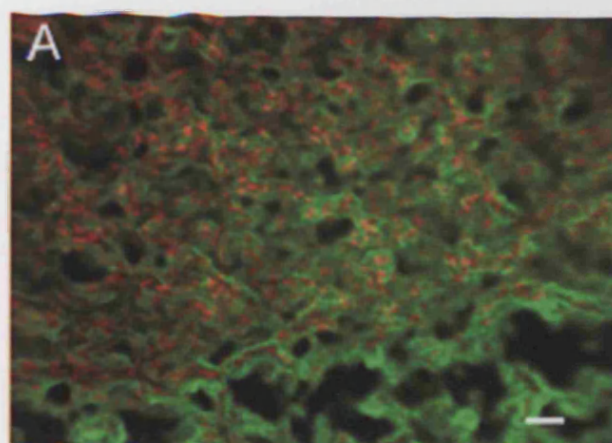


Figure 6.24 Long term large scale suspension cultures

Immunohistological labelling of **connexin 32** present in tendon fibroblast aggregates in 3-D culture system

A- 24hour cell aggregate

Connexin 32 is present in the cells aggregate after 24hours in culture, with a significant labelling at the periphery. Connexin 32 is restricted to the rim of the cells in the aggregate.

Scale bar=20µm

B- 7day cell aggregate

Connexin 32 is evident after 7 days in culture. There is no significant change in intensity, although the peripheral regions containing more flattened cells do not exhibit the same intensity than in the 24 hour cultures.

Scale bar=20µm

C- 14day cell aggregate

Connexin 32 remains present in all areas of the aggregate. Again there is an increase in labelling intensity at the peripheral regions of the aggregate and the characteristic wisp-like labelling pattern can be observed.

Scale bar=20µm

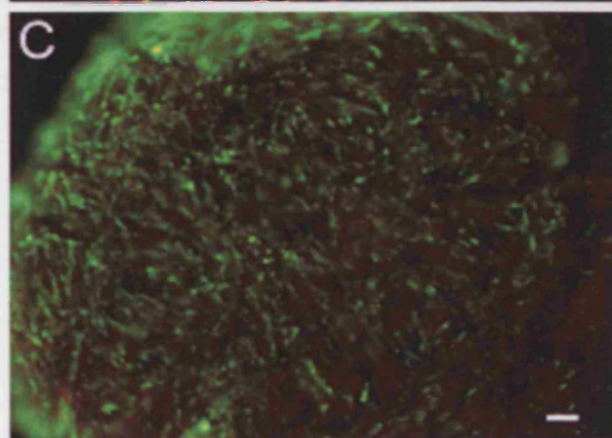
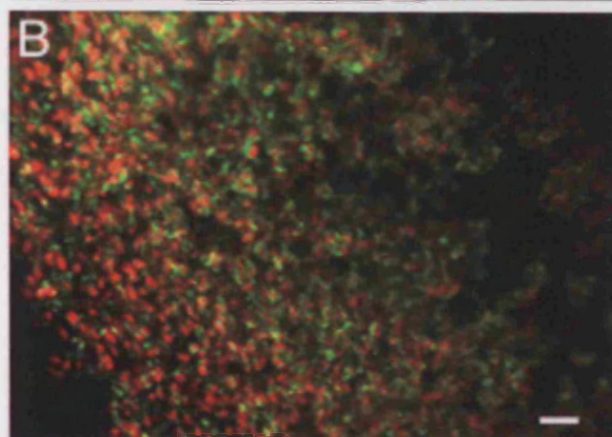
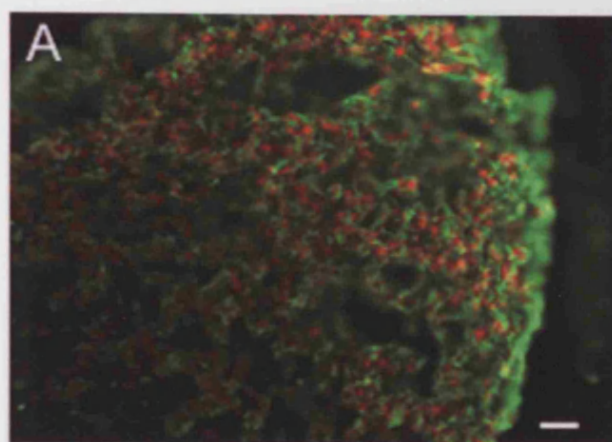


Figure 6.25 Long term large scale suspension cultures

Immunohistological labelling of **connexin 43** present in tendon fibroblast aggregates in 3-D culture system

A- 24hours cell aggregate

Connexin 43 is present, though sparse in its distribution. Foci are generally present at the periphery of the aggregates.

Scale bar=20µm

B- 7day aggregate

Increasingly limited connexin 43 is seen, although its presence is hard to identify.

Scale bar=20µm

C- 14 day aggregate

An increase in connexin 43 is apparent, although this is restricted to areas of the aggregate. There is background labelling creating a false positive image. The true connexin 43 label can be identified by the small punctate labelling.

Scale bar=20µm

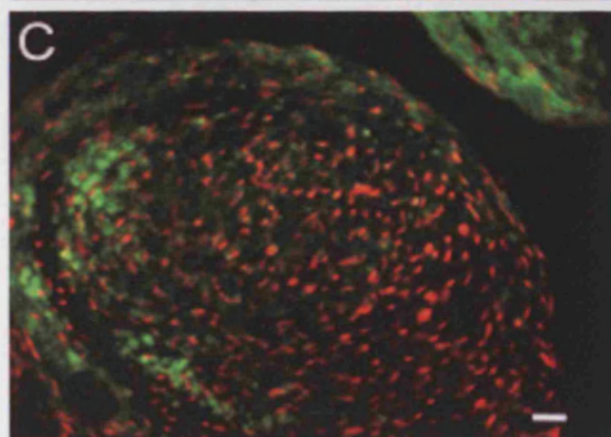
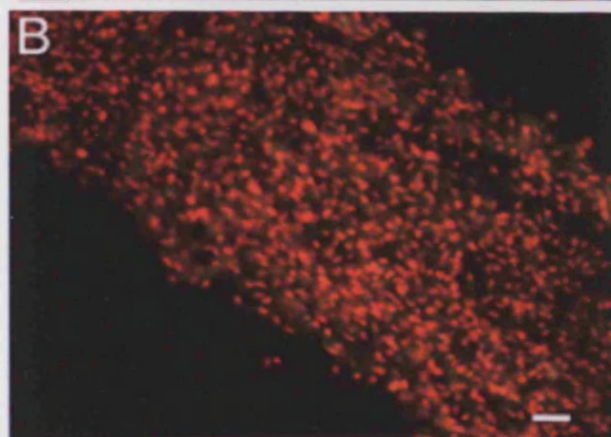
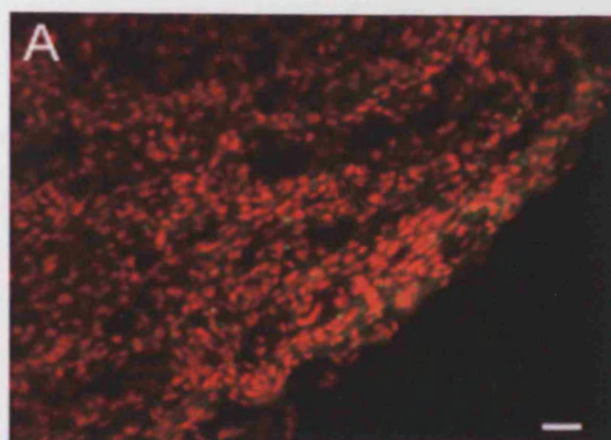


Figure 6.26 Embryonic tendon fibroblasts (E17) within 14 day cell aggregates.
Scale bars are measured in micrometers

Immunohistological labelling of ECM molecules present in tendon fibroblast aggregates in 3-D culture system.

A-Type I collagen label

Type I collagen label is positively labelled in these 14 day cell aggregates and there is no obvious orientation or any distinct collagen fibres to be determined.

B- Type II collagen label

Type II collagen label is negative throughout the cell aggregates.

C-Type III collagen label

Type III collagen label is present in the cell aggregates, although there is approximately one cell layer at the cell surface that labels negatively for type III collagen.

D- Tenascin label

Tenascin labels positively with an even distribution throughout the aggregate.

E- Decorin label

Decorin label shows a peripheral pattern of labelling within the aggregate.

F- Vimentin label

Vimentin is present throughout the cell aggregate.

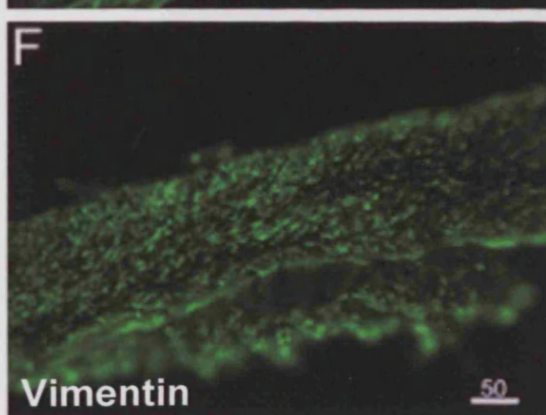
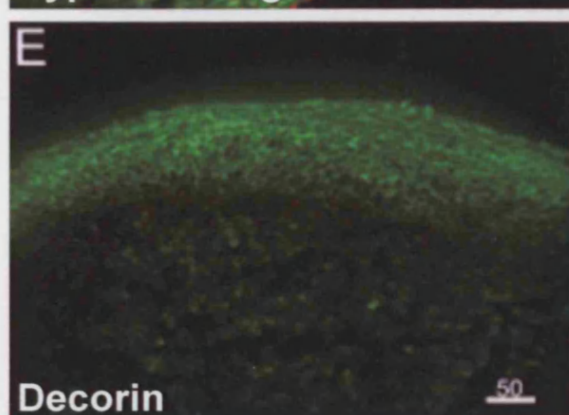
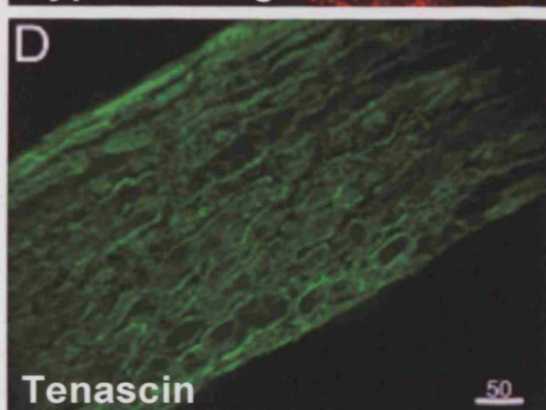
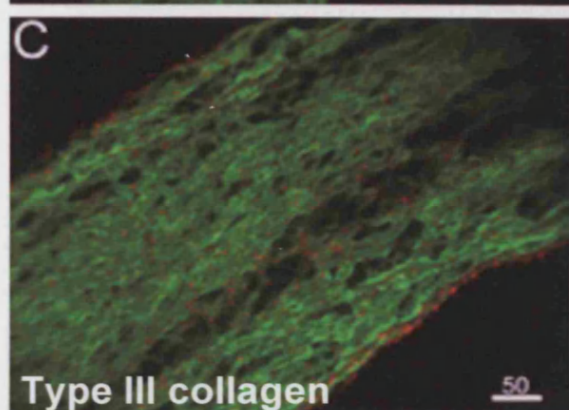
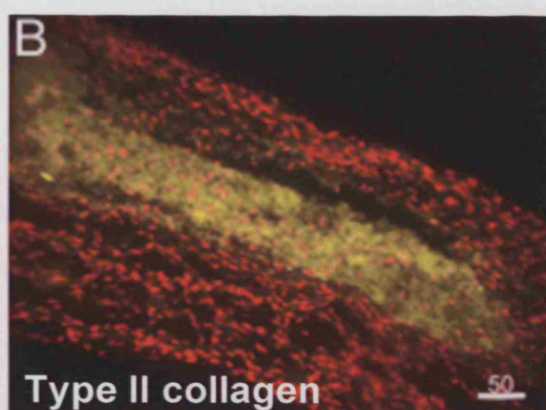
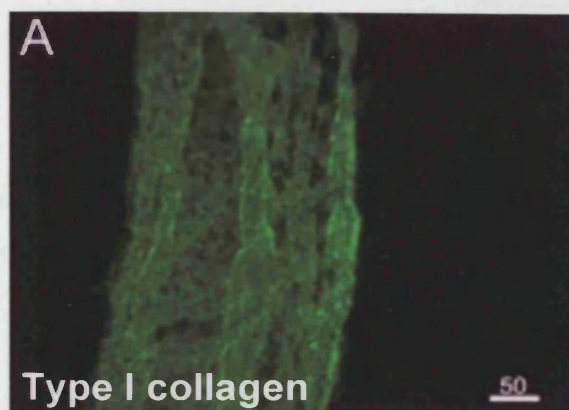


Figure 6.27 Embryonic tendon fibroblasts (E17) within 14 day cell aggregates.
Scale bars are measured in micrometers

Immunohistological labelling of cytoskeletal and cell junctional molecules present in tendon fibroblast aggregates in 3-D culture system.

A-Actin label

Phalloidin label for actin is positive and concentrated in the periphery of the cell aggregate. Cells are orientated perpendicularly to the aggregate surface and the actin appears to follow their orientation.

B- Vinculin label

Vinculin label is positive throughout the cell aggregate with a uniform distribution

C- pan-cadherin

pan-cadherin label is positive, showing a spotted pattern of labelling.

D- n-cadherin

n-cadherin shows a prominent speckled labelling pattern.

E- Connexin 32

Gap junctional protein connexin 32 is abundant label throughout the aggregate.

F- Connexin 43

Connexin 43 label is weak and is only found in isolated areas of the aggregate.

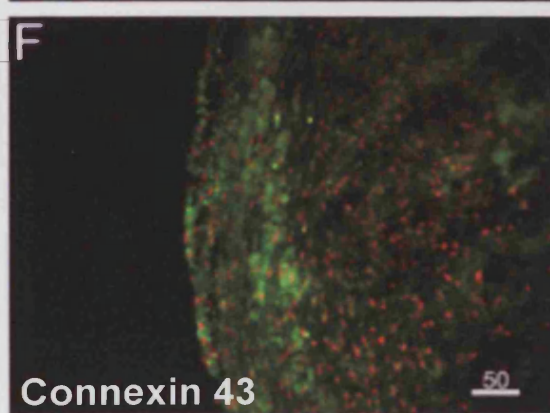
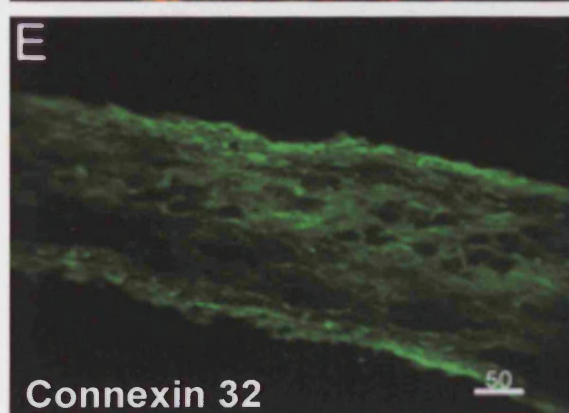
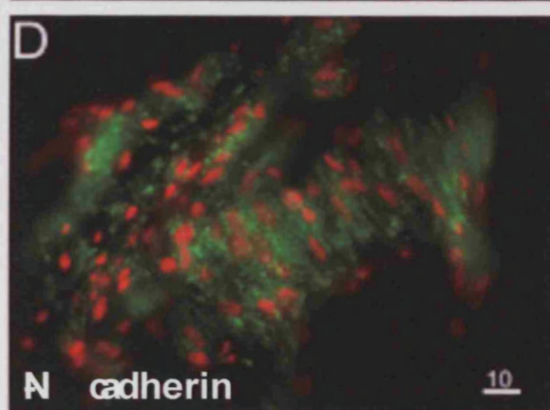
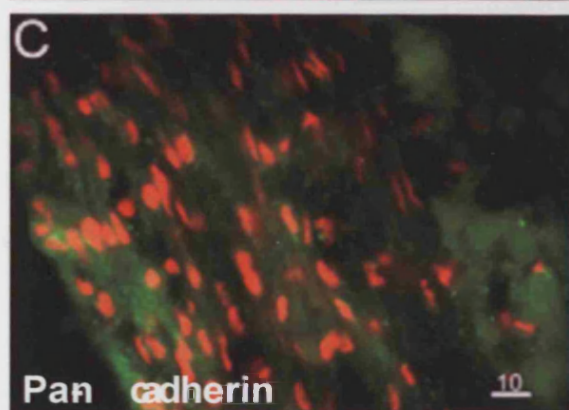
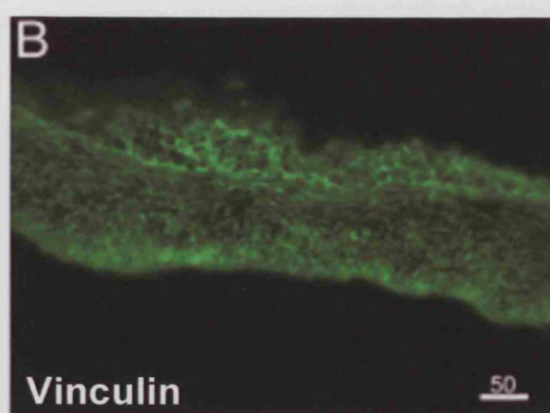
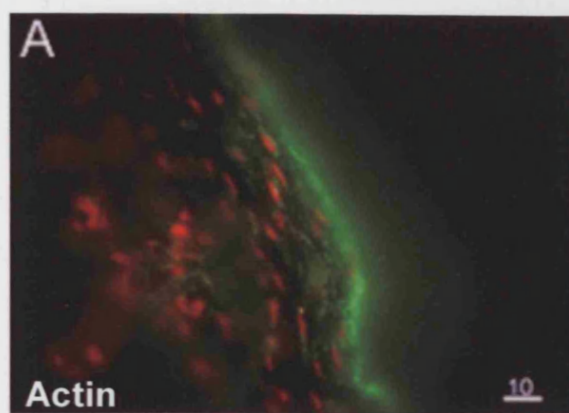


Figure 6.28 Tendon fibroblasts in 3-D roller culture cultures, DispoDialyzer tubes with 50 and 100 KDa MWCO.

Immunolabelled for types I, II and III collagen.

