Novel Strategies for Enhancing Tissue Integration in Cartilage Repair

Lindsay Davies

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Dedicated to my family:

Susan, Brian and Jo Davies

Thank you for your unconditional love, support and encouragement in everything I do

Special thanks to:

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For his love, motivation and true belief in me

I love you all, and could not have achieved this without you. Thank you.

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Abstract

Articular cartilage is found on the surface of opposing long bones within the synovial joint and aids to dissipate loads. Adult cartilage lacks the ability to initiate a repair response when injured due to its aneural and avascular nature.

Surgical techniques have been developed to enhance this repair process but none to date rival that of natural cartilage. Repair tissue is often of a fibrocartilaginous nature which does not have the biomechanical stability of articular cartilage. Integration between newly synthesised tissue and the endogenous cartilage is invariably poor as well, leading to degeneration of the repair tissue and surrounding cartilage with time.

The aim of this study was to develop strategies for the enhancement of integration between new and pre-existing articular cartilage to form a more biomechanically stable repair tissue. The migratory capacities of chondrocytes isolated from young and mature articular cartilage have been investigated using a Boyden chamber system, as well as the effects of different matrix substrates and chemoattractants on this process. Using this system the substrate dependent chemotactic effects of insulin-like growth factor 1 (IGF-1) and transforming growth factor beta 1 (TGFβ1) were only seen if the cells were seeded onto a fibronectin substrate. The inhibitory effect of chondroitin sulphate (CS) chains on chondrocyte migration was also demonstrated. Further experiments using an *in vitro* model of cartilage injury confirmed this, as it was demonstrated that chondrocytes migrated into chondroitinase ABC treated cartilage but not into untreated controls.

Biosynthesis studies were used to demonstrate the anabolic effects of IGF-1 and TGFβ1 on chondrocytes. Radiolabelled sulphate and proline were used as a measure of sulphated glycosaminoglycan and collagen biosynthesis respectively. These experiments demonstrated increased biosynthesis of both matrix molecules by chondrocytes from both age groups in response to these growth factors. In addition, these results suggest that the newly synthesised molecules were assembled into an extracellular matrix by the cells.

The results from both the migration and biosynthesis experiments demonstrated the potential of IGF-1 and TGFβ1 for enhancing cartilage repair, but also illustrated the limitations of their short half lives. Constructs encoding the mature peptides of IGF-1 and TGFβ1 were therefore developed for transfection into chondrocytes. The ability of these transfected cells to migrate was investigated on a chondroitinase ABC treated aggrecan substrate. Here a constant trend of increased migration with the transfected cells was seen when compared to controls.

This study demonstrates the ability of chondrocytes from both young and mature articular cartilage to migrate. This is the first study to report this using skeletally mature articular cartilage. In addition, the inhibitory effects of CS chains in chondrocyte migration have been shown using both the Boyden chamber system and an *in vitro* model of cartilage injury. The over-expression of IGF-1 and TGFβ1 in articular chondrocytes demonstrated the possibility of increasing both the migration of cells into articular cartilage as well as up-regulating their capacity for matrix biosynthesis, thereby creating an integrated repair tissue improving on current repair strategies.

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Abbreviations

α	Alpha
α2M	Alpha-2-macroglobulin
β	Beta
γ	Gamma
μCi	Microcurie
μg	Microgram
μl	Microlitre
μM	Micromolar
A_{260}	Absorbance at 260nm
A_{280}	Absorbance at 280nm
ABP	Actin binding protein
ACT	Autologous chondrocyte transplantation
ADAM	A disintegrin and metalloproteinase
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
ADF	Actin depolymerising factor
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
APMA	Amino phenyl mercuric acetate
APS	Ammonium persulphate
Arp 2/3	Actin related peptide 2/3
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BAPN	β-aminopropionitrile
bp	Base pair
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
C-4-S (0/6)	Chondroitin-4-sulphate (0/6)
CamKII	Calcium/calmodulin dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CCN	cyr61, ctgf, nov
cDNA	Complementary DNA
cFN	Cellular fibronectin
cGMP	Cyclic guanidine monophosphate
CILP	Cartilage intermediate layer protein
cm	Centimetre
CMFDA	5'chloromethylfluorescein diacetate
COMP	Cartilage oligomeric matrix protein
cpm	Counts per minute
CS	Chondroitin sulphate
CTGF	Connective tissue growth factor
DMEM	Dulbecco's modified Eagle Medium
DMMB	Dimethylmethylene blue
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribonucleotide triphosphates
DOPE	Dioleoyl phophotidylethanolamine

DOTMA	N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride
DS	Dermatan sulphate
DTT	Dithiothreitol
ECL™	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERK	Extracellular signal-related kinase
ERM	Ezrin, radixin and moesin
FACIT	Fibril associated collagens with an interrupted triple helix
FACS	Fluorescence-activated cell sorter
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FAT	Focal adhesion targeting
FBS	Foetal bovine serum
FCD	Fixed charge density
FERM	Band four point one, ezrin, radixin, moesin
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluoroisothiocynate
g	Gravitational force
G-actin	Globular actin
GAG	Glycosaminoglycan
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gdf5	Growth and differentiation factor 5
GDP	Guanidine diphosphate
GEF	Guanidine exchange factors
GPI	Guanidine protein inhibitors
Grb2	Growth factor receptor bound protein 2
GTP	Guanidine triphosphate
GTPase	Guanidine triphosphatase
HA	Hyaluronan
HBSS	Hank's balanced salt solution
HGF	Hepatocyte growth factor
Hox	Homeobox
HRP	Horseradish peroxidase
HS	Heparan sulphate
IF	Intermediate filaments
Ig	Immunoglobulin
IGD	Interglobular domain
IGF-1	Insulin-like growth factor 1
IGF-1R	Insulin-like growth factor 1 receptor
IGFBP	Insulin-like growth factor binding protein
IL-1 (6)	Interleukin-1 (6)
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRS 1	Insulin receptor substrate 1
I-Smad	Inhibitory Smad

ITS	Insulin, transferrin, selenium
JNK	c-jun N-terminal kinase
kb	Kilobase
Kd	Dissociation constant
KDa	Kilodaltons
KS	Keratan sulphate
LAP	Latency associated peptide
LB	Luria broth
LDH	Lactate dehydrogenase
LP	Link protein
LPS	Lipopolysaccharide
MACI	Matrix-induced chondrocyte implantation
MAP	Microtubule associated protein
MAPK	Mitogen activator of protein kinase
MCS	Multiple cloning site
mDIA	Diaphanous-profilin
MEK	Mitogen-activated kinase
MH2	MAD homology 2
MIF	Mullerian inhibitory factor
ml	Millilitre
MLCK	Myosin light chain kinase
mm	Millometre
MMP	Matrix metalloproteinase
MOPs	4-Morpholinepropanesulfonic acid
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
MT-MMP	Membrane type matrix metalloproteinase
NC4	Non-collagenous 4
N-CAM	Neural cell adhesion molecule
ng	Nanogram
NLS	Nuclear localisation signal
nm	Nanometre
OA	Osteoarthritis
OCD	Osteochondrosis Dessicans
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PET	Polyethylene terephthalate
pFN	Plasma fibronectin
PI3K	Phosphoinositide 3 kinase
PIP2	Phosphatidylinositol 3,4 bisphosphate
PKC	Protein kinase C
PLG	Phase lock gel
pmol	Picomoles
PRELP	Proline arginine-rich end leucine-rich repeat protein
PRG4	Proteoglycan 4
PVDF	Polyvinylidene fluoride
RGD	Arginine-glycine-aspartic acid
RNA	Ribonucleic acid

RNase	Ribonuclease
ROCK-LIMK	Rho activated kinase – Lin-II/Isl-1/Mec-3 domain containing protein kinase
RPM	Revolutions per minute
RSK	Ribosomal S6 kinase
R-Smad	Receptor regulated Smad
SARA	Smad anchor for receptor activation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sGAG	Sulphated glycosaminoglycan
SH2	Src homology domain
SLC	Small latent complex
SLRP	Small leucine-rich repeat proteoglycans
SMA	Smooth muscle actin
SZP	Superficial zone protein
Tanneal	Annealing temperature
TBE	Tris-Borate ethylenediaminetetraacetic acid
TBS	Tris buffered saline
TEMED	Tetra methylethylenediamine
TF	Transcription factor
TGFβ1	Transforming growth factor beta 1
TGFβR	Transforming growth factor beta receptor
TIMP	Tissue inhibitor of metalloproteinase
TNFα	Tumour necrosis factor α
TSP	Thrombospondin
TUNEL	Terminal deoxynucleotidyl-mediated dUTP-biotin nick end-labelling
V	Variable
v/v	Volume/volume
WASP	Wiskott-Aldrich Syndrome protein
WAVE	WASP family verprolin homolgous WAVE proteins
w/v	Weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

Chapter 1

Introduction

1.1 The Synovial Joint

"...the narrowest hinge in my hand puts to scorn all machinery." Walt Whitman 1892
(Buckwalter and Mankin, 1998)

This statement by Walt Whitman in 1892 succinctly portrays the intricate design of the synovial joint and its composite materials. Even today with the modern technology available, no man-made substitute can rival that of nature's creation. The synovial joint comprises two opposing long bones with a thin layer of articular cartilage at the interface region. This articular cartilage provides a functional surface for the frictionless movement needed within the joint and also helps aid the dissipation of loads, preventing excessive stress on the subchondral bone (see fig.1.1). Both the bones and associated ligaments are held together in a stable and secure manner by the fibrous joint capsule (Duance, 1998; Pacifici et al., 2005). Within the capsule, ligaments provide the strength to maintain the joint's alignment and stability. Ligaments outside of the capsule help in articulation of the joint and in maintaining alignment. Both sets of ligaments allow free movement when relaxed but prevent unwanted movement that may damage the joint when tense.

The synovial membrane lines the inside of the fibrous capsule and consists of between 1 and 3 layers of synoviocytes, which secrete and absorb synovial fluid (Benjamin, 1999; Nozawa-Inoue et al., 2003). This fluid provides both lubrication and nutrition to the joint. These cells are supported by vascular connective tissue and can be seen extending into the joint cavity as small villi or larger, fatty structures known as articular fat pads (Benjamin, 1999).

All the structures of the joint interact and provide the support and strength needed during impact or mechanical loading. Together they are able to stabilise and prevent damage to the joint.

1.2 Articular Cartilage

Articular cartilage forms the surface covering of opposing bones within the synovial joint cavity. Although functionally supported by numerous tissues such as ligaments and synovial tissue, articular cartilage centrally functions to aid the dissipation of loads. Its prevention of excessive stress on subchondral bone provides a surface for low friction movement. Differences in the composition and thickness of this tissue can be seen within the same joint as well as joints from the same and differing species. At most, articular cartilage is found to be a few millimetres thick, yet demonstrates impressive ability to resist compression and has phenomenal durability (Buckwalter et al., 2005).

Adult articular cartilage at a glance appears to be a simple and largely inactive tissue when compared to the metabolic activities of related connective tissues such as tendon. The chondrocyte is the sole cell type found within articular cartilage and is isolated from other cells by the dense matrix surrounding each chondrocyte. The tissue is avascular, aneural and alymphatic and relies on the diffusion of nutrients and the exchange of metabolites from the articular surface (Archer and Francis-West, 2003).

It is true that with skeletal maturity, cellular division and proliferation will decrease and within mature articular cartilage, chondrocytes most probably will never divide. In contrast to this rather simplistic view, detailed morphology has shown a highly organised structure with complex interactions between the chondrocytes and their surrounding matrix for the maintenance of the cartilage (Buckwalter and Mankin, 1998). The ability of isolated chondrocytes *in vitro* to proliferate demonstrates that they have not undergone terminal differentiation. It is therefore considered that *in vivo*, chondrocytes are within a 'resting state' of the cell cycle. The following section will describe the components of these cell-matrix interactions and look at their role in the maintenance of articular cartilage.