A- 14day cell aggregate. Cultured in **100KDa MWCO** DispoDialyzer tubes and labelled with type I collagen antibody. The elongated nature of the cells within the aggregate is identified by propidium iodide labelled nuclei. Positively labelled type I collagen is visible throughout the aggregate but lacks any discernable orientation. Scale bar=20µm

B- 14day cell aggregate. Cultured in **100KDa MWCO** DispoDialyzer tubes and labelled with type II collagen antibody. There is no positive label for type II collagen. Scale bar=20µm

C- 14day cell aggregate. Cultured in **100KDa MWCO** DispoDialyzer tubes and labelled with type III collagen antibody. Longitudinally arranged nuclei can be identified and type III collagen labels positively in a fibrous manner. There is little indication of matrix orientation, although some is identified running longitudinally between cells. Scale bar=20µm

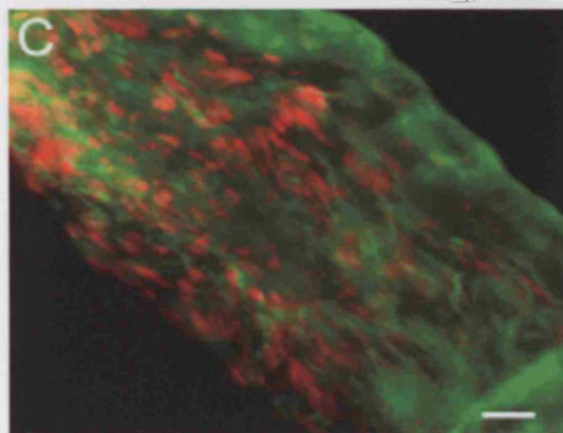
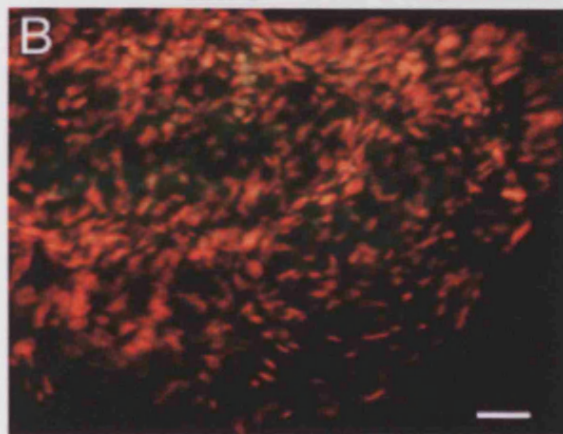
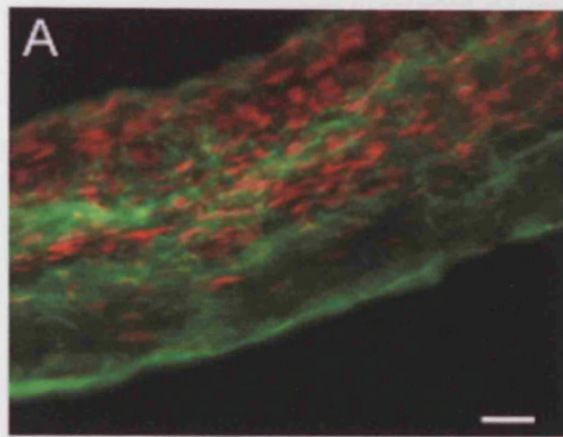


Figure 6.29 Tendon fibroblasts in 3-D roller culture cultures, DispoDialyzer tubes with 50 and 100 KDa MWCO.

Immunolabelled for tenascin, actin and vimentin.

A- 14day cell aggregate. Cultured in **50KDa MWCO** DispoDialyzer tubes and labelled for tenascin. The longitudinal and parallel orientation of the propidium iodide labelled cells is clear and the positively labelled tenascin can be identified. Thin fibrous streaks of label are seen between cells within the aggregate.

Scale bar=20µm

B- 14day cell aggregate. Cultured in **50KDa MWCO** DispoDialyzer tubes and labelled for actin. Hints of actin stress fibres can be seen with a suggestion of longitudinal orientation. Other positively labelled cells within the aggregate show the characteristic green hazy of label associated with actin filament networks.

Scale bar=20µm

C- 14day cell aggregate. Cultured in **50KDa MWCO** DispoDialyzer tubes and labelled for vimentin. Positively labelled vimentin can be observed throughout the cell aggregate, though little longitudinal orientation is identified. The labelling intensity is slightly increased at the aggregate periphery and in these areas labelling has an increased degree of organisation.

Scale bar=20µm

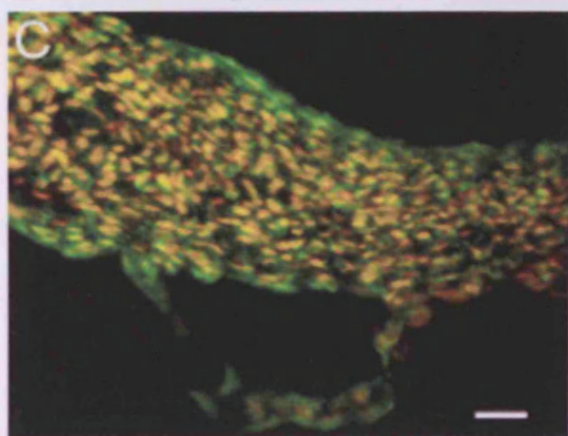
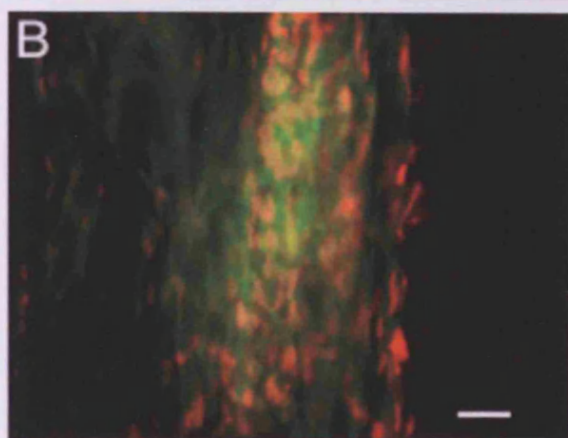
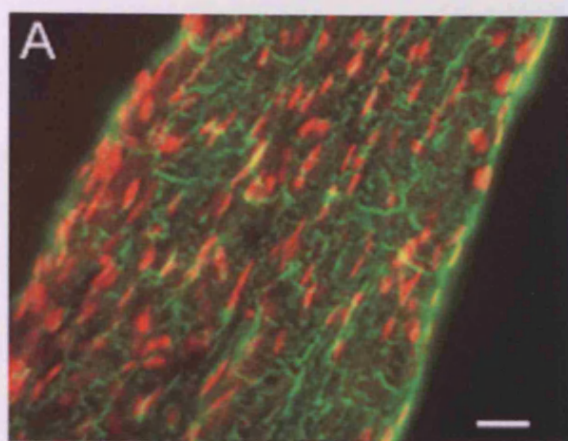


Figure 6.30 Tendon fibroblasts in 3-D roller culture cultures, DispoDialyzer tubes with 50 and 100 KDa MWCO.

Immunolabelled for n and pan-cadherin and vinculin.

A- 7day cell aggregate. Cultured in **50KDa MWCO** DispoDialyzer tubes and labelled for n-cadherin. Beautifully orientated cells show positively labelled n-cadherin after 7 days in roller culturing system. The pattern of labelling shows the elongated shape of cells within the aggregate, though the individual foci of labelling are difficult to identify here.

Scale bar=20µm

B- 14day cell aggregate. Cultured in **100KDa MWCO** DispoDialyzer tubes and labelled for pan-cadherin. Positively labelled pan-cadherin is present throughout the cell aggregate, though little orientation is seen here.

Scale bar=20µm

C- 14day cell aggregate. Cultured in **100KDa MWCO** DispoDialyzer tubes and labelled for vinculin. Areas of longitudinal and positively labelled vinculin are identified within the cell aggregate. Generally cells are labelled positively but a cloudy label predominates.

Scale bar=20µm

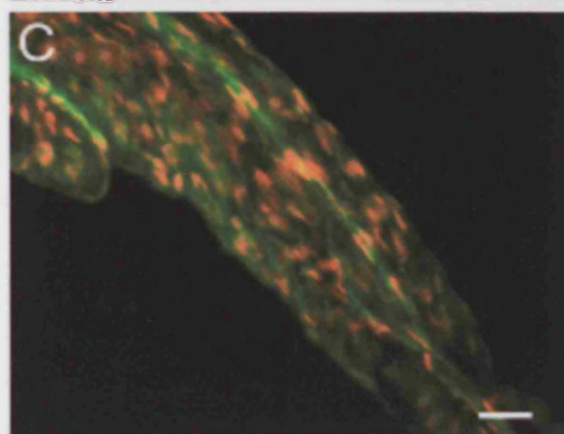
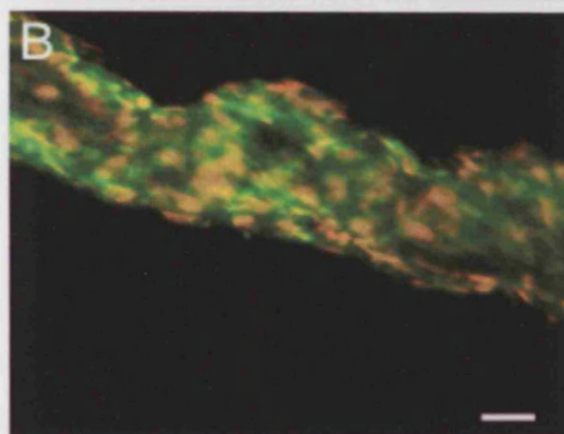
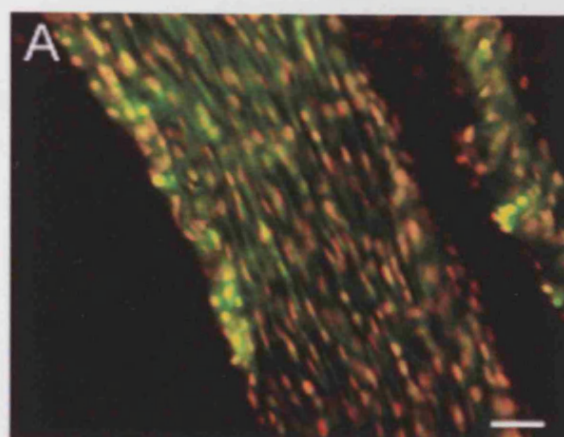


Figure 6.31 Tendon fibroblasts in 3-D roller culture cultures, DispoDialyzer tubes with 50 and 100 KDa MWCO.

Immunolabelled for connexin 32 and 43.

A- 7day cell aggregate. Cultured in **50KDa MWCO** DispoDialyzer tubes and labelled for connexin 32. Highly orientated cells and elongated nuclei label positively for connexin 32. The label is characteristically wisp-like and is identified in all cells.

Scale bar=20µm

B- 14day cell aggregate. Cultured in **100KDa MWCO** DispoDialyzer tubes and labelled for connexin 32. A less orientated section than in A, the connexin 32 remains constant and distributed throughout the aggregate.

Scale bar=20µm

C- 14day cell aggregate. Cultured in **100KDa MWCO** DispoDialyzer tubes and labelled for connexin 43. Typically difficult to find connexin 43 label shows a small amount of positively labelled points. There is no orientation to the sparse labelling.

Scale bar=20µm

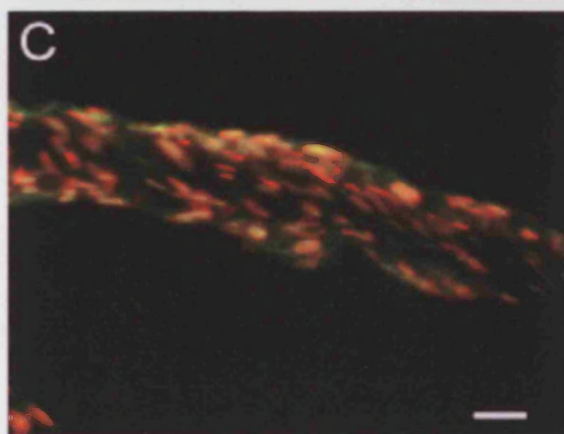
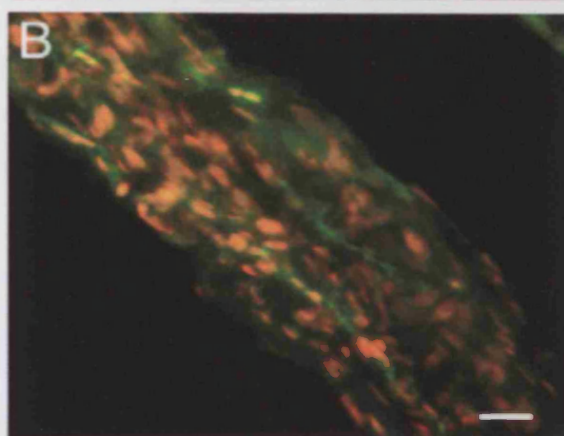
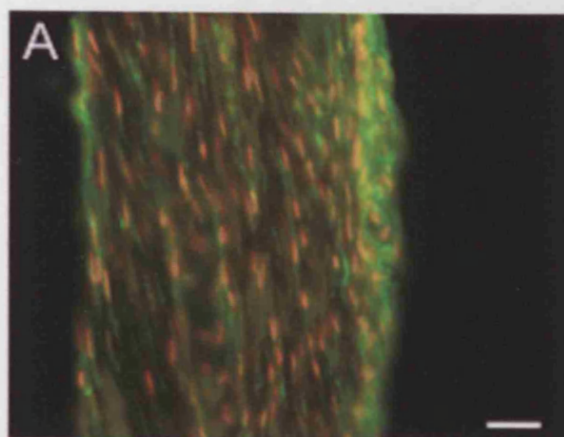


Figure 6.32- 7 day 3-D cultures, treated with growth factors.
Scale bars represent 20 micrometers, unless otherwise stated.

Immunohistological labelling of ECM molecules present in tendon fibroblast aggregates in 3-D culture system.

A-TGF β treated fibroblasts-labelled with type I collagen.

Type I collagen label is present throughout the cell aggregates treated with TGF β .

B-IGF treated fibroblasts-labelled with type I collagen

Positively labelled for type I collagen, showing extracellular labelling throughout the cell aggregate.

C- TGF β treated fibroblasts- Labelled with type III collagen

Type III collagen shows a more centralised pattern of labelling, taking on a fibrous arrangement. There are cell nuclei in the periphery labelling negatively for type III collagen.

D- IGF treated fibroblasts- Labelled with type III collagen

Labelling for type III collagen shows centralised labelling, taking on a convoluted pattern of fibrous labelling. There are several cell layers surrounding the type III collagen label, which label negatively for type III collagen.

E- TGF β treated fibroblasts- Labelled with tenascin.

Tenascin label is distributed evenly throughout the cell aggregate.

F- IGF treated fibroblasts- Labelled with tenascin.

Positively labelled tenascin is present extracellularly all the way through the cell aggregate.

TGF-beta

IGF-I

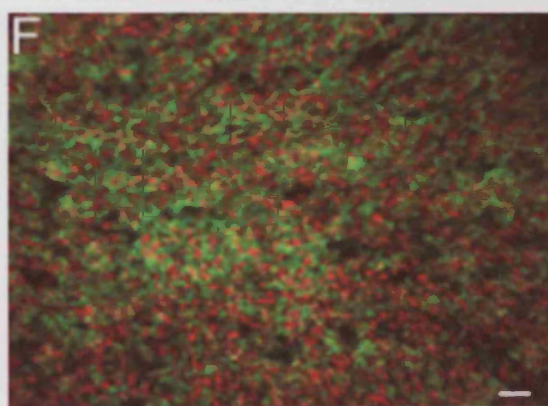
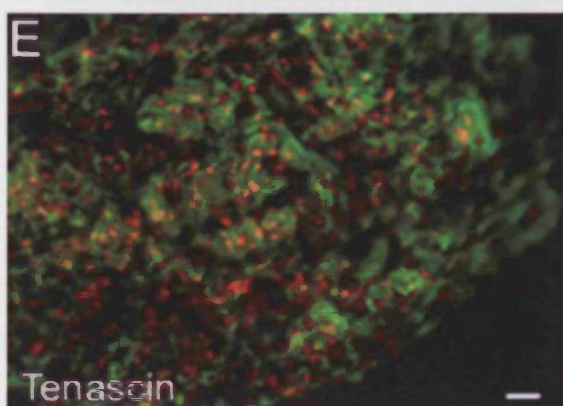
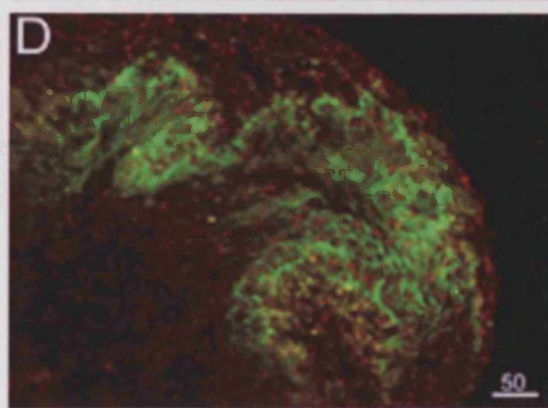
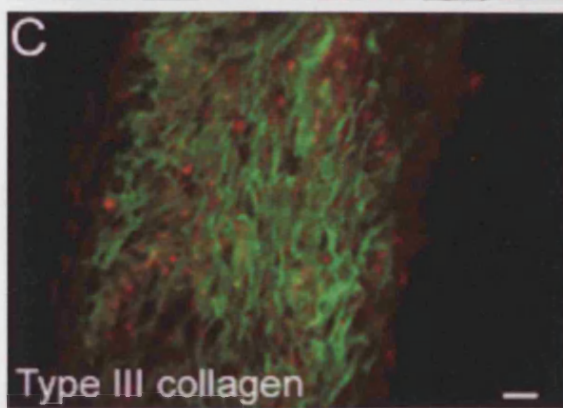
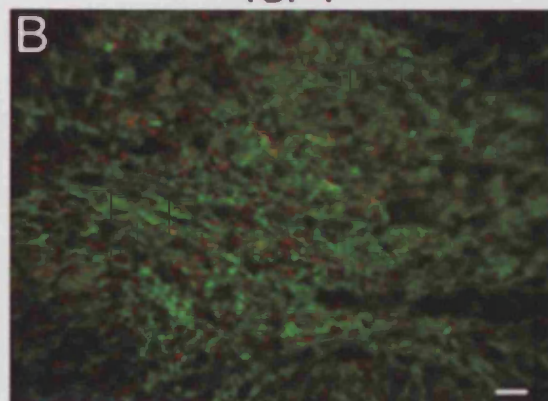
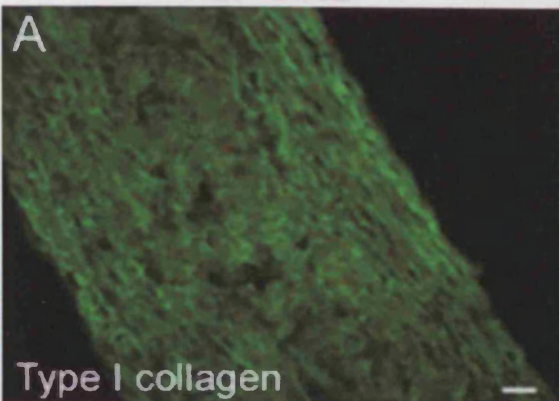


Figure 6.33- 7 day 3-D cultures, treated with growth factors.
Scale bars represent 20 micrometers, unless otherwise stated.

Immunohistological labelling of cytoskeletal and associated molecules present in tendon fibroblast aggregates in 3-D culture system.

A- TGF β treated fibroblasts- Labelled with phalloidin

Actin label is positive and actin stress fibres can be observed, but show no orientation.