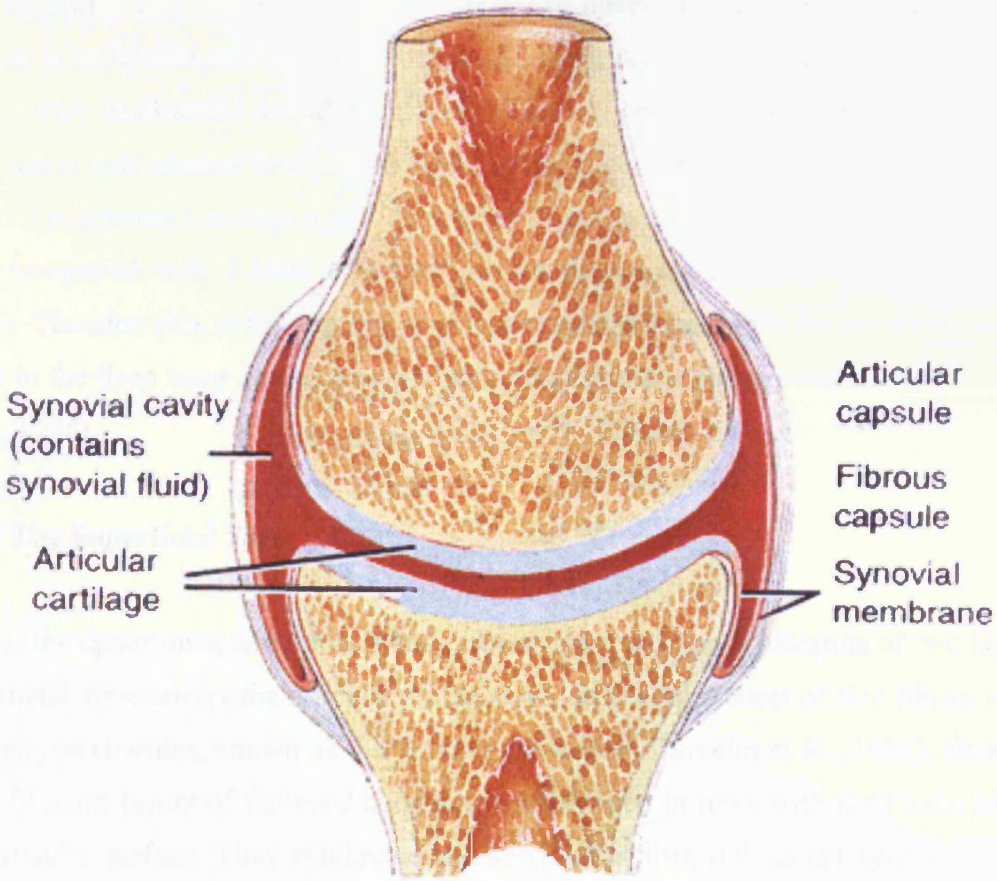


Fig.1.1 Frontal section of a generalised synovial joint

www.lactoferrin.com.au/regenr8.php

1.3 Articular Cartilage Morphology

The highly ordered structure of cells and their matrix proteins form the basis of articular cartilage with the differing arrangements of these components separating the tissue into four main zones; these being the superficial, transitional, deep and calcified cartilage zones (see fig.1.2). Cells within these layers appear to differ in size, shape and organisation as well as in metabolic activity (Aydelotte et al., 1988; Aydelotte and Kuettner, 1988; Bayliss et al., 1983; Buckwalter and Mankin, 1998; Ghadially et al., 1983; Kuettner et al., 1982). A decrease in cell number is seen with depth of cartilage, for example, it has been shown that in bovine articular cartilage a cell density of 54,000 cells/mm^3 was seen in the superficial zone compared with 30,000 cells/mm^3 in the deeper zones (Buckwalter and Hunziker, 1999). Chondrocytes in the superficial zone demonstrate one tenth the metabolic activity of those in the deep zone as well as only having half the cell volume and surface area (Wong et al., 1996).

1.3.1 The Superficial Zone

This is the upper most and thinnest zone of articular cartilage. Consisting of two layers, the superficial zone covers the articular surface with an acellular sheet of thin fibrils with very few polysaccharides, known as the '*lamina splendens*' (Jurvelin et al., 1996). Beneath this clear film are layers of flattened chondrocytes arranged in rows with their axes parallel to the articular surface. They synthesise and secrete a matrix rich in collagen but with only small quantities of proteoglycans (Poole, 2003). This is thought to be due to the ability of these chondrocytes to degrade proteoglycans much more efficiently than chondrocytes within the deeper zones (Buckwalter, 1999). The collagen content of the superficial zone is the highest of all the articular cartilage zones. Studies by Aydelotte and Kuettner (1988) demonstrated that chondrocytes within this zone proliferate slower and have a less defined matrix than those chondrocytes in the deeper zones. Studies have shown that chondrocytes within the superficial zone are unequally distributed (Schumacher et al., 2002). A recent report has suggested that within the superficial zone cells will form pairs which are linked together, possibly by gap junctions due to the expression of connexin 43 at the adjoining cell surfaces (Chi et al., 2004). Superficial zone chondrocytes also have a more irregular shape with numerous large processes, more vacuoles associated with the Golgi region and

small vesicles at the cell surface. This collagen fibril meshwork forms a barrier for molecules passing through the articular surface in either direction. Preventing the passage of large molecules, such as antibodies, through the superficial zone undoubtedly acts as an effective method of isolating healthy articular cartilage from the immune system (Buckwalter and Hunziker, 1999). Collagen fibrils contribute functionally to the resistance of shear and tensile forces at the articular surface from use of the joint (Buckwalter and Mankin, 1998).

1.3.2 The Transitional Zone

The transitional zone within articular cartilage lies directly beneath the superficial zone. This zone has a larger volume to that of the superficial zone and contains cells of a more spherical shape with greater amounts of endoplasmic reticulum (ER) and Golgi membranes, to the flattened cells previously described. The cells within this zone are more biosynthetically active (Poole et al., 2001). These cells appear to synthesise a higher concentration of proteoglycans but less collagen than those within the superficial zone. The collagen fibrils formed however are of a larger diameter. The water content in this zone is distinctly lower than seen in the superficial zone (Buckwalter and Hunziker, 1999).

1.3.3 The Deep/Radial Zone

The deep zone, also known as the radial zone, contains spherical chondrocytes as seen in the transitional layer, although here the cells tend to be organised into columns perpendicular to the articular surface (see fig.1.2). Extracellular matrix (ECM) within this zone contains the highest concentration of proteoglycans as well as the lowest concentration of water (Buckwalter and Hunziker, 1999). Collagen fibrils here have the largest diameter compared to those seen in other zones (Poole et al., 2001). Fibrils from the deep zone are seen extending into the interface between uncalcified and calcified cartilage. This region is known as the tidemark (Buckwalter and Hunziker, 1999).

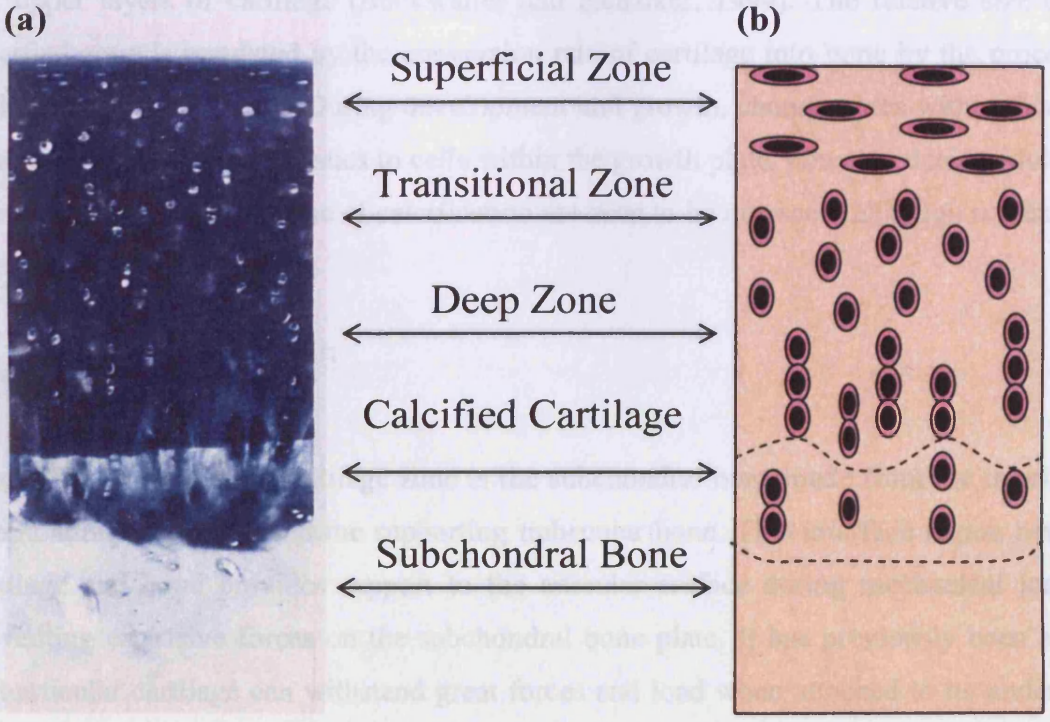


Fig.1.2 (a) Histological and (b) schematic section through articular cartilage illustrating its four composite zones

1.3.4 Calcified Cartilage Zone

The calcified cartilage zone forms an interface between the articular cartilage and the underlying subchondral bone. It is the point of attachment for cartilage to bone and functions to transmit force generated by mechanical loading (Oegema et al., 1997). Cells within this region have a much smaller volume and contain less synthetic organelles than in the upper layers of cartilage (Buckwalter and Hunziker, 1999). The relative size of the calcified zone is regulated by the conversion rate of cartilage into bone by the process of endochondral ossification. During development and growth, chondrocytes within this zone have very similar characteristics to cells within the growth plate, however during adulthood chondrocytes within the zone of calcification are seen to be quiescent although not inactive (Oegema et al., 1997).

1.3.5 Subchondral Bone

Underlying the calcified cartilage zone is the subchondral bone made from the interfacing subchondral bone plate and the supporting trabecular bone. This interface region between cartilage and bone provides support to the articular surface during mechanical loading, preventing excessive forces on the subchondral bone plate. It has previously been shown that articular cartilage can withstand great forces and load when attached to its underlying subchondral bone (Jeffrey et al., 1995). This junction between cartilage and bone is a site of active remodelling exhibiting extensive collagenase activity. This turnover process is required for a healthy interface region (Poole, 2003). The shape and curvature of the bone also provides a stable scaffold optimally designed for mechanical loading and also for the provision of smooth movement during articulation (Kawcak et al., 2001).

1.4 Components of Articular Cartilage

1.4.1 The Chondrocyte

As previously mentioned in section 1.2, the chondrocyte is the only cell type found within articular cartilage and comprises as little as 1% of the total tissue volume within adult cartilage (van Sickle and Evander, 1997). Isolated and surrounded by the ECM that they

synthesise and maintain, chondrocytes contain all the necessary organelles needed to perform this function, such as ER and Golgi apparatus (Archer and Francis-West, 2003). These cells are also characterised by the intracellular deposition of glycogen and the possession of short cilia protruding from the cell into the matrix, possibly with the role of detecting any minute changes within the matrix (Buckwalter and Mankin, 1998).

Within different zones of the cartilage, chondrocytes will exhibit differing morphology, changing their size, shape and organisation, with an accompanying change in metabolic activity. Mature chondrocytes are normally round in shape and are characterised by the synthesis of type II collagen, large aggregating proteoglycans and non-collagenous proteins. Ultimately it is the interaction of these components which forms the ECM (Buckwalter and Hunziker, 1999).

As articular cartilage is avascular, cellular metabolism is designed to function at a low oxygen tension, with levels between 10% at the articular surface to less than 1% in the deeper zones of the cartilage. This consequently means that the majority of the cell's energy requirements come from glycolysis (Archer and Francis-West, 2003; Grimshaw and Mason, 2000). Studies by Lane et al. (1977) showed that this was the preferred method of metabolism even in aerobic conditions.

1.4.2 The Extracellular Matrix

The ECM found surrounding the chondrocytes within articular cartilage is composed of tissue fluid and an array of structural macromolecules. Visualisation of the ECM sees its division into three circumferential sections found surrounding individual chondrocytes (see fig.1.3).