B- IGF treated fibroblasts- Labelled with phalloidin

Positively labelled actin is clearly visible and short stress fibres can be seen, although there is little or no organisation of the cells.

C- TGF β treated fibroblasts- Labelled with vimentin

Vimentin label is positive in the cell aggregate. Though the labelling is stronger in the periphery, there is positive label throughout the aggregates.

D- IGF treated fibroblasts- Labelled with vimentin

Positive labelling for vimentin is concentrated at the cell periphery, though lesser degrees of labelling are present in central regions of the aggregate.

E- TGF β treated fibroblasts- Labelled with vinculin

Vinculin is positively labelled throughout the cell aggregate.

F- IGF treated fibroblasts- Labelled with vinculin

Positive label for vinculin in the cell aggregates shows the characteristic pattern of labelling focused at the cell periphery.

TGF-beta

IGF-I

A

B

Actin

C

D

Vimentin

E

F

Vinculin

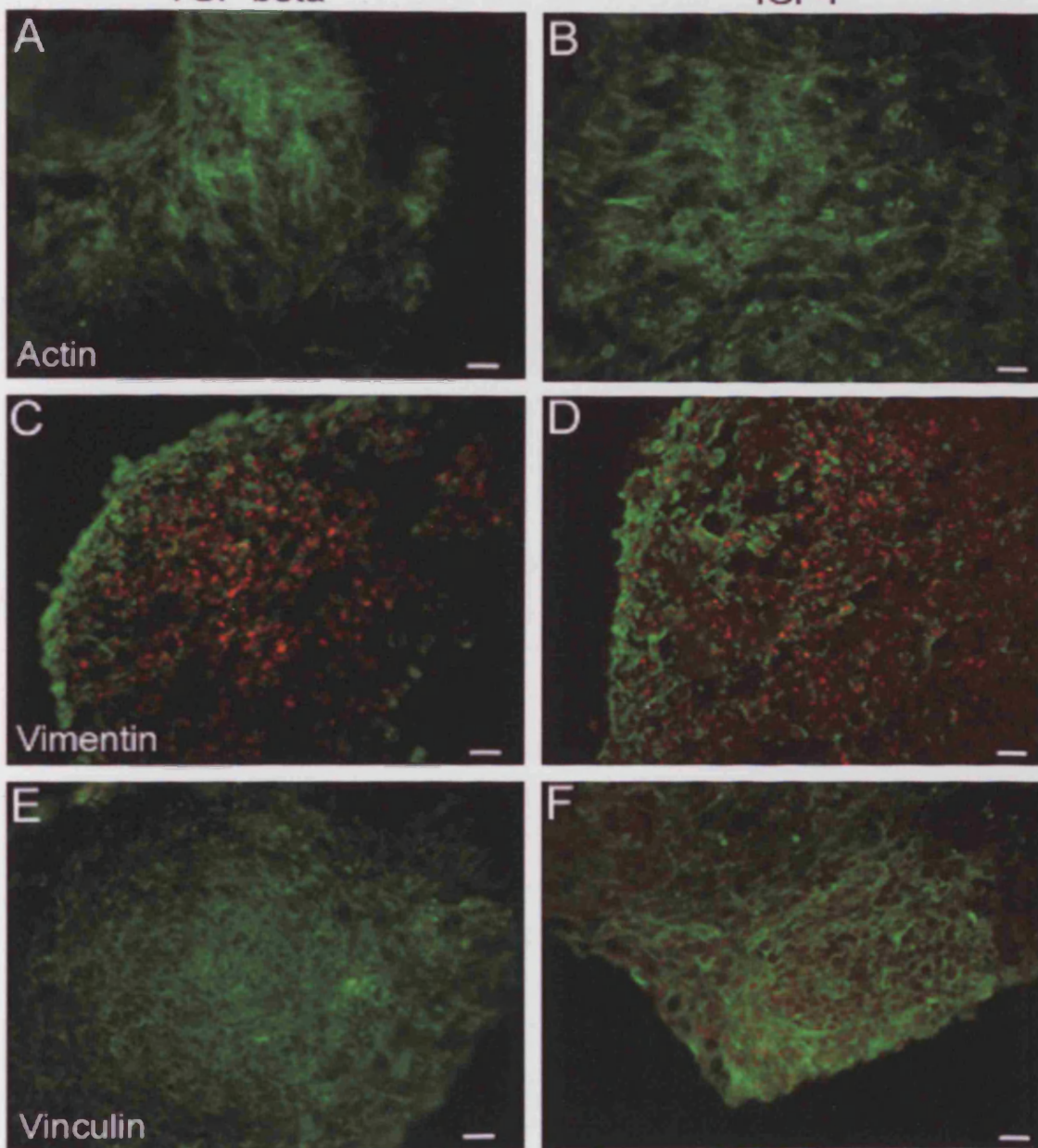


Figure 6.34- 7 day 3-D cultures, treated with growth factors.
Scale bars represent 20 micrometers, unless otherwise stated.

Immunohistological labelling of cell junctional molecules present in tendon fibroblast aggregates in 3-D culture system.

A- TGF β treated fibroblasts- Labelled with n-cadherin
n-cadherin label is positive in the cell aggregates with no notable organisation.

B- IGF treated fibroblasts- Labelled with n-cadherin
n-cadherin label was present though label was strongest at the edges of the cell aggregate.

C- TGF β treated fibroblasts- Labelled with connexin 32
Connexin 32 label is present throughout each cell aggregate. Areas nearest the aggregate periphery showed the strongest labelling.

D- IGF treated fibroblasts- Labelled with connexin 32
A similar labelling pattern as aggregates treated with TGF β , positive label observed throughout the cell aggregate but labelling focused at areas nearest the aggregate periphery.

E- TGF β treated fibroblasts- Labelled with connexin 43
Connexin 43 label shows negative label, though there are areas of auto-fluorescence.

F- IGF treated fibroblasts- Labelled with connexin 43
There is no label for connexin 43 present in the cell aggregates.

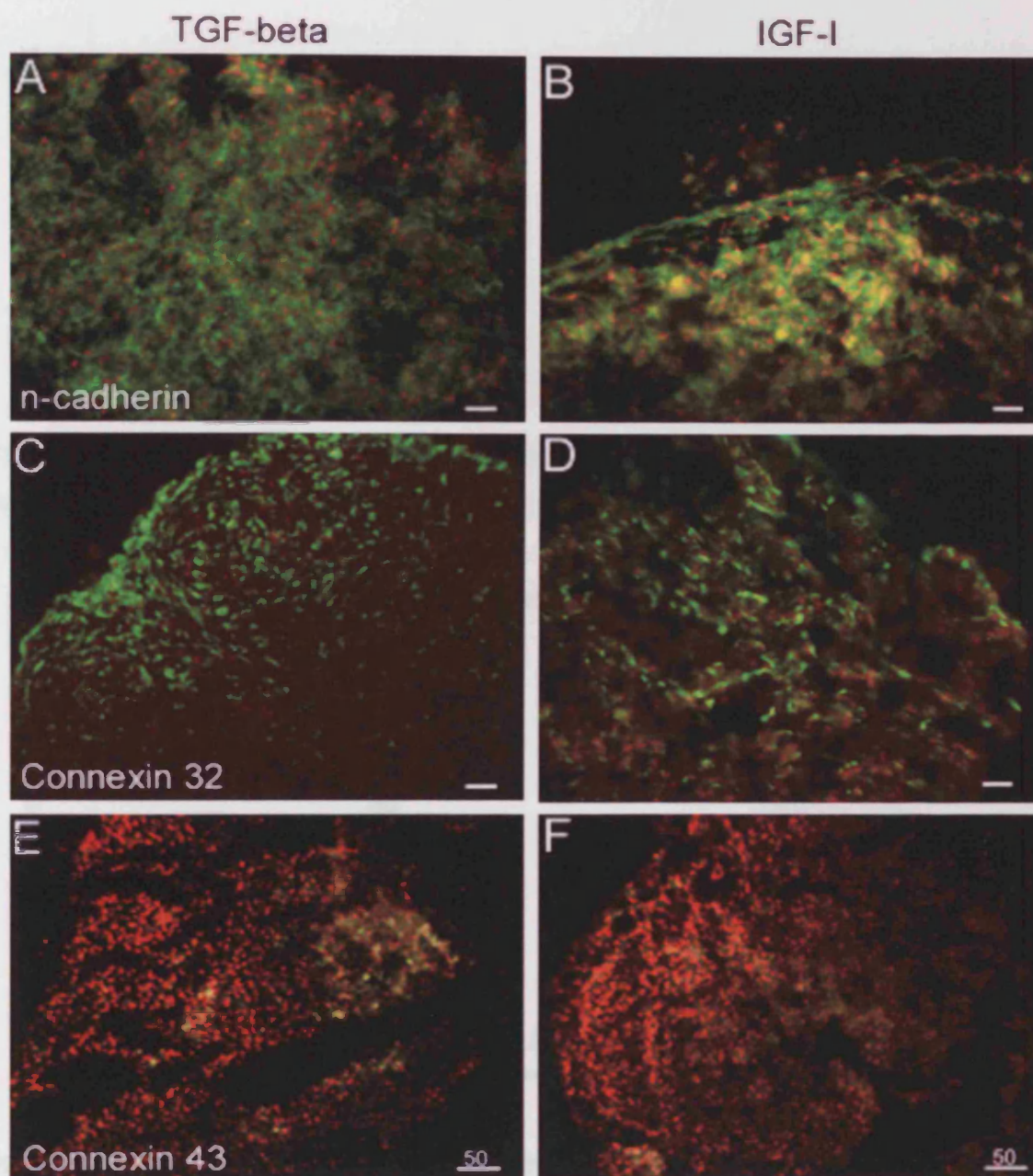


Figure 1. Immunofluorescence images of heart tissue showing the expression of TGF-beta, IGF-I, n-cadherin, Connexin 32, and Connexin 43.

Collagen produced by cells in suspension cultures

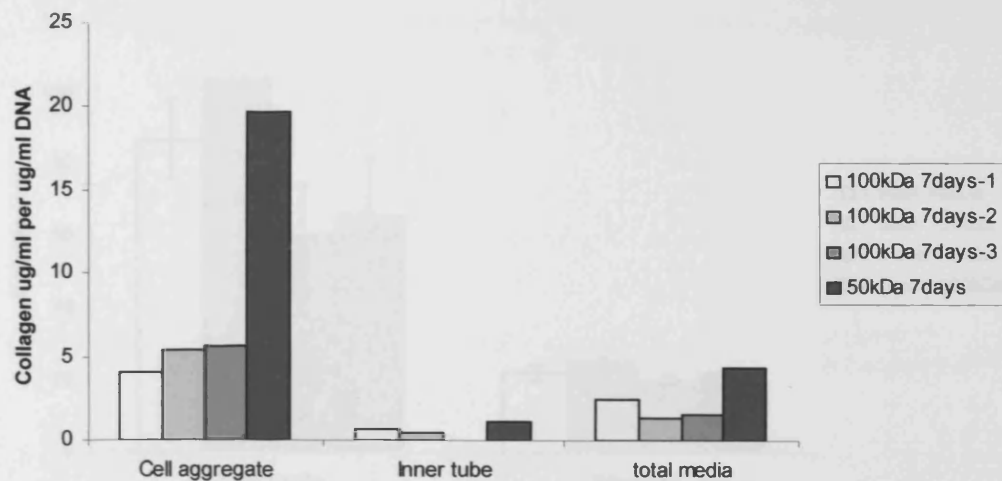


Figure 6.35. Shows collagen retained within the cell aggregate, released into the inner dialysis tubing and released into the total media. n=1.

Collagen produced by cells in suspension culture after 1 week

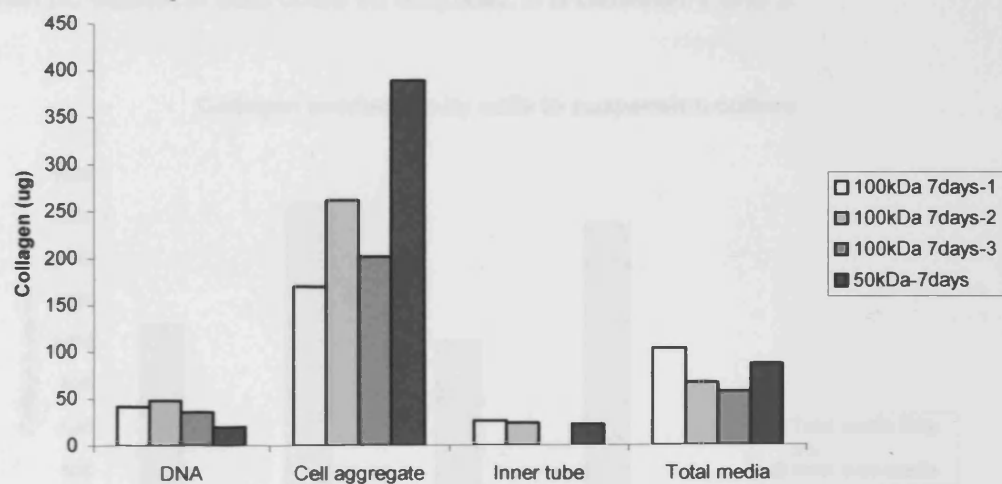


Figure 6.36. Shows total collagen retained within the cell aggregate, released into the inner dialysis tubing and released into the total media. n=1.

Collagen production from cells in long term suspension cultures: IGF-I treated

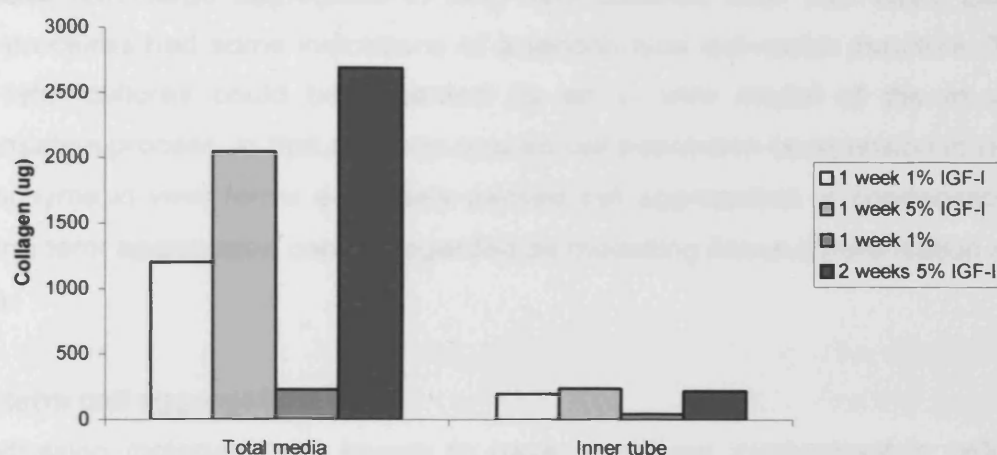


Figure 6.39. Shows the actual amounts of collagen released into the two media reservoirs of suspension cultures treated with IGF-I.

Collagen production from cells in long term suspension culture: TGF-beta treated

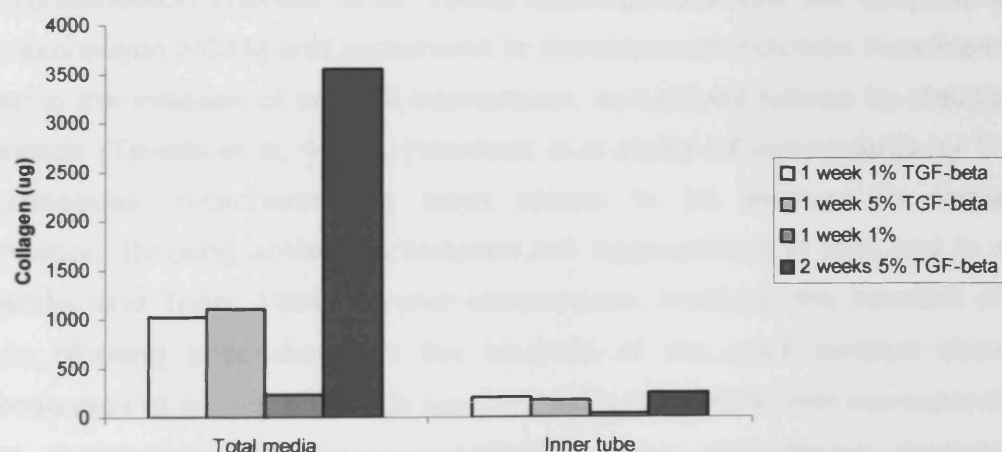


Figure 6.40. Shows the actual amounts of collagen released into the two media reservoirs of suspension cultures treated with TGF- β .

6.4 DISCUSSION

Results show firstly, that both tendon fibroblasts and chondrocytes formed substantial cell aggregates in 1-2 hours in suspension culture, and secondly that fibroblasts form large aggregates in long term cultures, over 1-21 days. Large scale structures had some indications of a tendon-type cell-matrix structure. The short term cultures could be regarded as an *in vitro* model of the *in vivo* condensation process, in that a widely spaced cell population (suspension *in vitro*, mesenchyme *in vivo*) forms a densely packed cell aggregation or condensation. The long term aggregation can be regarded as modelling tissue differentiation and growth.

Short term cell aggregation

Cell adhesion molecules are known to have significant involvement in cellular condensations of connective tissues, particularly n-cadherin and NCAM in cartilage development (Tavella et al, 1994). In the experiments described above n-cadherin was expressed by all cells in short term suspension cultures, but aggregation could not be blocked with specific function blockers. *In vivo* the timings of n-cadherin expression in cartilage correlates directly with the period of active condensation (Tavella et al, 1994). Investigations into the temporal and spatial expression NCAM and n-cadherin in development indicates n-cadherin is involved in the initiation of cell-cell interactions, and NCAM follows by stabilising the contacts (Tavella et al, 1994). However, in a series of experiments by Tuan and colleagues, n-cadherin has been shown to be involved in cartilage condensation. Blocking antibodies reduced cell aggregations *in vitro* and *in vivo* (Oberlender and Tuan, 1994). *In vivo* experiments, involving the injection of n-cadherin blocking antibodies into the hindlimb of the chick embryo showed chondrogenesis to be moderately to severely delayed together with corresponding skeletal deformities. Subsequent experiments using recombinant expression plasmids show overexpression of n-cadherin in micromass cultures enhances cellular condensation and chondrogenesis (DeLise and Tuan, 2002a), whereas, the use of deleted mutants and consequent reduction of n-cadherin expression did result in decreased cell condensation (DeLise and Tuan, 2002b).