1.4.2.1 The Pericellular Matrix

Collectively, the chondrocyte and its pericellular matrix are known as the chondron. The pericellular matrix is closest to the cell, linked by a transparent glycocalyx enclosed by the fibrillar pericellular capsule. This thin matrix provides protection to the chondrocyte during mechanical loading as well as preventing deformation of the cell (Huber et al., 2000). Type

IX collagen is found concentrated here, tightly regulating the fibril diameter of type II collagen. The presence of type VI collagen is thought to mediate interactions for pericellular architecture and receptor-mediated anchorage and signalling between the chondrocyte and its micro-environment. Type VI collagen is normally found located with the matrix glycoprotein fibronectin (Poole et al., 2001). These networks of collagen fibrils form a 'basket' around the contained cell.

High concentrations of the monomeric form of the proteoglycan aggrecan, hyaluronan (HA) and link protein (LP) in this region suggests the assembly of these components into aggregates within the pericellular capsule before moving into the outer two matrices. Other small proteoglycans such as decorin and biglycan may have roles in modulating the collagen-proteoglycan interactions here (Poole, 1997).

1.4.2.2 The Territorial Matrix

Cytoplasmic extensions from the chondrocyte, project into the pericellular matrix and also as far as the territorial matrix. The territorial region is well defined within all zones of articular cartilage. A reduced quantity of type IX collagen within this domain allows the development of thicker type II collagen fibrils. Collagen fibres arrange themselves into bundles and thereby forming a distinct border between the territorial and interterritorial matrices (Huber et al., 2000). The glycosaminoglycan (GAG), chondroitin sulphate (CS), is also more concentrated in this layer (Buckwalter and Hunziker, 1999; Poole, 1997).

1.4.2.3 The Interterritorial Matrix

The interterritorial matrix is furthest away from the cell, and contains the largest type II collagen fibrils (Hagg et al., 1998). This is accompanied by the low abundance of type IX collagen and the lowest concentration of type VI collagen within the 3 regions. Collagen fibres within this region arrange into vertical columns radiating up through the zones of cartilage, previously described as 'column arcades' by Benninghoff (Benninghoff, 1925), until they reach the superficial zone where they arrange parallel to the articular surface (Huber et al., 2000). The GAG keratan sulphate (KS) is concentrated in this region

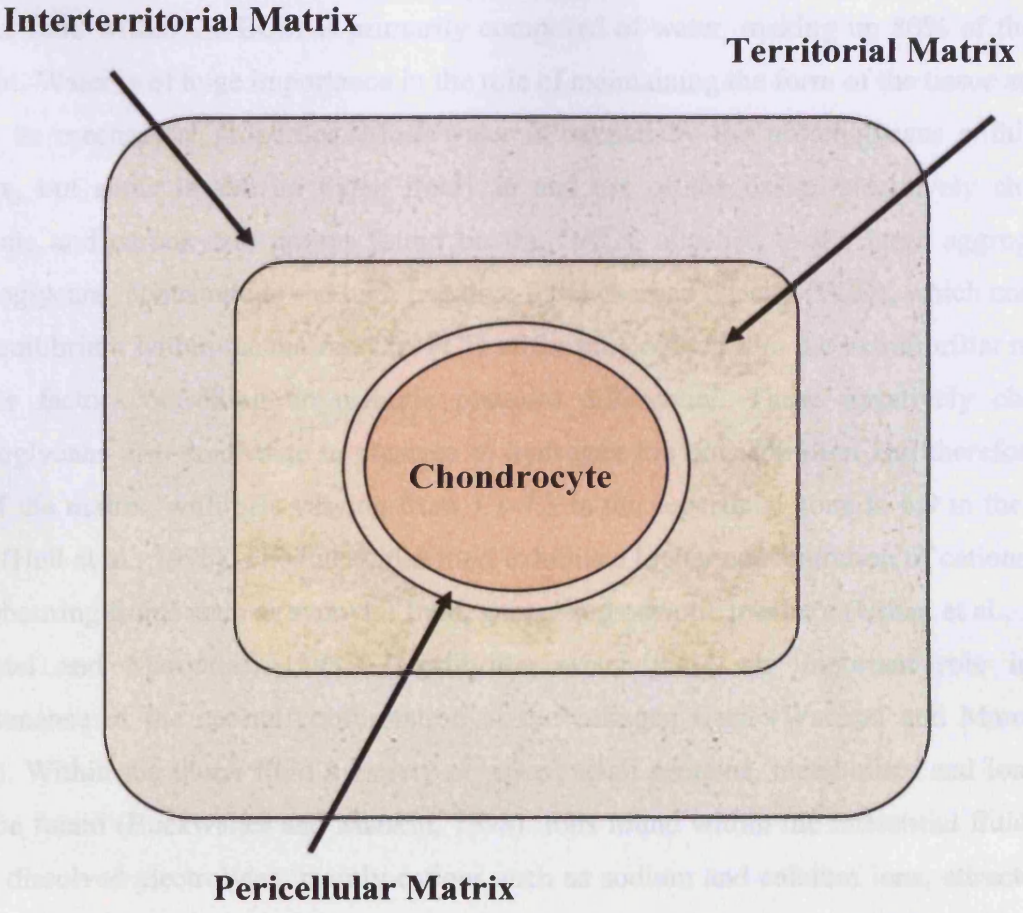


Fig.1.3 The chondrocyte and its ECM

(Aydelotte and Kuettner, 1988; Poole, 1997). These components work in co-operation to form a tissue able to withstand strong tensile and compressive forces.

1.4.3 Tissue Fluid

Tissue fluid within the ECM is primarily composed of water, making up 80% of the wet weight. Water is of huge importance in the role of maintaining the form of the tissue as well as in its mechanical properties. Most water is trapped by the proteoglycans within the matrix, but some is able to move freely in and out of the tissue. Negatively charged sulphate and carboxylate groups found on the GAGs, attached to the large aggregating proteoglycans, contribute to the high negative fixed charged density (FCD), which controls the equilibrium within the matrix. The FCD of the proteoglycans in the extrafibrillar matrix is one factor controlling an osmotic pressure differential. These negatively charged proteoglycans also contribute to changes in hydrogen ion concentration and therefore the pH of the matrix, with pHs varying from 7.1-7.3 in the superficial zone to 6.9 in the deep zone (Hall et al., 1996). The interstitial fluid exhibits a higher concentration of cations than neighbouring fluids such as synovial fluid, generating osmotic pressure (Urban et al., 1993; Wachtel and Maroudas, 1998). Intrafibrillar water plays an important role in the maintenance of the natural conformation of the collagen fibril (Wachtel and Maroudas, 1998). Within the tissue fluid a variety of gases, small proteins, metabolites and ions can also be found (Buckwalter and Mankin, 1998). Ions found within the interstitial fluid tend to be dissolved electrolytes, mainly cations such as sodium and calcium ions, attracted by the negative FCD. Chloride ions are also found though and collectively these ions are required to neutralise the charges fixed to the solid matrix (Mow et al., 1999).

The gaps between collagen fibres are relatively large, 100nm, and are filled with a fine, porous GAG-water gel (Maroudas, 1976). Movement of molecules from the synovial fluid into the ECM is regulated by this gel. The shear presence of the collagen fibrils and aggregating proteoglycans makes the flow of fluid difficult, resulting in a frictional resistance and therefore further decreasing the permeability of cartilage (Mow et al., 1999). Negatively charged GAGs within the gel restrict the flow of certain ions by their respective charges. The small pore sizes within the gel also discriminate against movement of large molecules. Proteins such as serum albumin are often excluded from the matrix, however

work by Maroudas (1976) suggests that some larger pores may be present for the regulated movement of these molecules. This would explain the ability of large aggregating proteoglycans to leave the matrix in normal ECM turnover. The observation further extends to that of degradative enzymes, some of which are smaller than 50KDa, and can freely diffuse in and out of the matrix. Their large inhibitors, such as alpha-2-macroglobulin (α2M), present within the joint fluid (Chu et al., 1994; Hadler et al., 1981) cannot enter the matrix though. Excessive degradation of the proteoglycans by proteolytic enzymes will increase these pore sizes though and diffusion of α2M molecules will be possible. Presence of α2M within the matrix will inhibit further proteolytic degradation of proteoglycans allowing their restoration. At this point α2M will once again be restricted from entering the matrix (Maroudas, 1976).

1.4.4 Extracellular Matrix Constituents

1.4.4.1 Proteoglycans

Proteoglycans provide compressive resistance to articular cartilage as well as enhancing and maintaining its durability. The specificity and concentration of proteoglycans varies between sites and zones of articular cartilage and are also affected by age, injury and disease (Buckwalter and Mankin, 1998). Generally proteoglycans consist of a core protein, to which numerous negatively charged GAG side chains are linked. These GAG chains attract water in order for the proteoglycan to carry out its function. The proteoglycans within cartilage can be divided into two subfamilies, namely the large aggregating proteoglycans and the small leucine-rich repeat proteoglycans (SLRPs). The function and distribution of these proteins are described below.

1.4.4.1.1 Large Aggregating Proteoglycans

There are four members of this family, namely aggrecan, versican, brevican and neurocan, although only the foremost three are found in articular cartilage. All members of the family have an amino terminal globular domain (G1) and a carboxyl terminal globular domain (G3) demonstrating lectin-like homology (Roughley, 2001).

Aggrecan

Aggrecan is by far the most abundant of the aggregating proteoglycans composing 90% of the total cartilage proteoglycan mass (Buckwalter and Hunziker, 1999). It is secreted as a monomer and forms aggregates with the GAG, HA. On aggregation, aggrecan becomes immobilised within the matrix due to the size of the complexes it forms. Each aggrecan: HA interaction is stabilised by one molecule of LP (Dudhia, 2005; Hascall and Heinegard, 1974; Poole, 1986). The length of HA restricts how many aggrecan monomers can bind to it, as does the abundance of HA molecules. The size of aggregates can therefore range from 2-5 aggrecan molecules where there is an excess of HA, to large aggregates of 400-800 aggrecan monomers where HA abundance is low (Hardingham and Fosang, 1995). Due to the large numbers of negatively charged GAG chains on aggrecan, these aggregates are hydrated providing cartilage with an exceedingly high water content aiding aggrecan's role in resisting compressive forces (Maroudas et al., 1969; Venn and Maroudas, 1977).

Aggrecan consists of a core protein which has an additional globular domain (G2) inserted between the amino terminal and carboxyl terminus (see fig.1.4). Located between the first and second globular domain is an interglobular domain (IGD), which is unique to aggrecan (Hardingham and Fosang, 1995). At the N-terminal the G1 domain is approximately 65KDa and contains 25% carbohydrate and some KS chains as well as oligosaccharides. This domain interacts specifically with HA in the formation of aggregates. In addition, the G1 domain may also play a role in regulating aggrecan biosynthesis by inhibiting GAG chain attachment to the core protein (Yang et al., 2000). The G2 domain is approximately 110KDa and contains more KS than G1. The G2 domain is separated from G3 by a KS and 2 CS attachment domains. There are over 110 CS and 30 KS attachments here which aid in aggrecan's function in resisting compression (Dudhia, 2005). The exact function of the G2 domain still remains unknown, but it is thought to inhibit the secretion of aggrecan prior to GAG modification in the Golgi (Luo et al., 1996). The G3 domain has lectin-like homology suggesting that it may interact with other ECM components and may also be involved in intracellular trafficking during aggrecan synthesis. Aggrecan molecules within cartilage often appear to have lost part of their C-terminal structure including the G3 domain due to proteolysis (Hardingham and Fosang, 1995; Roughley, 2001).