In the experiments performed here, cell aggregation could not be blocked. There are several possible reasons why this may have happened. It may be that the experiments to block cadherin-mediated interactions simply failed, however, as two different blocking techniques were attempted this seems unlikely. Other

Collagen production by cells in suspension culture

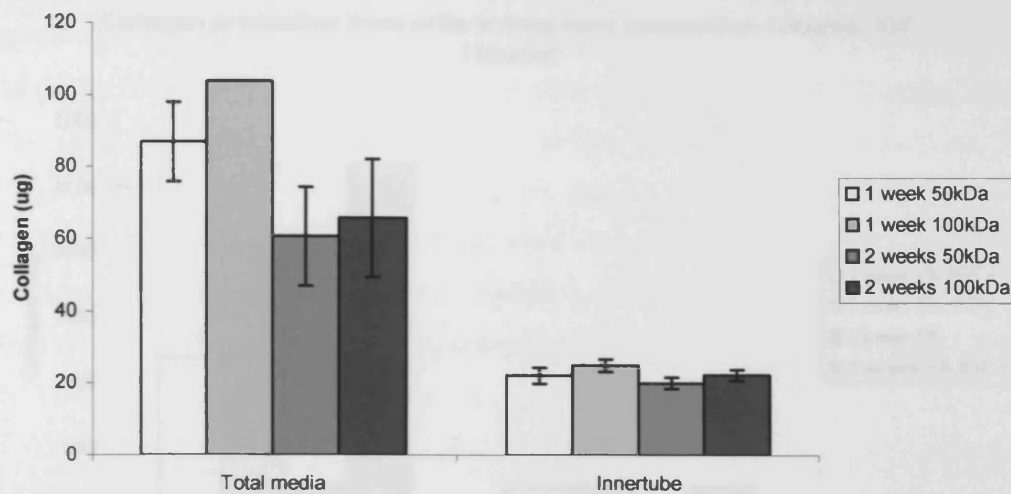


Figure 6.37. Shows the actual amounts of collagen released into the two media reservoirs. The first group of columns represent the total media used in the experiment, any media changes were kept and added. The second group of columns represent the media taken from inside the dialysis tubing. Small n values mean no statistical data could be acquired. n is between 1 and 3.

Collagen production by cells in suspension culture

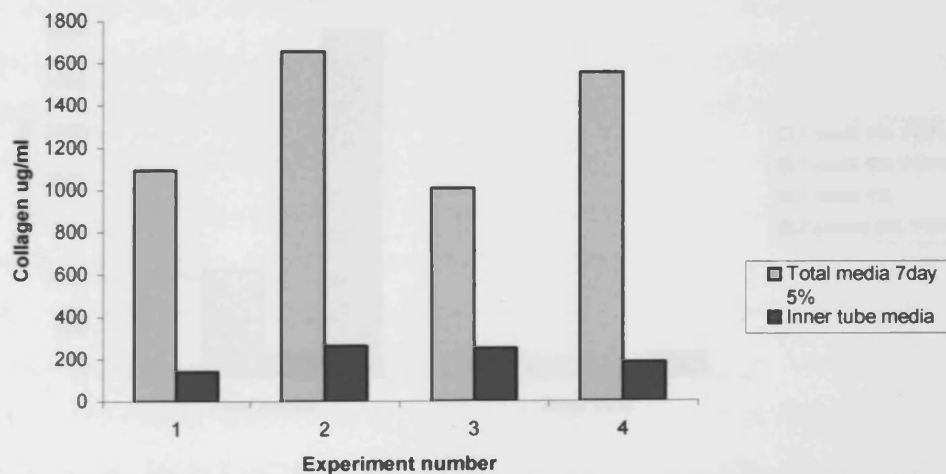


Figure 6.38. Shows the actual amounts of collagen released into the two media reservoirs. Blue columns represent collagen from the total media and the red columns the collagen from the media in the inner tube. Small n values mean no statistical data could be acquired. n is between 1 and 3.

investigations into cadherins have chosen HAV peptides or blocking antibodies to block cadherin-based cell interactions with success, including the epithelial cells of the proximal tubule, osteoblasts and osteoclasts, endothelial cells and neuronal cells (Bergin et al, 2000; Cheng et al, 1998; Ilvesaro, 1998; Chuah et al, 1991; Tang et al, 1998; Wanner and Wood, 2002; Harper et al, 1993; Gerhardt et al, 2000; Oberlender and Tuan, 2000). There are however, reports of blocking antibodies that do not cause complete obliteration of cadherin function, their conclusion was possibly a sub-optimal stoichiometry (Oberlender and Tuan, 1994). Other possibilities are that aggregation is not initially cadherin-based.

Some authors report that cell condensation mechanisms *in vivo* involve the activation of at least three pathways, none involving n-cadherin. The first requires the initiation of epithelial-mesenchymal interactions involving tenascin, growth factors, BMP-2, TGF β -1 and transcription factors, Msx-1 and -2 (Saxen and Thesleff, 1992; Hall and Miyake, 1992, 1995, 2000; Mackie, 1994; Dunlop and Hall, 1995), the second is the up-regulation of N-CAM by activin, a member of the TGF- β superfamily (Jiang et al, 1993) and the third is the up-regulation of fibronectin by TGF- β , further enhancing N-CAM accumulation (Chimal-Monroy et al, 1999). Reports concluded that it is by these three pathways that condensations are initiated and grow. It is important to note that n-cadherin is not indicated as part of these steps towards condensation, and the results described here suggest that it is not important in early condensation of tendon cells.

Cadherins may become important after the condensation process, with the formation of cell-cell junctions, either directly as adherens junctions or indirectly by mediating the formation of other cell-cell junctions. Certainly adherens junctions and gap junctions are characteristic features of interaction between adult tendon cells *in vivo* (McNeilly et al, 1996; Ralphs et al, 2002). Cells in suspension culture express major components of adherens and gap junctional complexes, including cadherins, vinculin and actin (Pavalko and Otey, 1994; Steinberg and McNutt, 1999). It is fair to acknowledge the formation of cell-cell junctions in this suspension culture; interestingly, adherens junctions are a pre-requisite for gap junctions (Gumbiner, 1987; Geipmans, 2004). The upregulation of E-cadherin has been shown to increase gap junctional communication and N-cadherin blocking antibodies can prevent adherens junction and gap junction formation in some cell types (Jongen et al, 1991; Meyer et al, 1992). This does not appear to be the case in the experiments of this study.

Other molecules indicated in early chondrogenesis, implying involvement in the cell condensation process are ECM components and integrins. Tenascin is present in the condensing mesenchyme of the limb but absent from the surrounding cells (Ros et al, 1995), and is known to be involved in cell-adhesion (Chiquet-Ehrismann et al, 1991; Riou et al, 1992; Chiquet-Ehrismann, 2004) and tendon development (Hurle et al, 1989,1990). Also COMP, or thrombospondin 5, has been found to enhance several early aspects of chondrogenesis by slowing the rate of cell growth (Kipnes et al, 2003). COMP contains an RDG sequence and is known to interact with fibronectin (DiCesare et al, 2002), indicating yet another possible role for integrin signalling in the condensation process. It would be interesting to know how blocking antibodies to integrin function or RGD peptides affected cell aggregation in the short term cultures.

Aggregation may involve cell surface receptors not tested, for example NCAM, as indicated above, but may also involve cell-matrix interactions. Areas of mesenchyme in the developing limb differentiate and produce elevated levels of fibronectin, induced by bFGF and TGF β (Hentschel et al, 2004). This in turn initiates adhesion mediated pre-skeletal condensations. If cells interact with fibronectin in the initiation of condensation, the cell-cell adhesion may involve integrins and cell-surface fibronectin. Cells held within this fibronectin scaffold then proliferate and increase cell-cell interactions (Hall and Miyake, 1995). An additional member of the TGF β family, GDF-5, is known to cause an increase in cell adhesion and cell proliferation when cultured in chondrocyte suspensions, consisting of isolated cells in a sealed bijoux tube on a roller and micromass cultures (Francis-West et al, 1999; Hatakeyama et al, 2004).

Gap junctions comprised of connexin 43 or 32 are prominent between tendon cells *in vivo* (McNeilly et al, 1996), but distribution and apparent amounts are different in the short term cultures. As in pellet cultures, connexin 32 was widespread and connexin 43 was present, although sparse. Connexin 32 label was in two positions within the cell, extensively at the cell surface at cell interfaces, and often intracellularly at a prominent perinuclear position. The former is a potential site of functional gap junction communication, whereas the latter is likely to be part of the intracellular trafficking system (Windoffer et al, 2000). Connexin 32 is turned over rapidly at the cell surface, with a half-life of around 3 hours. Connexin trafficking involves the golgi and connexin oligomerization and occurs in the golgi

membranes (Musil and Goodenough, 1993; Koval et al, 1997); it seems likely that the intracellular label involves a concentration of connexin 32 at this site.

Functionally, the two gap junctions appear to have different roles in tendon, as discussed in chapter 4. Briefly, signalling via connexin 32 is stimulatory, whereas connexin 43 is inhibitory to collagen under load (Waggett et al, submitted; Waggett et al, 2001). As in the pellet culture system and early developing tendons, the collagen stimulatory pathway appears to be favoured in conditions in which complete tissue architecture has yet to be established.

Type I collagen was labelled in most cells in suspension culture, including chondrocytes. Intracellular labelling was observed from time zero, suggesting that type I collagen seen at this stage was made by the cells when they were cultured as monolayers and retained after subculture, when cells were transferred to the high density culture. Its presence in chondrocytes was indicative of their de-differentiation in monolayer culture, with loss of type II collagen and onset of type I synthesis (Benya et al, 1978; Grundmann et al, 1980). The presence of extracellular type I collagen within three hours in the cell aggregates, taken along with the results of pellet cultures (chapter 5) demonstrates that the cells are able to rapidly lay down ECM after only a few hours in a high density 3-D culture. This is not the case for type III collagen which remained intracellular in the short term despite the fact that in longer term suspension cultures type III collagen is deposited (see below). It is interesting that type III collagen often precedes type I collagen deposition in development and in repair (Williams et al, 1980; Maffuli et al, 2000), but that this does not occur in all circumstances, i.e. in these short term intervals in suspension.

Collagen production from tendon fibroblasts in other type of suspension cultures showed that initial type I collagen production was at similar levels as *in vivo*, however, on an hourly basis this level dropped (Quinones et al, 1989). If taken together, these results imply the expression of type I collagen may fall, but the cells can retain the ability to deposit type I collagen in the correct environment, for example, a cell aggregate.

Long term aggregation

Long term cultures formed large aggregates, which showed a degree of tissue orientation and were responsive to growth factors TGF β and IGF-1. This was after cells having 3 passages in monolayer culture to expand cell numbers, indicating

some retention of tendon-like characteristics through the monolayer culture period. Some aggregates were of a size and shape relevant to potential tissue engineering application, being up to 3cm long. Tissue organisation, growth factor responsiveness and potential applications of these cultures are discussed below.

A variety of aggregate shapes were formed within cultures, with rod shapes, curved sheet-like structures and spherical or ovoid structures. These shapes must have been produced by a combination of influences, including cell interactions with one another and with the matrix they deposit, the rolling of the culture tube and the cells/aggregates hitting its walls, and by hydrodynamic forces involving fluid movements caused by the rolling and rocking of the tubes on the roller.

Cell aggregates interact with the rotating and rocking wall of the dialysis tube, but do not adhere to it. As the cell aggregate stays at the lower part of the tube, as it lies horizontally, they tend to be rolled into rounded structures in response to the circumferential motion of the tube, and moved longitudinally backwards and forwards in the tube as it rocks slightly, tending to form elongated structures. However, these movements cannot be isolated from hydrodynamic influences, as cells will be experiencing shear and rotational forces from fluid movements generated by the motion of the tube. Elongated aggregates tend to form in the main part of the tube and rounded one towards the ends, perhaps reflecting differences in liquid behaviour in these two regions. Fluid-flow stresses are known to have effects in a range of cell types, for example inducing differentiation of embryonic stem cells into endothelial cells, and increasing production of cell adhesion molecules, vascular endothelial (VE)-cadherin and PECAM-1 (Yamamoto et al, 2004). Endothelial cells subjected to a laminar flow can orientate themselves along the direction of flow (Yamamoto et al, 2003). In connective tissues fluid shear is involved in bone cell activation (Bacabac et al, 2004) and is known to enhance chondrogenesis (Buschmann et al, 1999). These mechanical and fluid stresses are important in the system as they do result in some mechanical stimulation of the cultures. They would therefore, appear to be important in the culture structure and organisation.

Cell-cell interactions appear to be stronger in tendon cells than in chondrocytes, as tendon cells are able to form structures in this system, whilst chondrocytes are not. This is clearly a departure from the early stage aggregation behaviour where tendon cells and chondrocytes behave identically. As described above, there is

evidence that this process is independent of n-cadherin; however it may be that this becomes more important in the large scale aggregates. N-cadherin is expressed in the long term cultures, whereas in cartilage cultures it is known to decrease with differentiation (Tavella et al, 1994). It may be that the tendon cells develop the cadherin based adherens junction contacts observed *in vivo* (Ralphs et al, 2002) as the cultures differentiate, whereas chondrocytes do not. Differentiated chondrocytes *in vivo* have few cell-cell contacts and are isolated by the matrix they deposit (Buckwalter, 1983), therefore lose contact with one another, whereas tendon cells *in vivo* are attached by cell junctions (McNeilly et al, 1996; Kannus, 2000; Ralphs et al, 2002).

Cell and matrix organisation in older cultures can resemble that of the mature tendon tissue (Kannus, 2000), with parallel rows of cells separated by orientated collagenous matrix. This was particularly prominent in the more elongated aggregates. It appears therefore that the cells have an intrinsic ability to reform *in vitro* the structures reminiscent of their original tissue in this culture system. Experiments using fibroblasts from different tissues showed that when seeded into collagen gels, tendon, dermal and cornea fibroblasts did retain a certain intrinsic 'memory' (Doane and Birk, 1991). Tendon fibroblasts formed parallel cell arrays within non-orientated collagen gels, corneal fibroblasts orientated orthogonally and dermal fibroblasts showed no apparent organisation. Even in the spherical aggregates of this study cells still formed an ordered structure, with layers of matrix and cells at the periphery and with areas of organised collagen in central regions of these aggregates. This also occurred in pellet cultures where the SEM images showed parallel orientation of cells at the pellet periphery.

Cells were evenly distributed in smaller rounded cell aggregates, rod-like structures and thin sheets. In larger aggregates cell distribution appears lower in the centre, which may be due to nutritional problems and/or oxygen deficiencies. Diffusion distances are relatively large to the centre of the aggregates, and thus low oxygen levels might be expected (Baltz and Biggers, 1991; Levick, 1995). Low oxygen tension enhances anaerobic metabolism and can be assessed by measuring the amount of lactate, a by-product of glycolysis, present in these areas (Malda et al, 2004). Evidence for some lactate tolerance of tendon cells has been shown (Klein et al, 2001), where cells resume synthesis of collagen levels corresponding to that *in vivo*. Cartilage is probably one of the most well known tissues to principally rely on glycolysis for its respiration (Otte, 1991; Archer and

Francis-West, 2003). This leads to the maintenance of a relatively small population of cells within the tissue (Buckwalter and Mankin, 1998). Similar, and perhaps even more extreme conditions can occur in the intervertebral disc. Cells in central parts of the disc can be between 7-8mm from the nearest blood supply (Urban and Roberts, 2003), in these areas oxygen-tension is low and lactate production increases. This results in a decrease in the rate of matrix synthesis and eventually cell death and degeneration of the disc (Urban and Roberts, 2003). These observations show that when culturing large cellular constructs, it is important to have a cell density appropriate to the nutritional conditions available, for example in cell populations with a restricted nutrient supply only a small number of cells can be supported. Many bioreactors have built-in oxygen chambers to counteract this (Leclerc et al, 2004) and perfusion systems have been shown to combat this problem (Radisic et al, 2003).

Cytoskeletal elements actin and vimentin can be seen in the cell aggregates. Vimentin is evident in spherical, sheet-like and rod-like cell aggregates with bright immunolabel. Vimentin is the typical intermediate filament of connective tissues (Wang and Stamenovic, 2002), associated with changes in mechanical load in chondrocytes (Durrant et al, 1999), and in tendons particularly associated with fibrocartilage (Ralphs et al, 1991). Vimentin expression within these cultures is a clear difference between the *in vivo*, rather weak expression in mature tensional tendon, and the *in vitro* situation. Vimentin is, however, present in developing chick flexor tendons, shown in chapter 3, which may indicate that the state of the tendon construct has similarities with embryonic tendon. In addition, some aspect of the fluid flow or other physical situation of the culture system may be causing cells to express vimentin. There was no evidence of chondrogenic differentiation, as occurs in fibrocartilage (Ralphs et al, 1991; Rufai et al, 1992), in these cells as examined by labelling for chondrocytic products.

Prominent labelling of actin fibres occurred only in organised regions of sheet-like and rod-like shaped aggregates and not in spherical ones. It is striking that these are in the regions that most resemble tendons in structure, which also contain prominent actin fibres similar to tendon cells *in vivo* (Ralphs et al, 2002). In large, rounded aggregates only the green 'haze' of labelling was observed indicating the presence of a filamentous actin network, but no distinctive actin fibres. At the periphery of the rounded aggregates were areas of positively labelled actin fibres, where the cells were orientated in parallel sheets around the aggregate. This is

similar to the periphery of the pellets, where orientated cells also occur therefore, there is an association of actin fibres and orientated cells. Fibroblasts are able to generate tensile force, involving actin stress fibres, when cultured in collagen gels (e.g. Halliday and Tomasek, 1995; Schneider et al, 1999; Gentleman et al, 2004), and so it may be that cells are able to do so in the large scale cultures. Actin stress fibres are characteristic of tendon cells experiencing or exerting tensile loading (Stephens et al, 1992; Halliday and Tomasek, 1995; Ralphs et al, 2002) and it may be that such loads exist where there are orientated tendon fibroblasts. Cell orientation and actin fibres together suggest intrinsic generation of tension and organised parts of the cultures.

Extracellular matrix molecules and the cytoskeleton have a reciprocal relationship where the ECM can induce effects on cell shape and function (Mooney et al, 1995) and cytoskeleton effects matrix organisation. The actin cytoskeleton is known to be associated with fibronectin deposition (Schwarzbauer and Sechler, 1999) and the involvement of both is implicit in cell differentiation (Antras et al, 1989). In addition, the actin cytoskeleton can affect orientation of developing tissues. Actin stress fibres orientate cells and the ECM of the intervertebral disc in development (Hayes et al, 1999). Disc cell alignment, induced by the actin cytoskeleton, was observed before a matrix was deposited corresponding with the cell orientation. It may be therefore, that in some culture regions the actin fibres are involved in cell orientation and matrix deposition. This would correspond with results from chapter 3, showing longitudinally orientated actin fibres present in early tendon development.

N-cadherin and vinculin are involved in connecting actin stress fibres between tendon cells longitudinally *in vivo* (Ralphs et al, 2002). In cultures derived from adult cells both labels were bright but diffuse at the cell surface, rather than being present as punctate, high intensity spots between cells. Interestingly, these were observed in the cultures derived from embryonic cells, indeed this was the only observed difference between adult and embryonic cells in all of the culture experiments. It is not clear why there should be such a difference, as the *in vitro* morphogenic situation would appear to be the same. It could be that label intensity in adults is affected by expression of additional cadherins types, as suggested by increased pan-cadherin labelling masking n-cadherin based junctions, but fundamental reasons for this difference remain obscure.

Connexin distribution, as discussed above, was similar to pellets and the short term aggregation experiments, with similar implications to collagen synthesis (Waggett et al, submitted; Waggett et al, 2001). Even though cultures showed a degree of orientation, the organisation of connexins was not the same as *in vivo* (McNeilly et al, 1996). As in the pellet and short term cultures connexin 32 was much more widespread than connexin 43, with an indication that connexin 43 was becoming more prominent in older cultures. As indicated above, and in chapters 3 and 4, connexin 32 represents a stimulatory pathway for collagen synthesis and connexin 43 an inhibitory one. It appears that connexin 32 dominates whilst tissue is being established, and connexin 43 appears later. Connexin 43 is mechanically regulated (Banes et al, 1995) and its expression and phosphorylation is upregulated in response to load. It may be that with cell and matrix organisation and stress fibre formation, the extent of mechanical stimulation to cells in these older cultures is sufficient to promote connexin 43 expression. Therefore, the stimulatory pathway appears to be active when tissue is being constructed *in vivo* and *in vitro*, and negative regulation appears when tissue starts to be stressed.

Immunolabelling shows abundant ECM was produced by cells within 24 hours of culture with label appearing to increase over 7 and 14 days in culture. In 7 and 14 day cultures, regions of aggregates showing cell organisation also showed matrix organisation, with types I and III collagen between orientated rows of tendon cells. Type III collagen was present throughout the matrix, and was thus perhaps more like the situation in developing or repairing tendon (Birk and Mayne, 1997; Maffulli et al, 2000). Label for type I collagen, whilst positioned between cell rows, did not display a strong fibrous orientation like type III collagen. It may be that type I collagen fibres were too fine to be resolved and thus just appeared as a finely textured fluorescence, whereas the type III collagen formed larger structures. Certainly it was clear from the TEM studies of pellet cultures that the fibroblasts were capable of laying down orientated bundles of fine collagen fibres. *In vivo*, type III collagen is characteristic of the material around tendon fascicles of type I collagen, and the fascicular structure is established early in tendon differentiation (Birk and Mayne, 1997). An intriguing possibility is that cells were making some kind of fascicular structure visible only in immunolabel for type III collagen. This happens around day 14 in development (Birk and Mayne, 1997), with some evidence that it may begin earlier (see chapter 3), with deposition of type III collagen around groups of cells.

The effects of serum and growth factors showed that cultures treated with low serum formed no cell aggregates. Experiments using 5% serum, TGF β and IGF-1 resulted in well-structured cell/matrix assemblies. There were no discernable qualitative effects on matrix synthesis or cell adhesion in aggregates fed with 5%FBS/DMEM and either growth factor treatment, therefore presence of the growth factors enabled compensation for a reduction in serum content. TGF β is known to increase the synthesis of n-cadherin and integrins, matrix synthesis and cell proliferation (Massagué et al, 1985; Moses et al, 1987; Fine and Goldstein, 1993; Klein et al, 2002; Heinemeier et al, 2003). IGF-I is known to increase matrix synthesis (James and Bradshaw, 1984; Roberts et al, 1985; Massagué et al, 1985; Molloy et al, 2003; Grazul-Bilska et al, 2003) and could promote cell adhesion; the upregulation of IGF-1 receptor increases expression of e-cadherin in tumour cells (Mauro et al, 2001, 2003), therefore both cells can enhance features important in forming these structures, i.e. molecules involved in cell-cell adhesion, and the ECM. Cell aggregates produced in dialysis membranes with smaller pore sizes, MWCO of 50kDa and 100kDa, showed no qualitative differences in ECM molecules retained by the cells, cell junctional proteins or connexins expressed implying the pore sizes were of no significance in this system. Interestingly, although pellet cultures grown in DMEM containing 1% serum were able to synthesise and secrete a collagenous matrix, cells within these long term suspension cultures were unable to do so. The significant difference between these systems is motion. It is unclear as to why pellet cultures appeared relatively unaffected by the low serum content, possibly because they are encouraged into contact with one another by centrifugation and are able to establish a stable environment. Whereas, there is a significant difference in the cells ability to adhere to one another in these suspension cultures when fed with 1% serum. The cells are unable to adhere and consequently fail to form any significant structure, whether the presence of low serum levels affects cell surface adhesion protein expression is unclear.

Quantitative studies were disrupted by the commercial decision of SpectraPor to discontinue the production of the DispoDialyzer tubes with the 300kDa MWCO membranes. Because of this, data for the 300kDa MWCO tubes is incomplete as it was planned to do a complete series of quantitative analysis of the cell aggregate, media of the inner tube and the outer growth medium. Experiments with 50kDa and 100kDa MWCO dialysis tubes were performed, but the cell aggregate had to be used for morphological studies for comparison with the 300kDa MWCO

membranes. In consequence the overall data is incomplete and most investigations have very low numbers. Statistical analysis is not therefore possible. However, the results have been incorporated here for interest, information and discussion even though they cannot be regarded as statistically valid.

In experiments where collagen was measured in the cell aggregates, inner tube media and total growth media, collagen within the aggregates was high, in excess of 60% of the total. The implication from these results is that this system encourages collagen retention by the aggregates to greater levels than found in the pellet cultures in chapter 5 (15-25% of collagen was retained). It is likely that if these cultures were put under any mechanical strain the collagen amounts would be increased, and the cell aggregates may retain larger amounts of collagen (Chiquet et al, 1996).

The only comparisons to be made between the 50kDa, 100kDa and 300kDa MWCO tubes, is that collagen released into the total growth media is relatively higher in 300kDa MWCO cultures than in the 50kDa and 100kDa MWCO tubes. This corresponds with the large molecular weights of collagens, described above, collagen fragments from cultures grown in 300kDa MWCO membranes are able to pass through the membrane easily. It is also possible that nutrients have increased access to the cell aggregates again due to the larger pore size, enabling the production of elevated levels of collagen. The experiments with 50kDa and 100kDa MWCO tubes show that collagen is still released to the main culture medium, even though fibrillar collagens have molecular weights of approximately 95kDa per alpha chain (Dehm and Kefalides, 1978; reviewed Bornstein and Sage, 1980), so an assembled molecule would be approximately 285kDa. These would only just fit through the 300kDa MWCO membrane and then only with the correct orientation. This indicates collagen released into the media has smaller molecular weights and consequently that the collagen is being degraded. This implies the presence of matrix metalloproteases (MMPs) that digest collagen *in vivo* (Harper, 1980). MMPs have a substantial role in matrix degradation and are known to cleave collagens into very small pieces, essentially down to tripeptides (reviewed Overall, 2002; Stamenkovic, 2003). In the context of this culture system MMPs may have a role in degrading collagen fragments synthesised by the cells, enabling released collagen to pass through the membrane into the growth media.

Growth factors added to these long term suspension cultures showed a compensation for lower serum levels as previously described, and both growth factors have similar effects on collagen synthesis compared to the 5% serum controls. IGF-I appeared to increase collagen production over TGF- β treatment. Similar results would have been expected from each growth factor treatment. The growth factor TGF- β however, is known to have a short active life *in vitro* (Coffey et al, 1987; Wakefield et al, 1990). It is possible the initial effects of IGF-I have a knock-on effect on the cells and its application enabled lasting effects on the cells. It is also possible that cells within the cultures were synthesising their own supply of growth factors, either intrinsically or that IGF-I induced the production of other growth factors (Banes et al, 1995; Tsuzaki et al, 2000; Dalhgren et al, 2005).

The definitions of tissue engineering are varied (Langer and Vacanti, 1993), but most incorporate words to the effect 'Tissue engineering is the development and subsequent replacement of diseased or damaged living tissue with living tissue designed and constructed for the needs of each individual to restore, maintain and improve function'. Tissue engineering in tendon has rapidly evolved (reviewed in chapter 1). Other attempts to engineer tendon have focused on cell-seeded scaffolds, mainly because of the strain the replacement tissue would be under instantly upon being implanted. The scaffolds used in these situations are growing rapidly and can be made of natural material or synthetic, biodegradable or not (DeFranco, 2004). Overall, this large scale culture system shows some promise as a method for producing fibrous connective tissue structures in the absence of any exogenous scaffold structures. Tendon cells have been shown to produce organised and orientated cell-matrix structures sharing some features in common with both adult and embryonic tendon structure. Substitution of serum with growth factors, albeit in the presence of a small serum component, suggests that a defined culture medium could be developed for use with them. The absence of scaffold and a potentially defined culture medium would be of considerable advantage in a tissue-engineering context. Further possible developments of this system are discussed in chapter 7.

CHAPTER 7
General Discussion

7. GENERAL DISCUSSION

Overall, the work described in this thesis has been aimed at examining how events in the development of tendons may be replicated *in vitro* with particular reference to cell-cell interactions and matrix deposition. More specifically, evidence is presented that 3-D cell organisation, with the increased possibility for cell interaction provided, appears to enhance the ability of tendon cells to lay down an ECM compared to conventional monolayer culture. As described in the chapters 4, 5 and 6, this ECM retention could relate to there being cell-bound spaces into which cells can deposit their matrix, which would be very limited in monolayer. However, there remains a considerable loss of collagenous material into the culture medium, indicating the pellet system is not ideal. One possibility is that the aggregates are not adequately 'sealed'. Perhaps the presence of tendon macrostructure such as epitenon and fascicle-bounding endotenon is necessary, or the presence of tight, sealing junctions between cells and their processes. An alternative possibility is that this pellet system models matrix synthesis and catabolism, rather than actual matrix assembly. The degradation of connective tissue ECM is important in matrix turnover and remodelling (Rees et al, 2000; review Overall and Lopez-Oten, 2002; Archambault et al, 2002a, b; Stamenkovic, 2003). MMPs initiate the extracellular degradation of collagen and are known to increase expression after injury, aiding in the removal of debris and remodelling deposited scar tissue (Cleutjens et al, 1995; Ishiguro et al, 1996; Maricourt et al, 1995). Their role in this system is yet to be investigated. There are tantalising clues that the large scale cultures may be an improvement in the deposition and retention of collagen compared to monolayers, micromass and pellet cultures, although statistical difficulties mean that this is not conclusive.

Cells in the long term 3-D roller culture system essentially express the same cell junction molecules and ECM as the embryonic tendons. One key observation suggesting that this 3-D system does indeed model tendon development better than a monolayer is the expression of gap junction proteins. Early embryonic tendon and the long term 3-D culture system both show strong expression of connexin 32 and weak or absent connexin 43 label. In all of these systems it may be that connexin 32 is associated with the promotion of collagen synthesis, and connexin 43 with inhibition (Waggett et al, submitted, Waggett et al, 2001), suggesting that under condition of low matrix, stimulatory pathways are enhanced. Monolayers do not exhibit this differential expression, perhaps correlating with a poor ability to deposit matrix. Cell-cell junctions and their components are known

to be important in many developmental processes, including chondrogenesis (Oberlender and Tuan, 1994; Reviewed in Pizette and Niswander, 2001; Hoffman et al, 2003; Tuan, 2003), osteogenesis (Katagiri and Takahsi, 2002; Bouletreau et al, 2002) and possibly tendon development (Edom-Vovard and Duprez, 2004).

As indicated in chapter 6, the large scale cultures show potential for tissue engineering applications. This potential is the main focus of the remainder of this discussion. As described in chapter 6, the definition of tissue engineering stands as: 'Tissue engineering is the development and subsequent replacement of diseased or damaged living tissue with living tissue designed and constructed for the needs of each individual to restore, maintain and improve function' (Langer and Vacanti, 1993). In the context of tendons, it is of potential value in repair of injury or severe rheumatic disease. A number of approaches have been attempted in experimental systems. These may involve the creation of materials to be implanted to augment tendon function with the aim of having them invaded and populated by host tissues. One scaffold based construct involved in tendon repair to have been accepted at clinical level is GraftJacket (Wright Medical, Arlington, TN), a biological scaffold used in the augmentation of tendon repair (Lee, 2004). It consists of a mesh of ECM macromolecules including type I, III, IV and VII collagen, elastic, proteoglycans, laminin, tenascin and bFGF and has been used in rotator cuff tendon reinforcement (Lee, 2004). It can be revascularised between 4-7 days with host cell infiltration complete at 14 days. The cell population measured within the construct is the same as the host tissue surrounding it (Chaplin et al, 1999). Following this re-population, histochemical studies show the protein framework is slowly incorporated and replaced by normal host tissue, undergoing gradual remodelling (Chaplin et al, 1999). There is no host immunological response (Brigido et al, 2004) and the construct does not undergo processing that would result in cross-linking and subsequent inflammation (Chaplin et al, 1999). The strength of GraftJacket has also been tested and found that it has a comparably high suture retention load of approximately 120N (Rubin et al, 1999). This may be a promising material with a range of uses, but it remains far from a complete tendon replication *in vitro*. Other scaffolds used clinically with similar functions include Restore Orthobiologic Implant (DePuy, Warsaw, IN), Permacol (Zimmer, Warsaw, IN) and OrthoMend (Stryker, Mahwah, NJ), however, regular failures remain a significant problem with these structures. Also, theoretically, this is not tissue engineering, as the constructs have no cells when they are implanted and therefore, the construct is not a 'living tissue'.

Alternatively, scaffold structures may be seeded with host cells, either tissue specific cells or mesenchymal stem cells and then implanted (Bosch and Krettek, 2002; Jorgensen et al, 2004; Sun et al, 2005; Uematsu et al, 2005). Mesenchymal stem cells have been used in efforts to create almost every type of tissue *in vitro*, including tendon (Awad et al, 1999; Kall et al, 2004), each relying heavily on a scaffold component and each having limited success. A further approach involves injecting cells directly into the injury site (Yamada et al, 2004; Crevensten et al, 2004), with the aim of the cells augmenting the natural tendon healing response. All methods have advantages and disadvantages. Scaffold-based systems involve placing a material into a host, which may elicit immune a response, or generate unwelcome breakdown products. Cells may not behave as anticipated, for example implanted cells may die or proliferate abnormally. Cells injected into damaged tissue may help to repair it, but will be of little use if defects are large or lengths of tendon are absent. Whatever the method, an integration of the engineered tissue with the host is vital. It is difficult to synthesise a construct without an original scaffold.

The large structures developed in chapter 6 could be regarded as scaffold-free tissue engineered constructs of tendon. They have, at best, a good degree of orientation and possibly a useful amount of organised matrix deposition. If they could be developed further they may provide a solution for some tissue repair solutions. Theoretically, large scale structures could be prepared from autologous tissue, thus reducing the need for a scaffold and eliminating the chance of a host immune reaction. Also, the natural assembly forces within the developing aggregates are unlikely to interfere with cell regulatory networks (Kelm et al, 2004). A different scaffold-free approach to tendon tissue engineering has recently been published, which although initially based as a monolayer, shows some promise in developmental and mechanical tendon characteristics (Calve et al, 2004). Therefore, scaffold-free tissue engineering approaches appear potentially viable. It is important therefore, to examine possible ways of improving such culture systems. Further developments would have to be made in several areas; consistency in the production of tendon-shaped aggregates, with fewer cells lost to incorrectly shaped structures; nutrition of large scale aggregates; strength of aggregates and specific structure and composition of aggregates to match potential implant sites.

Increasingly the reliability of appropriately shaped tendon-like cell aggregates probably requires alteration to the roller culture system. As indicated in chapter 6, the aggregate shapes are created by cell adhesion followed by interaction with the rotating and rocking walls of the inner dialysis tube and by hydrodynamic movements of medium within the tube. Modification of rotation and tilt rates may well result in the incorporation of more cells into rod shaped aggregates, with less waste to other shapes. There is also the intriguing possibility that modifying such parameters and changing shapes of the culture vessel could be used to produce different shapes altogether. Flattened, rounded culture vessels could be used to produce annular and disc shaped aggregates, for example, for other repair situations.

An important aspect of developing large scale culture constructs is in provision of nutrition. The system used here was derived to allow interaction of large numbers of cells in the long term, without disturbance by media changes. This relatively simple response may have its nutritive limitations, however, as there was evidence of reduced cell density in central regions of large aggregates. Within the limitations of the study, changing the pore size of the dialysis tubing had little effect on the structure and composition of the cell aggregates, so some other modification may be necessary. One simple approach may simply be to change the feeder media more frequently, increasing nutrient supply and removing waste. Alternatively, a more nutrient rich medium might be used, with additional supplements and possibly gassed oxygen, although this would have to be carefully controlled given tendon cells sometime anaerobic metabolism (Klein et al, 2001). A more sophisticated approach would be to develop a continuous flow through medium supply, although this inevitably increases the complexity of the system. This approach is used in other systems including cells in dialysis tubing to produce cell products, for example the 'Technomouse' used to produce monoclonal antibodies (Pannell and Cesar, 1995; Nagel et al, 1999) or some of the larger scale bioreactors systems (Falkenberg, 1994).

An alternative approach to increasing nutrient access to inner parts of large aggregates would be to try to form a vascular network within the aggregates. Microvascular endothelial cells will form tubular capillary-like structures under the appropriate situations in the correct ECM environment (Madri et al, 1988; Tada et al, 1994; Faitova et al, 2004). It would be interesting to determine whether seeding the initial tendon cell population with a proportion of microvascular endothelial cells

would allow the formation of a capillary-like network within the constructs, possibly with treatment using angiogenic factors, such as VEGF (reviewed in Patan, 2004). Such a network could improve access to the deeper parts of cells aggregates for exchange of materials and in the ultimate development, potentially link with the host vasculature if constructs were implanted. The development of microvascular networks in tissue engineering has recently been reviewed by Kannan et al (2005). Some of the constructs produced in the work described in chapter 6 had labelling features that could be interpreted as attempts to recreate a fascicular structure i.e. the expression and distribution of type III collagen. The fascicular structure is intimately associated with the vascular system of a tendon (Brockis, 1953; Fenwick et al, 2002) and thus could become involved in vascularisation of the constructs.

The 3-D hierarchical architecture of the tendon is also important in its ability to resist mechanical loading. The development of a fascicular system could therefore, also be important in functional aspects of tendon replacement. Measurements on whole tendons *in vivo* reveal strains under load of up to 7-8% (Abrahams, 1967; Woo et al, 1982; Baer et al, 1988). However, if strains such as this are applied to tendon samples *in vitro*, strain measurements subjected to cells do not exceed 1.2% (Screen et al, 2004). This is related to the sliding mechanism of fascicles under load. Replication of fascicular architecture would therefore be valuable from two points of view: provision of nutrition, and protection of cells from excessive strain.

For constructs to work in tissue engineering applications they must be strong enough to be handled and to perform a function once implanted. Scaffold-based systems would appear to have an advantage here as the necessary actual strength can be designed into the scaffold. For a scaffold-free approach to be successful, its strength would have to be at an appropriate level for handling and implantation. Increasing the strength of scaffold-free structures must involve increasing deposition and organisation of the extracellular matrix. Various aspects of this could be addressed by the application of mechanical load and treatment with growth factors. Cyclic tensile loading is known to increase types I and III collagen and tenascin expression by fibroblasts (Chiquet et al, 1996; Waggett et al, 1998) and improve collagen organisation in scar tissue (Zimmerman et al, 2000). The challenge is in applying mechanical load to the cell aggregates with the culture system. There are some devices designed to apply tensile strain to relatively small tissue constructs, for example the strain device used to load

tendon fascicles (Screen et al, 2004) or the flexercell 'tissue train' device (Garvin et al, 2003). These could almost certainly be adapted to loading the elongate cell aggregates with an appropriate cyclic loading regime. However, the disadvantage is that material would have to be removed from the culture vessel, introducing new handling stages and giving increased possibilities of contamination.