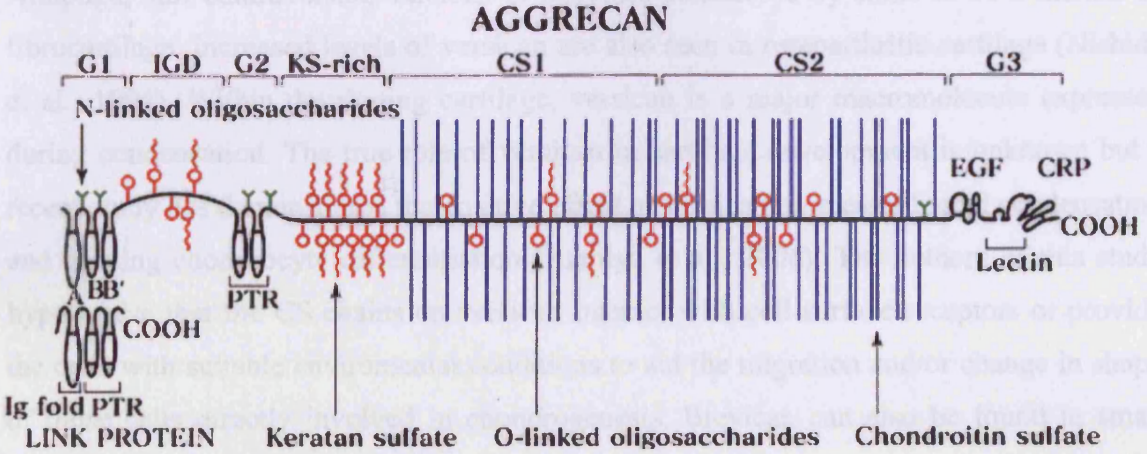


Fig.1.4 A schematic representation of the large aggregating proteoglycan, aggrecan

www.glycoforum.gr.jp/science/hyaluronan/HA05/HA05E.html

Versican and Brevican

Versican, so named because of its highly interactive and versatile nature, encodes two exons for CS attachment within its core protein and is thought to have roles in cell adhesion, proliferation, migration and ECM assembly. It is found in mature articular cartilage, although only as a minor constituent and is found mainly within a variety of soft tissues. With increasing passage, chondrocytes expanded in monolayer culture for use in cartilage grafts express versican within the deep zone of the graft, suggesting dedifferentiation of the chondrocytes (personal communication with Dr. Anthony Hayes). Although, still controversial, versican is therefore considered by some to be a marker of fibrocartilage. Increased levels of versican are also seen in osteoarthritic cartilage (Nishida et al., 1994). Within developing cartilage, versican is a major macromolecule expressed during condensation. The true role of versican in cartilage development is unknown but a recent study has demonstrated the positive effect of versican on mesenchymal condensation and ensuing chondrocyte differentiation (Kamiya et al., 2006). The authors of this study hypothesise that the CS chains on versican interact with cell surface receptors or provide the cells with suitable enviromental conditions to aid the migration and/or change in shape of those cells directly involved in chondrogenesis. Brevican can also be found in small quantities in cartilage, but is primarily located in neuronal tissues like neurocan (Wight, 2002).

1.4.4.1.2 Small Leucine-Rich Repeat Proteoglycans

The SLRPs interact with the fibrillar collagens of the ECM and are characterised by a central domain composed of a series of adjacent leucine-rich repeats bordered at each end by disulphide bonded domains. These repeats provide an ideal conformation for binding to other matrix proteins. The SLRPs are unique in that they flank their leucine-rich repeats with cysteine residues. These non-aggregating proteoglycans do not contribute a great deal to the volume of the tissue or its mechanical function. It is thought that these molecules are present to regulate cellular behaviour through interactions with other matrix macromolecules (Buckwalter and Hunziker, 1999). This family of proteoglycans can be subdivided into three classes (Iozzo, 1998).

Class I SLRPs

This class of SLRPs comprises two members within cartilage, namely decorin and biglycan. They exist with one or two dermatan sulphate (DS) or CS chains in the N-terminus of their core protein respectively (Iozzo, 1998; Roughley, 2001). Despite their structural similarities they are thought to have dissimilar functions due to their differing expression patterns (Hocking et al., 1998). Decorin is found normally in association with type II collagen regulating fibril diameter and also interacting with adjacent proteoglycan molecules. Biglycan may also regulate type II collagen interactions but can be found most concentrated within the pericellular matrix interacting with type VI collagen (Buckwalter and Mankin, 1998). Biglycan possesses the unique ability to organise type VI collagen fibrils into hexagonal-like networks (Wiberg et al., 2003). Both decorin and biglycan bind to the N-terminus of type VI collagen through the formation of complexes with matrilins 1, 3 and 4. Complexes between these SLRPs and matrilin-1 can also link them to aggrecan and LP (Wiberg et al., 2003). Both these SLRPs have growth factor binding capabilities, especially to transforming growth factor beta 1 (TGFβ1). Decorin and biglycan have both been shown to bind TGFβ1 through their core protein, and that their GAG attachments hinder this process (Hildebrand et al., 1994). Decorin's interaction with TGFβ1 occurs independently of the SLRP binding to collagen and it is hypothesised that the TGFβ1-SLRP interaction may be a means of storing the growth factor within the ECM, thereby modulating the interactions with its receptor (Schonherr et al., 1998).

Class II SLRPs

This group is further divided into 2 subfamilies. Fibromodulin and lumican comprise the first subfamily and keratocan, Proline Arginine-Rich End Leucine-Rich Repeat Protein (PRELP) and chondroadherin the second (Hocking et al., 1998; Iozzo, 1998). All members of this family have distinct structural homology and are therefore also classified as the KS proteoglycans. Within cartilage the more important members of this group are fibromodulin, which is found within articular cartilage throughout life but only contains KS chains in embryonic and juvenile tissues and lumican, which is only found in adult cartilage as a glycoprotein (Roughley, 2001). Both however are up-regulated in cartilage with ageing. Both fibromodulin and lumican have been shown to interact with fibrillar collagens

and inhibit fibrillogenesis (Rada et al., 1993). Proteins in this family can be distinguished from the class I SLRPs due to the tyrosine sulphation seen at the N-terminal. Tyrosine sulphation has been implicated in protein-protein interactions during intracellular transporting and secretion. It has also been suggested to play a role in ligand recognition and proteolytic processing (Hocking et al., 1998).

PRELP was first isolated from articular cartilage and has been shown to encode a 382 amino acid protein (Bengtsson et al., 1995). PRELP is unique in that its N-terminal is rich in arginine and proline. This proteoglycan has been shown to promote chondrocyte attachment but does not enhance spreading. This function was similarly described for the 36KDa protein, chondroadherin (Hocking et al., 1998). Chondroadherin's protein structure consists of ten leucine-rich repeats and nine cysteine residues and has been shown to interact with chondrocytes via their $\alpha 5\beta 1$ integrin receptor (Camper et al., 1997).