An alternative approach is to treat the cells with growth factors to enhance their matrix productions, to go some way towards mimicking the effects of mechanical loading by biochemical treatment. In the relatively simple experiments described in chapters 5 and 6, PDGF, bFGF, TGF β and IGF individually and in combination showed some abilities to at least replace unknown serum-based factors in promoting collagen secretion, although no treatment proved better than serum. These experiments would have to be extended to look at the dose response, different combinations and different factors to determine whether a significant enhancement could be made. An important aspect of such studies would be to aim towards producing a defined medium, without serum supplements so that the system can be fully defined for regulatory, commercial and legislative purposes. Assuming some enhancement in strength and size can be made, it may be possible to design a treatment regime where a construct from the culture system is implanted, and then mechanically conditioned in situ, by controlled re-mobilisation procedures for the patient to strain the implant and then gradually enhance its properties as treatment progresses.

If such implants were to be grafted into patients, consideration would have to be given to integration to host tissues. With tendon implants this means integrating with pre-existing tendon, with muscle and/or with bone. With tendon, one might hope that fairly minor variations on suturing techniques used in standard tendon repairs (Wilson and Sammut, 2003) may be applicable. Followed by the standard biological tendon repair process assisted by treatments, such as continuous passive motion and careful physiotherapy. At bone attachments, if a normal pattern of enthesis development were to occur, then bone must be encouraged to grow into tendon (Benjamin and Ralphs, 2000; Benjamin et al, 2002; Milz et al, 2002). Since this occurs by a process somewhat analogous to endochondral ossification, with metaplasia to fibrocartilage followed by fibrocartilage hypertrophy, vascular invasion and bone formation, perhaps some form of chondrogenic treatments with BMP or TGF β family (Schmitt et al, 2003; Grunder et al, 2004; Goldberg et al, 2005) members may be appropriate at the bone attachment, along

with VEGF to encourage angiogenesis (Molloy et al, 2003). A further point is that at other sites, the tendon construct must be encouraged not to integrate, i.e. it must not form adhesions along its route to the paratenon or tendon sheath. This is a significant problem if tendons are immobilised *in vivo* (Taras and Lamb, 1999; Feldscher and Schneider, 2003; Smith et al, 2004) and is likely to be a serious consideration with tissue engineered materials. At least the observation that the cell constructs appear to make an epitenon-like structure may indicate that progress could be made with this problem.

Overall, the large scale culture system could have tissue engineered applications. It has some important merits; it is simple, based on standard disposable items and therefore cheap and sterile. One could envisage creating 1 pack of disposable systems for growing tendon, or ligament implants if various issues were solved. Against it is that it is difficult to apply mechanical load to cells, which would probably result in a better construct, and there are some nutritive problems whose solution may increase the complexity of the system and therefore negate some of the benefits. Nevertheless, this simple system needs further development to assess the extent to which it could be used to form reasonably sized, well organised and useable connective tissue implants.

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Appendices

Absorbance Values

0.076	0.083	0.082	0.089	0.094	0.099	0.115	0.103	0.111	0.113	0.123	0.084
0.076	0.084	0.089	0.1	0.103	0.099	0.104	0.11	0.109	0.113	0.118	0.086
0.097	0.094	0.093	0.097	0.102	0.099	0.104	0.104	0.107	0.106	0.103	0.109
0.108	0.11	0.107	0.109	0.113	0.108	0.107	0.106	0.107	0.109	0.106	0.112
0.114	0.118	0.111	0.109	0.112	0.107	0.103	0.104	0.1	0.104	0.103	0.105
0.096	0.097	0.093	0.104	0.111	0.107	0.095	0.095	0.113	0.112	0.11	0.116
0.109	0.122	0.108	0.113	0.115	0.108	0.113	0.106	0.112	0.111	0.109	0.111
0.105	0.12	0.111	0.108	0.11	0.112	0.113	0.106	0.112	0.129	0.127	0.111

standard abs log st logOD

1	0.084	0	-1.07831 slope	0.16	1.3	1.2	2.0	2.8	3.9	10.1	5.0	8.0	9.0	15.4
2	0.086	0.30103	-1.06803 intercept	-1.10	1.4	2.0	4.1	5.0	3.9	5.3	7.6	7.2	9.0	11.8
3	0.095	0.477121	-1.02457 rsq	0.93										
4	0.099	0.60206	-1.00656											
5	0.099	0.69897	-1.00436		1.3	1.6	3.1	3.9	3.9	7.7	6.3	7.6	9.0	13.6 average
6	0.110	0.778151	-0.96059		1	2	3	4	5	6	7	8	9	10 standard
7	0.107	0.845098	-0.97265											
8	0.110	0.90309	-0.95861											
9	0.113	0.954243	-0.94692											
10	0.121	1	-0.91901											

Actual values

0.7	1.3	1.2	2.0	2.8	3.9	10.1	5.0	8.0	9.0	15.4	1.4
0.7	1.4	2.0	4.1	5.0	3.9	5.3	7.6	7.2	9.0	11.8	1.6
3.4	2.8	2.6	3.4	4.7	3.9	5.3	5.3	6.4	6.0	5.0	7.2
6.8	7.6	6.4	7.2	9.0	6.8	6.4	6.0	6.4	7.2	6.0	8.5
9.5	11.8	8.0	7.2	8.5	6.4	5.0	5.3	4.1	5.3	5.0	5.6
3.2	3.4	2.6	5.3	8.0	6.4	3.0	3.0	9.0	8.5	7.6	10.6
7.2	14.6	6.8	9.0	10.1	6.8	9.0	6.0	8.5	8.0	7.2	8.0
5.6	13.2	8.0	6.8	7.6	8.5	9.0	6.0	8.5	20.8	18.8	8.0

Test 1-way between subjects ANOVA
 Total
 Comparison 2week: 0.01, 0.05, FGF+PDGF, FGF+TGF, TGF+PDGF
 Performed by Laura Haughton

Date 24 January 2005

n 35

2week	n	Mean	SD	SE
0.01	7	30.860	12.846	4.8554
0.05	7	46.646	7.041	2.6611
FGF+PDGF	7	26.211	6.917	2.6144
FGF+TGF	7	19.186	6.784	2.5641
TGF+PDGF	7	45.141	18.864	7.1300

Source of variation	SSq	DF	MSq	F	p
2week	4012.791	4	1003.198	7.55	0.0002
Within cells	3985.954	30	132.865		
Total	7998.745	34			

Contrast	Difference	Tukey 95% CI	
0.01 v 0.05	-15.786	-33.657 to 2.085	
0.01 v FGF+PDGF	4.649	-13.223 to 22.520	
0.01 v FGF+TGF	11.674	-6.198 to 29.545	
0.01 v TGF+PDGF	-14.281	-32.153 to 3.590	
0.05 v FGF+PDGF	20.435	2.563 to 38.306	(significant)
0.05 v FGF+TGF	27.460	9.588 to 45.331	(significant)
0.05 v TGF+PDGF	1.505	-16.367 to 19.376	
FGF+PDGF v FGF+TGF	7.025	-10.846 to 24.896	
FGF+PDGF v TGF+PDGF	-18.930	-36.801 to -1.059	(significant)
FGF+TGF v TGF+PDGF	-25.955	-43.826 to -8.084	(significant)

Growth factor single treatments

Collagen present in 1 day cultures

Pellets			Media						Total							
1%																
Hydroxypr	Collagen	DNA	Hydroxypr	Collagen/DNA	Hydroxypr	Collagen	DNA	Hydroxypr	Collagen/DNA	Total Hydr	collagen	DNA	Total Hydr	Collagen/DNA		
1.35	9.42	3.24	0.42	2.91	1.08	7.43	3.24	0.33	2.29	2.41	16.85	3.24	0.74	5.20		
1.37	9.58	4.18	0.33	2.29	1.32	9.24	4.18	0.32	2.21	2.89	18.82	4.18	0.64	4.51		
1.32	9.27	3.37	0.39	2.75	1.08	7.58	3.37	0.32	2.25	2.41	16.86	3.37	0.71	5.00		
1.39	9.74	4.60	0.30	2.12	1.29	9.06	4.60	0.28	1.97	2.89	18.80	4.60	0.58	4.09		
1.35	9.43	5.01	0.27	1.88	1.01	7.07	5.01	0.20	1.41	2.36	16.50	5.01	0.47	3.29		
0.05																
Hydroxypr	Collagen	DNA	Hydroxypr	Collagen/DNA	Hydroxypr	Collagen	DNA	Hydroxypr	Collagen/DNA	Total Hydr	collagen	DNA	Total Hydr	Collagen/DNA		
1.38	9.66	2.24	0.82	4.31	1.47	10.29	2.24	0.66	4.60	2.85	19.95	2.24	1.27	8.91		
1.40	9.82	3.65	0.38	2.69	1.33	9.29	3.65	0.36	2.54	2.73	19.11	3.65	0.75	5.23		
1.37	9.58	1.23	1.11	7.79	1.54	10.78	1.23	1.25	8.78	2.91	20.36	1.23	2.36	16.55		
1.41	9.90	3.84	0.37	2.58	1.52	10.63	3.84	0.40	2.77	2.93	20.53	3.84	0.76	5.34		
1.29	9.04	3.50	0.37	2.59	1.30	9.07	3.50	0.37	2.59	2.59	18.11	3.50	0.74	5.18		
1.24	8.67	3.84	0.32	2.26	1.29	9.06	3.84	0.34	2.36	2.53	17.73	3.84	0.66	4.62		
1.43	9.98	4.79	0.30	2.08	1.46	10.19	4.79	0.30	2.13	2.88	20.17	4.79	0.60	4.21		
FGF																
Hydroxypr	Collagen	DNA	Hydroxypr	Collagen/DNA	Hydroxypr	Collagen	DNA	Hydroxypr	Collagen/DNA	Total Hydr	collagen	DNA	Total Hydr	Collagen/DNA		
1.43	9.99	3.96	0.36	2.52	1.24	8.69	3.96	0.31	2.20	2.87	18.88	3.96	0.67	4.72		
1.37	9.59	8.03	0.17	1.19	0.86	6.00	8.03	0.11	0.75	2.23	15.60	8.03	0.28	1.94		
1.44	10.09	5.62	0.26	1.79	0.85	5.97	5.62	0.15	1.06	2.29	16.06	5.62	0.41	2.86		
1.42	9.91	4.80	0.29	2.06	0.81	5.69	4.80	0.17	1.19	2.23	15.59	4.80	0.46	3.25		
1.38	9.67	5.67	0.24	1.71	0.88	6.14	5.67	0.15	1.08	2.26	15.82	5.67	0.40	2.79		
1.28	8.97	4.46	0.29	2.01	0.93	6.51	4.46	0.21	1.46	2.21	15.48	4.46	0.50	3.47		
1.67	11.71	2.73	0.61	4.30	0.85	5.92	2.73	0.31	2.17	2.52	17.63	2.73	0.92	6.46		
TGF																
Hydroxypr	Collagen	DNA	Hydroxypr	Collagen/DNA	Hydroxypr	Collagen	DNA	Hydroxypr	Collagen/DNA	Total Hydr	collagen	DNA	Total Hydr	Collagen/DNA		
1.38	9.63	4.43	0.31	2.18	1.86	13.04	4.43	0.42	2.94	3.24	22.66	4.43	0.73	5.12		
1.77	12.40	4.55	0.39	2.73	0.95	6.66	4.55	0.21	1.46	2.72	19.06	4.55	0.60	4.19		
5.50	38.49	5.85	0.94	6.58	0.90	6.29	5.85	0.15	1.07	6.40	44.78	5.85	1.09	7.65		
1.48	10.35	4.88	0.30	2.12	0.97	6.81	4.88	0.20	1.39	2.45	17.16	4.88	0.50	3.51		
1.50	10.52	3.86	0.39	2.73	0.97	6.80	3.86	0.25	1.76	2.48	17.33	3.86	0.64	4.49		
1.34	9.38	5.21	0.26	1.80	0.92	6.42	5.21	0.18	1.23	2.26	15.80	5.21	0.43	3.03		
PDGF																
Hydroxypr	Collagen	DNA	Hydroxypr	Collagen/DNA	Hydroxypr	Collagen	DNA	Hydroxypr	Collagen/DNA	Total Hydr	collagen	DNA	Total Hydr	Collagen/DNA		
1.75	12.23	6.28	0.28	1.95	1.09	7.60	6.28	0.17	1.21	2.83	19.83	6.28	0.45	3.16		
1.41	9.86	5.52	0.25	1.78	1.90	13.27	5.52	0.34	2.40	3.30	23.13	5.52	0.60	4.19		
1.49	10.43	3.98	0.37	2.62	1.10	7.71	3.98	0.28	1.94	2.59	18.14	3.98	0.65	4.56		
1.55	10.86	5.65	0.27	1.92	1.14	8.00	5.65	0.20	1.42	2.69	18.85	5.65	0.48	3.34		
1.73	12.13	4.77	0.36	2.54	1.01	7.10	4.77	0.21	1.49	2.75	19.23	4.77	0.58	4.03		
1.54	10.76	2.35	0.66	4.59	0.59	4.12	2.35	0.25	1.76	2.13	14.88	2.35	0.91	6.34		
1.59	11.11	4.11	0.39	2.70	0.57	3.99	4.11	0.14	0.97	2.16	15.09	4.11	0.52	3.67		

Growth factor single treatments

Collagen present in 7 day cultures

1%														
Pellet			Media			Total collagen			DNA			Total colla		
Hydroxy	Collagen in pellets	DNA	Hydroxy	Collagen/DNA	Collagen in media	DNA	Hydroxy	Collagen/DNA	Total collagen	DNA	Total colla	Collagen/DNA		
1.18	8.24	12.02	0.10	0.89	1.99	13.92	0.17	1.18	3.17	22.18	12.02	0.26	1.84	
1.11	7.76	7.74	0.14	1.00	1.48	10.37	0.19	1.34	2.59	18.13	7.74	0.33	2.34	
1.12	7.81	7.61	0.15	1.03	2.00	13.99	0.26	1.84	3.12	21.81	7.61	0.41	2.86	
1.44	10.11	15.85	0.09	0.84	2.08	14.39	0.13	0.91	3.50	24.50	15.85	0.22	1.55	
1.07	7.46	9.12	0.12	0.82	1.76	12.30	0.19	1.35	2.82	19.77	9.12	0.31	2.17	
0.98	6.84	8.26	0.12	0.83	1.82	12.73	0.22	1.54	2.80	19.57	8.26	0.34	2.37	
1.07	7.52	7.75	0.14	0.97	1.41	9.84	0.18	1.27	2.48	17.36	7.75	0.32	2.24	
0.05														
Hydroxy	Collagen in pellets	DNA	Hydroxy	Collagen/DNA	Collagen in media	DNA	Hydroxy	Collagen/DNA	Total collagen	DNA	Total colla	Collagen/DNA		
1.17	8.22	8.51	0.18	1.26	2.31	16.18	0.35	2.48	3.48	24.39	8.51	0.53	3.74	
1.01	7.07	11.70	0.09	0.80	3.07	21.49	0.28	1.84	4.08	28.56	11.70	0.35	2.44	
1.04	7.26	9.18	0.11	0.79	2.29	16.01	0.25	1.74	3.33	23.28	9.18	0.36	2.53	
1.06	7.41	8.35	0.13	0.89	2.37	16.58	0.28	1.99	3.43	23.99	8.35	0.41	2.87	
0.98	6.88	8.09	0.12	0.85	2.81	18.24	0.32	2.25	3.59	25.12	8.09	0.44	3.10	
1.00	6.97	8.72	0.11	0.80	2.52	17.85	0.29	2.02	3.52	24.63	8.72	0.40	2.82	
1.02	7.17	8.11	0.13	0.88	2.53	17.73	0.31	2.19	3.56	24.90	8.11	0.44	3.07	
FGF														
Hydroxy	Collagen in pellets	DNA	Hydroxy	Collagen/DNA	Collagen in media	DNA	Hydroxy	Collagen/DNA	Total collagen	DNA	Total colla	Collagen/DNA		
1.03	7.23	4.57	0.23	1.58	2.29	16.03	0.50	3.51	3.32	23.28	4.57	0.73	5.09	
0.89	6.22	10.17	0.09	0.61	2.94	20.59	0.29	2.02	3.83	28.81	10.17	0.38	2.84	
1.03	7.22	7.15	0.14	1.01	2.58	18.07	0.38	2.53	3.81	25.29	7.15	0.51	3.54	
1.08	7.42	5.14	0.21	1.44	2.91	20.34	0.56	3.95	3.97	27.78	5.14	0.77	5.40	
1.17	8.21	10.24	0.11	0.80	3.33	23.31	0.33	2.28	4.50	31.52	10.24	0.44	3.08	
1.13	7.92	10.23	0.11	0.77	2.39	18.74	0.23	1.64	3.52	24.66	10.23	0.34	2.41	
1.60	11.22	10.97	0.15	1.02	2.55	17.86	0.23	1.63	4.15	29.08	10.97	0.38	2.85	
TGF														
Hydroxy	Collagen in pellets	DNA	Hydroxy	Collagen/DNA	Collagen in media	DNA	Hydroxy	Collagen/DNA	Total collagen	DNA	Total colla	Collagen/DNA		
1.22	8.52	5.45	0.22	1.56	1.87	13.08	0.34	2.40	3.09	21.60	5.45	0.57	3.96	
1.29	9.06	9.29	0.14	0.98	3.11	21.75	0.33	2.34	4.40	30.81	9.29	0.47	3.32	
1.18	8.24	5.52	0.21	1.49	3.70	25.89	0.67	4.89	4.88	34.13	5.52	0.88	6.18	
1.26	8.81	7.91	0.16	1.11	4.16	29.12	0.53	3.68	5.42	37.93	7.91	0.89	4.80	
1.15	8.02	4.64	0.25	1.73	3.32	23.24	0.71	5.00	4.47	31.26	4.64	0.96	6.73	
1.68	11.74	3.54	0.47	3.32	3.85	26.93	1.09	7.81	5.52	38.87	3.54	1.56	10.93	
1.14	7.98	8.34	0.14	0.96	3.33	23.32	0.40	2.80	4.47	31.30	8.34	0.54	3.75	
PDGF														
Hydroxy	Collagen in pellets	DNA	Hydroxy	Collagen/DNA	Collagen in media	DNA	Hydroxy	Collagen/DNA	Total collagen	DNA	Total colla	Collagen/DNA		
1.13	7.88	6.25	0.18	1.26	4.57	32.01	0.73	5.12	5.70	39.90	6.25	0.91	6.38	
1.10	7.71	7.15	0.15	1.08	2.55	17.87	0.36	2.50	3.85	25.57	7.15	0.51	3.57	
1.19	8.35	11.36	0.11	0.74	3.25	22.74	0.29	2.00	4.44	31.10	11.36	0.39	2.74	
1.12	7.87	8.01	0.14	0.98	2.70	18.87	0.34	2.38	3.82	26.73	8.01	0.48	3.34	
1.13	7.92	9.51	0.12	0.83	2.97	20.78	0.31	2.18	4.10	28.70	9.51	0.43	3.02	
1.04	7.29	10.36	0.10	0.70	2.19	15.36	0.21	1.48	3.24	22.85	10.36	0.31	2.19	
1.19	8.35	4.89	0.24	1.71	1.55	10.83	0.32	2.22	2.74	19.17	4.89	0.56	3.93	

Growth factor single treatments

Collagen present in 14 days cultures

Pellet			Media			Total		
1%								
Hydroxypr	Collagen in pellets	DNA	Hydroxypr	Collagen /DNA	Hydroxypr collagen	DNA	Total Hydr Collagen	DNA
1.79	12.56	2.52	0.71	4.98	4.78	33.48	2.52	2.81
1.85	12.94	3.49	0.53	3.71	5.10	35.72	3.49	1.99
1.72	12.04	1.92	0.90	6.29	4.31	30.17	1.92	3.15
1.62	11.35	3.32	0.49	3.42	5.94	41.56	3.32	2.28
1.68	11.74	3.96	0.42	2.96	6.66	46.63	3.96	2.10
1.90	13.27	2.76	0.69	4.81	6.51	45.54	2.76	3.05
1.86	13.05	3.35	0.56	3.90	3.74	26.16	3.35	1.67
0.05								
Hydroxypr	Collagen	DNA	Hydroxypr	Collagen /DNA	Hydroxypr Collagen	DNA	Total Hydr Collagen	DNA
1.75	12.23	4.80	0.36	2.55	6.64	46.47	4.80	1.75
1.99	13.90	4.87	0.41	2.86	7.69	53.85	4.87	1.99
2.03	14.24	5.75	0.35	2.48	6.66	46.63	5.75	1.51
1.91	13.37	6.63	0.29	2.02	6.66	46.64	6.63	1.29
1.86	13.05	5.82	0.32	2.24	10.32	72.27	5.82	2.09
1.85	12.94	2.65	0.70	4.88	12.10	84.73	2.65	5.26
1.95	13.68	5.27	0.37	2.60	9.15	64.03	5.27	2.11
FGF								
Hydroxypr	Collagen	DNA	Hydroxypr	Collagen /DNA	Hydroxypr Collagen	DNA	Total Hydr Collagen	DNA
2.70	18.90	3.68	0.73	5.13	5.94	41.56	3.68	2.35
1.80	12.63	3.77	0.48	3.35	5.52	38.62	3.77	1.94
1.96	13.74	0.82	2.40	16.82	4.57	31.97	0.82	7.99
1.82	12.74	3.48	0.52	3.67	4.80	33.58	3.48	1.90
1.59	11.16	2.52	0.83	4.43	4.44	31.10	2.52	2.40
1.55	10.87	4.79	0.32	2.27	4.91	34.37	4.79	1.35
1.85	12.96	3.07	0.60	4.22	4.77	33.37	3.07	2.16
TGF								
Hydroxypr	Collagen	DNA	Hydroxypr	Collagen /DNA	Hydroxypr Collagen	DNA	Total Hydr Collagen	DNA
1.66	11.62	2.48	0.87	4.69	4.41	30.86	2.48	2.45
1.72	12.03	1.56	1.10	7.69	8.12	56.82	1.56	6.29
1.87	13.07	2.58	0.72	5.06	4.77	33.42	2.58	2.57
1.80	11.22	0.96	1.67	11.69	3.57	24.99	0.96	5.39
1.80	12.62	2.49	0.72	5.06	3.07	21.52	2.49	1.96
1.65	11.52	2.15	0.77	5.36	3.69	25.84	2.15	2.49
1.78	12.43	3.06	0.58	4.07	3.67	25.69	3.06	1.78
PDGF								
Hydroxypr	Collagen	DNA	Hydroxypr	Collagen /DNA	Hydroxypr Collagen	DNA	Total Hydr Collagen	DNA
1.66	11.62	3.97	0.42	2.93	2.13	14.89	3.97	0.95
1.63	11.42	4.13	0.40	2.77	4.50	31.47	4.13	1.48
1.69	11.83	4.43	0.38	2.67	3.58	25.05	4.43	1.19
1.49	10.46	3.89	0.38	2.69	3.33	23.29	3.89	1.24
1.82	12.76	3.98	0.46	3.21	3.49	24.42	3.98	1.33
1.76	12.30	4.13	0.43	2.98	12.20	85.37	4.13	3.38
1.85	12.94	5.58	0.33	2.32	8.72	61.04	5.58	1.89

Growth factor combination

Collagen in 1 day cultures

Pellets			Media						Total					
1%														
Hydroxypr	collagen	DNA	Hydroxypr	Collagen/DNA	Hydroxypr	collagen	DNA	Hydroxypr	Collagen/DNA	Total Hydr	collagen	DNA	Total Hydr	Collagen/DNA
0.86	6.02	4.55	0.19	1.32	1.14	8.00	4.55	0.25	1.78	2.00	14.01	4.55	0.44	3.08
0.57	4.01	4.84	0.12	0.83	1.14	7.99	4.84	0.24	1.85	1.71	12.00	4.84	0.35	2.48
0.68	4.75	3.57	0.19	1.33	1.07	7.47	3.57	0.30	2.09	1.75	12.22	3.57	0.49	3.42
0.64	4.46	5.66	0.11	0.79	1.21	8.48	5.66	0.21	1.50	1.85	12.94	5.66	0.33	2.29
0.68	4.73	4.36	0.15	1.08	1.83	12.79	4.36	0.42	2.93	2.50	17.51	4.36	0.57	4.01
0.54	3.75	4.41	0.12	0.85	1.02	7.14	4.41	0.23	1.62	1.56	10.89	4.41	0.35	2.47
0.55	3.84	5.66	0.10	0.68	0.88	6.16	5.66	0.16	1.09	1.43	10.00	5.66	0.25	1.77
0.05														
Hydroxypr	collagen	DNA	Hydroxypr	Collagen/DNA	Hydroxypr	collagen	DNA	Hydroxypr	Collagen/DNA	Total Hydr	collagen	DNA	Total Hydr	Collagen/DNA
0.57	4.01	5.83	0.10	0.69	1.01	7.10	5.83	0.17	1.22	1.59	11.11	5.83	0.27	1.91
0.43	3.00	4.78	0.09	0.63	1.29	9.05	4.78	0.27	1.90	1.72	12.06	4.78	0.36	2.52
0.80	4.19	5.24	0.11	0.80	2.25	15.76	5.24	0.43	3.01	2.85	19.95	5.24	0.54	3.81
0.56	3.92	4.51	0.12	0.87	1.81	12.68	4.51	0.40	2.81	2.37	16.61	4.51	0.53	3.68
0.59	4.10	4.43	0.13	0.93	1.64	11.47	4.43	0.37	2.59	2.22	15.57	4.43	0.50	3.52
0.65	4.58	5.62	0.12	0.82	1.26	8.80	5.62	0.22	1.57	1.91	13.38	5.62	0.34	2.38
0.56	3.92	5.61	0.10	0.70	1.42	9.92	5.61	0.25	1.77	1.98	13.85	5.61	0.35	2.47
FGF+PDGF														
Hydroxypr	collagen	DNA	Hydroxypr	Collagen/DNA	Hydroxypr	collagen	DNA	Hydroxypr	Collagen/DNA	Total Hydr	collagen	DNA	Total Hydr	Collagen/DNA
0.56	3.92	4.22	0.13	0.93	1.75	12.28	4.22	0.42	2.91	2.32	16.21	4.22	0.55	3.84
0.48	3.39	5.22	0.09	0.65	2.18	15.27	5.22	0.42	2.93	2.67	18.66	5.22	0.51	3.58
0.56	3.95	4.54	0.12	0.87	1.41	9.90	4.54	0.31	2.18	1.98	13.65	4.54	0.44	3.05
0.88	6.15	5.12	0.17	1.20	1.65	11.54	5.12	0.32	2.28	2.53	17.88	5.12	0.49	3.46
0.76	5.32	4.67	0.16	1.14	1.98	13.89	4.67	0.43	2.98	2.74	19.21	4.67	0.59	4.11
0.64	4.49	5.05	0.13	0.89	1.85	12.92	5.05	0.37	2.56	2.49	17.41	5.05	0.49	3.45
1.12	7.85	3.95	0.28	1.99	1.57	11.00	3.95	0.40	2.79	2.69	18.85	3.95	0.68	4.78
FGF+TGF														
Hydroxypr	collagen	DNA	Hydroxypr	Collagen/DNA	Hydroxypr	collagen	DNA	Hydroxypr	Collagen/DNA	Total Hydr	collagen	DNA	Total Hydr	Collagen/DNA
0.71	5.00	7.85	0.09	0.64	1.25	8.78	7.85	0.16	1.12	1.97	13.78	7.85	0.25	1.76
0.62	4.37	7.54	0.08	0.58	1.68	11.73	7.54	0.22	1.56	2.30	16.10	7.54	0.31	2.14
0.53	3.71	5.67	0.09	0.65	1.86	13.04	5.67	0.33	2.30	2.39	16.75	5.67	0.42	2.95
0.62	4.36	6.92	0.09	0.63	1.38	9.64	6.92	0.20	1.39	2.00	14.00	6.92	0.29	2.02
0.73	5.12	10.47	0.07	0.49	1.53	10.70	10.47	0.15	1.02	2.26	15.82	10.47	0.22	1.51
0.43	3.02	5.82	0.07	0.52	1.06	7.44	5.82	0.18	1.26	1.50	10.47	5.82	0.26	1.80
0.50	3.53	5.92	0.09	0.60	1.72	12.02	5.92	0.29	2.03	2.22	15.55	5.92	0.38	2.63
TGF+PDGF														
Hydroxypr	collagen	DNA	Hydroxypr	Collagen/DNA	Hydroxypr	collagen	DNA	Hydroxypr	Collagen/DNA	Total Hydr	collagen	DNA	Total Hydr	Collagen/DNA
0.80	5.58	6.04	0.13	0.92	2.99	20.90	6.04	0.49	3.46	3.78	26.47	6.04	0.63	4.38
0.50	3.51	3.62	0.14	0.97	6.33	44.31	3.62	1.75	12.23	6.83	47.82	3.62	1.89	13.20
0.65	4.53	7.01	0.09	0.65	5.02	35.15	7.01	0.72	5.02	5.67	39.69	7.01	0.81	5.66
0.47	3.26	4.85	0.10	0.67	3.15	22.08	4.85	0.65	4.55	3.62	25.34	4.85	0.75	5.22
0.65	4.52	5.78	0.11	0.78	4.34	30.35	5.78	0.75	5.25	4.98	34.87	5.78	0.86	6.03
0.51	3.58	4.65	0.11	0.77	1.61	11.27	4.65	0.35	2.42	2.12	14.85	4.65	0.46	3.19
0.70	4.89	5.27	0.13	0.93	2.25	15.74	5.27	0.43	2.99	2.95	20.63	5.27	0.56	3.92

growth factor combination

collagen content in 7 day cultures

Pellet			Media						Total						
1%															
Hydroxypr	Collagen	DNA	Hydroxypr	Collagen/DNA	Hydroxyproline in me	Collagen	DNA	Hydroxypr	Collagen/DNA	Total Hydr	Collagen	DNA	Total Hydr	Collagen/DNA	
1.48	10.38	2.70	0.55	3.85	1.28	8.99	17.99	2.70	0.48	6.87	3.98	27.87	2.70	1.48	10.34
1.22	8.57	2.62	0.47	3.26	1.75	12.26	24.52	2.62	0.67	9.34	4.38	30.63	2.62	1.67	11.67
1.53	10.70	1.95	0.78	5.49	2.93	20.48	40.95	1.95	1.50	21.01	4.87	34.12	1.95	2.50	17.51
1.43	10.02	2.86	0.50	3.50	1.33	9.29	18.59	2.86	0.46	6.50	4.19	29.31	2.86	1.46	10.25
1.58	11.08	2.51	0.63	4.41	1.17	8.20	16.40	2.51	0.47	6.53	3.68	25.78	2.51	1.47	10.27
1.54	10.77	2.36	0.65	4.56	2.88	20.15	40.31	2.36	1.22	17.06	5.24	36.70	2.36	2.22	15.53
1.53	10.70	2.48	0.62	4.31	1.54	10.75	21.50	2.48	0.62	8.98	4.02	28.13	2.48	1.62	11.33
0.05															
Hydroxypr	Collagen	DNA	Hydroxypr	Collagen/DNA	Hydroxyproline in me	Collagen	DNA	Hydroxypr	Collagen/DNA	Total Hydr	Collagen	DNA	Total Hydr	Collagen/DNA	
1.29	9.04	2.74	0.47	3.30	2.41	16.84	33.68	2.74	0.88	12.28	5.15	36.03	2.74	1.88	13.14
1.15	8.05	3.32	0.35	2.43	1.46	10.25	20.51	3.32	0.44	6.18	4.78	33.48	3.32	1.44	10.09
1.22	8.53	3.27	0.37	2.61	1.55	10.83	21.66	3.27	0.47	6.63	4.81	33.70	3.27	1.47	10.31
1.09	7.61	2.83	0.38	2.69	2.09	14.64	29.27	2.83	0.74	10.36	4.92	34.41	2.83	1.74	12.18
1.12	7.81	3.11	0.36	2.52	2.07	14.46	28.92	3.11	0.66	9.31	5.17	36.21	3.11	1.66	11.65
1.25	8.77	3.50	0.36	2.51	1.91	13.37	26.74	3.50	0.55	7.65	5.41	37.85	3.50	1.55	10.82
1.61	11.27	3.48	0.46	3.24	2.39	16.75	33.49	3.48	0.69	9.82	5.87	41.12	3.48	1.69	11.81
FGF+PDGF															
Hydroxypr	Collagen	DNA	Hydroxypr	Collagen/DNA	Hydroxyproline in me	Collagen	DNA	Hydroxypr	Collagen/DNA	Total Hydr	Collagen	DNA	Total Hydr	Collagen/DNA	
1.64	11.45	4.16	0.39	2.75	1.11	7.74	15.49	4.16	0.27	3.72	5.27	36.89	4.16	1.27	8.86
1.26	8.80	4.66	0.26	1.81	1.39	9.75	19.50	4.66	0.29	4.01	6.25	43.78	4.66	1.29	9.01
2.92	20.41	4.28	0.68	4.77	1.32	9.26	18.52	4.28	0.31	4.33	5.60	39.20	4.28	1.31	9.17
1.54	10.78	5.16	0.30	2.09	1.08	7.54	15.09	5.16	0.21	2.93	6.24	43.65	5.16	1.21	8.46
1.74	12.16	3.51	0.50	3.47	1.28	8.98	17.97	3.51	0.37	5.12	4.79	33.53	3.51	1.37	9.56
2.03	14.24	4.08	0.50	3.49	1.32	9.26	18.52	4.08	0.32	4.54	5.40	37.80	4.08	1.32	9.27
2.22	15.53	4.68	0.47	3.32	1.50	10.51	21.01	4.68	0.32	4.49	6.18	43.28	4.68	1.32	9.24
FGF+TGF															
Hydroxypr	Collagen	DNA	Hydroxypr	Collagen/DNA	Hydroxyproline in me	Collagen	DNA	Hydroxypr	Collagen/DNA	Total Hydr	Collagen	DNA	Total Hydr	Collagen/DNA	
2.05	14.38	3.52	0.58	4.08	1.46	10.25	20.50	3.52	0.42	5.82	4.98	34.89	3.52	1.42	9.91
2.11	14.77	4.90	0.43	3.02	1.50	10.50	21.01	4.90	0.31	4.29	6.40	44.78	4.90	1.31	9.15
1.63	11.42	4.53	0.36	2.52	1.39	9.75	19.49	4.53	0.31	4.30	5.93	41.48	4.53	1.31	9.15
2.48	17.33	4.38	0.56	3.95	1.31	9.19	18.37	4.38	0.30	4.19	5.70	39.88	4.38	1.30	9.10
1.62	11.32	3.25	0.50	3.48	1.27	8.87	17.73	3.25	0.39	5.45	4.52	31.83	3.25	1.39	9.73
1.38	9.68	3.58	0.39	2.71	1.04	7.25	14.51	3.58	0.29	4.06	4.81	32.29	3.58	1.29	9.03
1.82	12.73	3.69	0.49	3.45	1.35	9.43	18.85	3.69	0.37	5.11	5.03	35.23	3.69	1.37	9.56
TGF+PDG															
Hydroxypr	Collagen	DNA	Hydroxypr	Collagen/DNA	Hydroxyproline in me	Collagen	DNA	Hydroxypr	Collagen/DNA	Total Hydr	Collagen	DNA	Total Hydr	Collagen/DNA	
2.16	15.15	5.82	0.37	2.60	1.58	11.03	22.06	5.82	0.27	3.79	7.39	51.76	5.82	1.27	8.90
1.72	12.03	4.48	0.38	2.88	2.17	15.16	30.32	4.48	0.48	6.77	6.65	46.54	4.48	1.48	10.38
1.87	13.11	3.45	0.54	3.80	1.57	10.96	21.92	3.45	0.45	6.35	5.02	35.12	3.45	1.45	10.18
2.52	17.67	3.73	0.68	4.74	1.58	11.05	22.09	3.73	0.42	5.92	5.31	37.17	3.73	1.42	9.96
2.03	14.22	4.09	0.50	3.48	1.40	9.83	19.66	4.09	0.34	4.81	5.49	36.44	4.09	1.34	9.41

growth factor combination

Collagen content in 14 day cultures

Pellet			Media			Total			
1%									
Hydroxypr	Collagen in pellets	DNA	Hydroxypr collagen/DNA	Hydroxypr Collagen in media	DNA	Hydroxypr collagen/DNA	Total Hydr Collagen	DNA	Total Hydr collagen/DNA
2.20	15.39	4.55	0.48 3.38	23.57 184.99	4.55	5.17 36.22	25.77 180.38	4.55	5.68 39.60
2.44	17.07	4.84	0.50 3.53	18.88 132.13	4.84	3.90 27.32	21.31 149.20	4.84	4.41 30.85
2.81	19.65	3.57	0.79 5.50	20.26 141.84	3.57	5.67 39.70	23.07 181.48	3.57	6.46 45.20
2.81	19.65	5.68	0.50 3.47	21.40 149.81	5.68	3.78 26.45	24.21 169.45	5.68	4.27 29.92
2.81	19.85	4.38	0.64 4.50	23.53 184.71	4.38	5.39 37.75	26.34 184.35	4.38	6.04 42.26
2.84	19.85	4.41	0.64 4.50	7.78 54.48	4.41	1.78 12.34	10.62 74.33	4.41	2.41 16.84
2.73	19.08	5.66	0.48 3.37	6.46 45.19	5.66	1.14 7.98	9.18 64.28	5.66	1.62 11.35
0.05									
Hydroxypr	Collagen in pellets	DNA	Hydroxypr collagen/DNA	Hydroxypr Collagen in media	DNA	Hydroxypr collagen/DNA	Total Hydr Collagen	DNA	Total Hydr collagen/DNA
3.70	25.87	5.83	0.63 4.44	33.66 235.62	5.83	5.77 40.41	37.36 281.50	5.83	6.41 44.84
2.32	16.27	4.78	0.49 3.41	23.58 185.03	4.78	4.94 34.58	25.90 181.30	4.78	5.42 37.97
2.10	14.72	5.24	0.40 2.81	38.05 286.33	5.24	7.28 50.80	40.15 281.04	5.24	7.68 53.61
2.37	16.59	4.51	0.52 3.67	27.92 195.41	4.51	6.18 43.29	30.29 212.00	4.51	6.71 46.96
2.16	15.13	4.43	0.49 3.42	28.40 198.79	4.43	6.41 44.89	30.56 213.92	4.43	6.90 48.30
3.09	21.60	5.62	0.55 3.85	27.64 193.49	5.62	4.92 34.46	30.73 215.08	5.62	5.47 38.30
3.48	24.37	5.61	0.62 4.34	41.84 292.89	5.61	7.46 52.20	45.32 317.26	5.61	8.08 56.54
FGF+PDGF									
Hydroxypr	Collagen in pellets	DNA	Hydroxypr collagen/DNA	Hydroxypr Collagen in media	DNA	Hydroxypr collagen/DNA	Total Hydr Collagen	DNA	Total Hydr collagen/DNA
2.50	17.52	4.22	0.59 4.15	12.07 84.48	4.22	2.86 20.01	14.57 101.99	4.22	3.45 24.16
2.89	18.85	5.22	0.52 3.81	7.77 54.40	5.22	1.49 10.43	10.46 73.25	5.22	2.01 14.04
4.00	28.03	4.54	0.88 6.17	16.73 117.14	4.54	3.69 25.80	20.74 145.17	4.54	4.57 31.98
3.46	24.23	5.12	0.68 4.74	14.43 101.00	5.12	2.82 19.74	17.89 125.24	5.12	3.50 24.48
3.33	23.28	4.67	0.71 4.99	13.46 94.24	4.67	2.88 20.18	16.79 117.52	4.67	3.80 25.17
5.23	36.58	5.05	1.03 7.24	14.75 103.27	5.05	2.92 20.45	19.98 139.84	5.05	3.96 27.69
6.61	46.29	3.95	1.68 11.73	13.66 95.63	3.95	3.46 24.24	20.27 141.92	3.95	5.14 35.97
FGF+TGF									
Hydroxypr	Collagen in pellets	DNA	Hydroxypr collagen/DNA	Hydroxypr Collagen in media	DNA	Hydroxypr collagen/DNA	Total Hydr Collagen	DNA	Total Hydr collagen/DNA
1.58	11.03	7.85	0.20 1.41	13.17 92.21	7.85	1.88 11.75	14.75 103.23	7.85	1.88 13.16
1.38	9.64	7.54	0.18 1.28	14.14 98.99	7.54	1.88 13.13	15.52 108.63	7.54	2.06 14.41
1.21	8.48	5.67	0.21 1.49	13.42 93.95	5.67	2.37 16.56	14.63 102.44	5.67	2.58 18.06
1.31	9.14	6.92	0.19 1.32	14.37 100.60	6.92	2.08 14.55	15.68 109.74	6.92	2.27 15.87
2.13	14.91	10.47	0.20 1.42	24.50 171.51	10.47	2.34 16.38	26.83 186.41	10.47	2.54 17.80
2.53	17.73	5.82	0.43 3.04	25.07 175.49	5.82	4.30 30.13	27.60 193.22	5.82	4.74 33.18
1.38	9.66	5.92	0.23 1.63	17.08 119.58	5.92	2.89 20.20	18.46 129.24	5.92	3.12 21.83
TGF+PDGF									
Hydroxypr	Collagen in pellets	DNA	Hydroxyproline in pellet/DNA	Hydroxypr Collagen in media	DNA	Hydroxypr collagen/DNA	Total Hydr Collagen	DNA	Total Hydr collagen/DNA
2.58	18.09	6.04	0.43 3.00	18.03 126.22	6.04	2.99 20.90	20.62 144.31	6.04	3.41 23.90
2.12	14.85	3.62	0.59 4.10	30.19 211.35	3.62	8.34 58.35	32.31 226.20	3.62	8.92 62.45
1.99	13.96	7.01	0.28 1.99	14.07 98.46	7.01	2.01 14.05	16.06 112.42	7.01	2.29 16.04
2.25	15.76	4.85	0.46 3.25	41.88 293.01	4.85	8.63 60.42	44.11 308.77	4.85	9.10 63.67
2.41	16.88	5.78	0.42 2.92	43.05 301.37	5.78	7.45 52.13	45.46 318.24	5.78	7.86 55.04
1.82	12.71	4.65	0.39 2.73	34.02 238.17	4.65	7.32 51.24	35.84 250.88	4.65	7.71 53.97
2.18	15.23	5.27	0.41 2.89	28.62 200.33	5.27	5.43 38.02	30.79 215.66	5.27	5.84 40.91

MATRIX DEPOSITION BY TENDON CELLS IN SUSPENSION CULTURE

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INTRODUCTION: Tendons connect muscles to bone, and so must transmit high tensile loads. They consist of longitudinally running parallel bundles of collagen with rows of tendon cells between them. The tendon cells have an intimate relationship with one another and with the collagen bundles¹. Recent *in vitro* studies indicate that the two gap junctions that are important in regulation of tendon cells response to mechanical loading; connexin 32 containing gap junctions upregulates collagen synthesis, whereas connexin 43 gap junctions does the opposite².

An important aim of tissue engineering of tendons is to replicate such environments *in vitro*. Here we describe the behaviour of tendon cells in a novel 3-dimensional culture system designed to allow cells to establish cell-cell contacts and deposit matrix in the absence of scaffold components and without disturbance by medium changes.

METHODS: Tendon fibroblasts were isolated by sequential protease and collagenase digestion from 50-day-old chicken feet³. Grown to passage 3 in Dulbecco's modified eagle medium (DMEM) with 5% foetal calf serum, 1% antibiotic, 1% L-glutamine at 37°C, 5% CO₂. Cells were suspended at 3x10⁷ cells/ml and 1 ml placed in a DispoDialyzer tube and sealed. This was placed into a 50 ml centrifuge tube with 40 ml DMEM and ascorbate (1mg/ml). After 24 hours, 7, 14 and 21 days the cell aggregates were fixed in 90% methanol (4°C), washed in PBS and rapidly frozen on dry ice in Cryo-M-Bed embedding medium (Cryo-M-Bed Ltd.)

Sections were cut at 10-15 µm, collected on histobond slides and immunolabelled. Sections labelled with polyclonal type I collagen were pretreated with hyaluronidase/chondroitinase. Sections were labelled with monoclonal type II and III collagen, actin, vimentin and decorin, connexins 32 and 43, vinculin, Pan cadherin and N-cadherin. Polyclonal antibody binding was detected using FITC conjugated secondary antibody goat anti-rabbit. Monoclonal antibody binding was detected using Alexa488 conjugated goat anti-mouse immunoglobulins. Labelled sections were mounted using Vectashield with propidium iodide. Sections were examined on a Leica Labrolux 12 epifluorescence microscope and photographs taken on a digital camera.

RESULTS: Cells in suspension culture form elongated aggregates up to 3cm long. Immunolabels showed that at 7 days type I and collagens were present, predominantly in periphery. At 14 days the collagens were uniformly distributed throughout the aggregate and showed a degree of organisation. It is clear from propidium iodide label that the nuclei have distinct areas of alignment. The aggregates labelled positively for actin stress fibres, N-cadherin, decorin and connexin. Type II collagen and connexin 43 showed conclusive label.

DISCUSSION & CONCLUSIONS: The suspension cultures clearly show that tendon cells are capable of assembling an extracellular matrix in culture, rather than just releasing the collagen to the media. The structures formed were remarkably similar to tendons in their cell and matrix organisation, with parallel longitudinal rows of tendon cells interspersed with longitudinal collagen fibres. The amount of collagen produced by the cells is affected by time though quantification must be obtained.

The differences in connexin label may relate to the growth and deposition of matrix. From antisense knockout studies we know that connexin 32 is stimulatory to collagen synthesis and connexin 43 inhibitory². It is important to note the orientation of the cell nuclei corresponding to that of the matrix orientation.

These results suggest that this system is influenced by growth factor combinations and that matrix deposition could be further enhanced and controlled.

REFERENCES: 1. McNeilly et al, (1996). *Anat. Dec*;189 (pt3) pp 593-600. 2. Waggett et al (2001). *Trans. Orth. Res. Soc.* 26. pp 700. Banes et al, (1988). *J. Orthop. Res.* 6 pp 83-94

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Matrix deposition by tendon cells in suspension culture

Introduction. Tendons consist of longitudinally running parallel bundles of collagen with rows of tendon cells between them. The tendon cells are linked to one another via gap junctions¹ and cytoskeletally associated adherens junctions. Recent *in vitro* studies indicate that the two types of gap junction present, made of connexin 32 and connexin 43, regulate tendon cell responses to mechanical load². In view of the importance of cell-cell interactions in tendon cell behaviour, we describe the behaviour of tendon cells in a novel 3-dimensional culture system designed to allow cells to establish cell-cell contacts and deposit matrix in the absence of scaffolds, which would favour cell-substrate interactions, and without disturbance by medium changes.

Methods. Tendon fibroblasts were isolated from chicken tendons by protease and collagenase digestion³, grown to passage 3 in HEPES buffered DMEM/5% foetal calf serum/1% antibiotic/1% L-glutamine at 37°C, in 5% CO₂ in air. Cells were suspended at 3x10⁷ cells/ml and 1 ml placed in a Spectrapor DispoDialyzer™ tube (MW cutoff 300,000). This was then placed into a 60 ml centrifuge tube with 40 ml DMEM containing ascorbate (1mg/ml) on a roller. Medium in the large tube was changed every 2 days. At 24 hours, 7, 14 and 21 days the cell aggregates were fixed in 90% methanol (4°C), frozen on dry ice and cryosections cut at 10-15 µm. Sections were labelled by indirect immunofluorescence with monoclonal type I, II, and III collagen, actin, vimentin and decorin, connexins 32 and 43, vinculin, Pan cadherin and N-cadherin.

Results. Cells in suspension culture formed elongated aggregates up to 3cm long. Immunolabels showed that at 7 days type I and III collagens were present, predominantly in the periphery. At 14 days the collagens were uniformly distributed throughout the aggregates and showed parallel longitudinal organisation. It is also clear from propidium iodide label that the cell nuclei have distinct areas of alignment. The aggregates labelled positively for actin stress fibres, N-cadherin, decorin and connexin 32. Type II collagen and connexin 43 showed no conclusive label.

Discussion. The suspension cultures clearly show that tendon cells can form large scale, organised structures in this culture system, and are capable of assembling an organised extracellular matrix in the absence of a scaffold to support them. The structures formed were similar to tendons in their cell and matrix organization. The amount of collagen deposited by the cells increases over time. Therefore, tendon cells could be used in a tissue engineering context to form well organised tissue in the absence of scaffolds in suitable culture systems. The presence of connexin 32 gap junctions and absence of connexin 43 shows the cell aggregates favouring matrix synthesis pathways – in cell cultures connexin 32 junctions promote collagen synthesis whereas connexin 43 inhibits it².

REFERENCES: 1. McNeilly et al, (1996). J. Anat. Dec;189 (pt3) pp 593-600. 2. Waggett et al, (2001). Trans. Orth. Res. Soc. 26. pp 700. 3. Banes et al, (1988). J. Orthop. Res. 6 pp 83-94

Matrix deposition by tendon cells in suspension culture

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Intro-Tendons consist of longitudinally running parallel bundles of collagen with rows of tendon cells between them. The cells are linked via gap junctions¹ and cytoskeletally associated adherens junctions. In view of the importance of cell-cell interactions in tendon cell behaviour, we describe the behaviour of tendon cells in a novel 3-D culture system, allowing cells to establish cell-cell contacts and deposit matrix in the absence of scaffolds.

Methods-Tendon fibroblasts were isolated from chicken tendons by protease and collagenase digestion³. At P3 cells were suspended at 3×10^7 cells/ml and 1 ml placed in a Spectrapor DispoDialyzerTM tube. Then placed in a 50 ml centrifuge tube with 40 ml DMEM containing ascorbate (1mg/ml) on a roller. At 24 hours, 7, 14 and 21 days the cell aggregates were fixed and cryosections taken. Sections were immunolabelled.

Results-Cells in suspension culture formed elongated aggregates up to 3cm long. Immunolabels show at 7 days type I and III collagens were present, predominantly in the periphery. At 14 days the collagens were distributed throughout the aggregates and showed longitudinal organisation. Propidium iodide label shows that the cell nuclei have distinct areas of alignment. The aggregates labelled positively for actin, N-cadherin, decorin and connexin 32. Type II collagen and connexin 43 showed no conclusive label.

Discussion-The suspension cultures show that tendon cells can form large organised structures in this system. They are capable of assembling an organised ECM similar to tendon, in the absence of a scaffold. Therefore, tendon cells are able to form organised tissue in the absence of scaffolds in suitable culture systems.

References: 1. McNeilly et al, (1996). J. Anat. Dec; **189** (pt3) pp 593-600. 2. Waggett et al, (2001). Trans. Orth. Res. Soc. **26**. pp 700. 3. Banes et al, (1988). J. Orthop. Res. 6 pp 83-94.

MATRIX DEPOSITION BY TENDON CELLS IN SUSPENSION CULTURE

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Introduction-Tendons are made up of longitudinally running parallel bundles of collagen fibres, with rows of tendon cells between them. The tendon cells have an intimate relationship with one another and with the collagen bundles [1]. *In vivo*, tendon cells have prominent lateral cell processes that allow contact between cells of adjacent rows and the collagen bundles, which pass longitudinally from cell to cell. The cell processes and cell interactions create spaces between cells into which the organised collagen matrix is deposited [1,2] Where cells meet, cell membranes contain gap junctions, this indicates the presence of a 3-dimensional communicating network [1,3]. Cells are connected longitudinally by adherens junctions linking the longitudinal actin stress fibres between cells [4]. Recent *in vitro* studies indicate that the two gap junctions that are important in the regulation of tendon cell response to mechanical loading; communication via connexin 32 containing gap junctions upregulates collagen synthesis, whereas via connexin 43 gap junctions does the opposite [5].

An important aim of tissue engineering of tendons is to replicate such environments *in vitro*. In monolayer culture, tendon cells release the majority of the collagen produced to the culture medium [5]. We hypothesise that this is because the monolayer cultures lack a 3-dimensional organisation of cells, ie the cells can interact with one another in the plane of the culture, but there are no cell-enclosed spaces for them to deposit and organise their extracellular matrix as occur *in vivo* [1,2]. Here we describe the behaviour of tendon cells in a novel 3-dimensional culture system designed to allow establish cell-cell contacts and deposit matrix in the absence of scaffold components and without disturbance by medium changes.

Method- Tendon fibroblasts were isolated by sequential protease and collagenase digestion from 50-day-old chicken feet [6]. Grown to passage 3 in Dulbecco's modified eagle medium (DMEM) with 5% foetal calf serum (FCS), 1% antibiotic, 1% L-glutamine at 37°C, 5% CO₂. Cells were suspended at 3x10⁷ cells/ml and 1 ml placed in a DispoDialyzer tube and sealed. This was placed into a 50 ml centrifuge tube with 40 ml DMEM and ascorbate (1mg/ml).

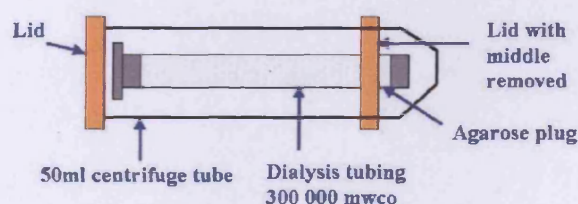


Fig1. Three-dimensional culturing equipment

TGF-β (5ng/ml) and IGF (50ng/ml) were added to the media every 3 days. After 24 hours, 7, 14 and 21 days the cell aggregates were fixed in 90% methanol (4°C), washed in PBS and rapidly frozen on dry ice in Cryo-M-Bed embedding medium (Cryo-M-Bed Ltd.)

Sections were cut at 10-15 μm, collected on histobond slides and immunolabelled. Sections labelled with polyclonal type I collagen (Col-1, Sigma) were pretreated with hyaluronidase/chondroitinase. Sections were labelled with monoclonal type II, III and V collagen (3B2, CIIC1 and M1-B4, DSHB), actin, vimentin and decorin (JLA20, AMF-17b and CB-1, DSHB), connexins 32 and 43 (Chemicon), vinculin, Pan cadherin and N-cadherin (hVIN1, CH-19 and ACAM, Sigma). Polyclonal antibody binding was detected using FITC conjugated secondary antibody goat anti-rabbit. Monoclonal antibody binding was detected using Alexa488 conjugated goat anti-mouse immunoglobulins. Labelled sections were mounted using Vectashield with propidium iodide (Vectashield Laboratories, Inc). Sections were examined on a Leica Labrolux 12 epifluorescence microscope and photographs taken on a digital camera.

Results- Cells in suspension culture formed elongated aggregates up to 3cm long. Immunolabels showed that at 7 days type I and III collagens were present, predominantly in the periphery. At 14 days the collagens labels were uniformly distributed throughout the aggregates and showed a degree of organisation. It is also clear from propidium iodide label that the cell nuclei have distinct areas of alignment.

Immunolabels of aggregates treated with growth factors showed similar amounts of type I and III collagen label. The aggregates labelled positively for actin stress fibres, N-cadherin, decorin and connexin 32. Connexin 43 showed no conclusive label.

Experiments using 1% FCS in the culture medium showed that tendon cells do not form large aggregates. However, when growth factors were added to the 1% FCS DMEM the cells formed large aggregates comparable to when they were cultured in 5% FCS.



Fig.2 a photograph of a 14-day cell aggregate untreated with growth factors. NB. The ball of cells at one end and the thin, elongated structure of the rest of the cells. This aggregate has a total length of 3 cm.

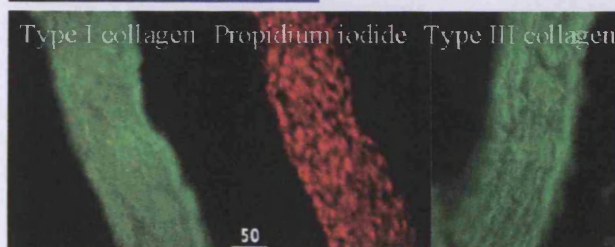


Fig.3. The immunolabels show types I and III collagen counterstained with propidium iodide. Cultures show cell orientation, organisation and matrix orientation similar to tendon. Scale bar in microns.

Discussion- The suspension cultures clearly show that tendon cells are capable of assembling an extracellular matrix in culture, rather than just releasing their collagen to the media. The structures formed were remarkably similar to tendons in their cell and matrix organisation, with parallel longitudinal rows of tendon cells interspersed with longitudinal collagen fibres. The amount of collagen produced by the cells is affected by time and the presence of growth factors, though quantification must be obtained.

The differences in connexin label may relate to the growth and deposition of matrix. From antisense knockout studies we know that connexin 32 is stimulatory to collagen synthesis and connexin 43 inhibitory [5]. It is important to note the orientation of the cell nuclei corresponding to that of the matrix orientation.

These results suggest that this system is influenced by growth factor combinations and that matrix deposition could be further enhanced and controlled.

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