Class III SLRPs

This class consists of the proteoglycans epiphycan and osteoglycin. The former is found exclusively within epiphyseal cartilage and has therefore been implicated in cartilage differentiation (Iozzo, 1998). Osteoglycin was first isolated from bone (Bentz et al., 1989) and has subsequently been shown to play a major role in the regulation of collagen fibrillogenesis in many connective tissues, but is especially present within the cornea (Tasheva et al., 2002). Both these SLRPs have a core protein containing 6 leucine-rich repeats flanked by cysteine residues (Hocking et al., 1998).

1.4.4.2 Glycosaminoglycans

Many of the functional properties of proteoglycans are as a result of their GAG attachments. GAGs are divided into categories dependent on their saccharide composition (see fig.1.5). Within each category they are then further sub-divided according to their sulphation pattern and chain length. Within cartilage the major sulphated GAG (sGAG) chains are CS and KS, with a small quantity of DS, as well as the non-sulphated GAG, HA (Bayliss et al., 1999). Their acidic and hydrophilic nature provides a net negative charge thereby attracting water molecules and providing compressive strength to the tissue. They

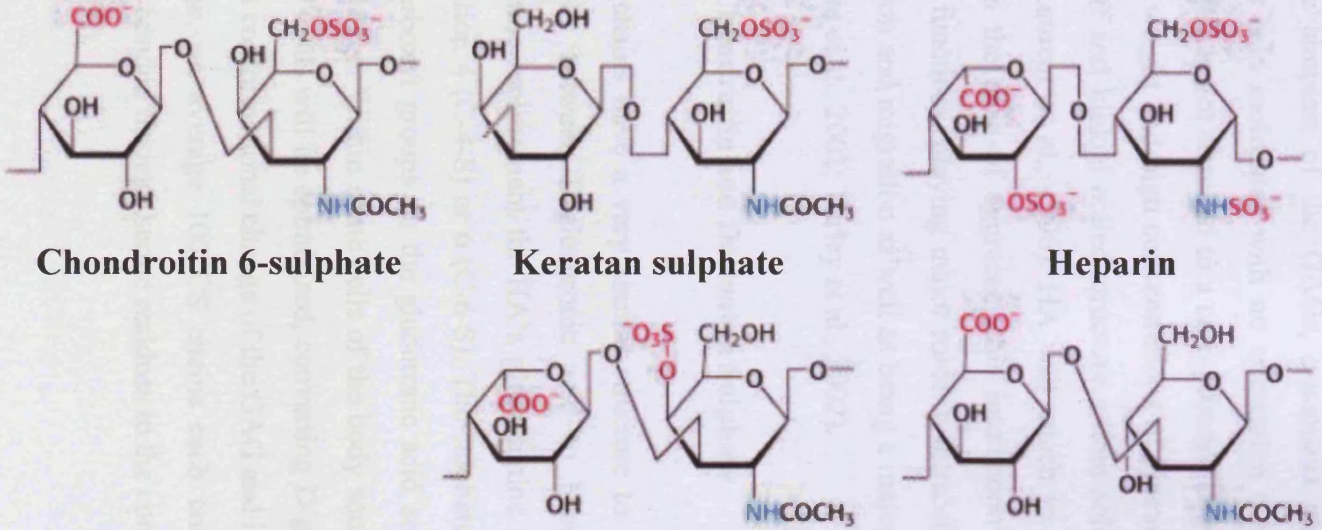


Fig.1.5 Chemical structures of the GAG family

http://oregonstate.edu/instruction/bb450/stryer/ch11/slide47.jpg

(Adapted from Stryer (1995))

are also involved in binding to growth factors, enzymes, cell surface receptors and ECM associated proteins (Gallagher, 1989).

1.4.4.2.1 Hyaluronan

HA is the simplest of the GAGs, composed of alternating N-acetylglucosamine and glucuronic acid molecules with no sulphation (see fig.1.5). Unlike other GAGs within cartilage, HA is not attached to a core protein (Day and Prestwich, 2002). Due to its large molecular weight and high concentrations of glucuronic acid within its structure, HA will form a stiff and kinked coiled structure within solution occupying a large hydrodynamic volume (Laurent et al., 1996). HA will attach to other molecules through non-covalent linkage. In the case of aggrecan, this interaction is stabilised by LP. HA has diverse biological functions, playing major roles in intracellular signalling, providing a matrix for cell adhesion and migration as well as being a major component of cartilage (Tammi et al., 2002; Toole et al., 2002; Turley et al., 2002).

1.4.4.2.2 Chondroitin and Dermatan Sulphate

CS GAG chains have a very similar structure to HA in that they are constructed from (1→3) links between D-glucuronic acid to N-acetylgalactosamine (see fig.1.5). The galactosamine replacement for HA's glucosamine residues are differentially sulphated in either position 4 (C-4-S) or 6 (C-6-S). The sulphate groups on the galactosamine residues and the carboxyl groups on the glucuronic acid are ionised and therefore result in a net negative charge. Within some cells of the body some of the carboxyl groups found on the glucuronic acids will be epimerised, converting D-glucuronic acid to L-iduronic acid. This results in a conformational change of the GAG and is now referred to as DS. In tissues such as cartilage, on average 100 CS chains each containing 50-60 disaccharide units are covalently bonded through serine residues to the core protein of aggrecan (Bali et al., 2001; Róden, 1980).

The pattern of sulphation of CS found within articular cartilage is dependent on age, topography of the joint and the zone of the cartilage. Immature cartilage contains high concentrations of C-4-S cartilage, but as the joint and therefore cartilage matures, C-6-S

will become the predominant form. This change in sulphation coincides with the time that the chondrocyte is most actively synthesising GAG chains. Concentrations of this GAG will also be much higher within the deeper zones of articular cartilage than those closer to the articular surface (Bayliss et al., 1999). CS can be catabolised within the ECM by chondroitinases and glycosidases. The former is able to destroy the osidic bond between the N-acetylgalactosamine and glucuronic acid (Bali et al., 2001).

1.4.4.2.3 Keratan Sulphate

Two main classes of KS have been characterised, originally named KSI and KSII dependent on their distribution in cornea and cartilage respectively. Both consist of a repeated disaccharide of N-acetylglucosamine and galactose (see fig.1.5), although KSI is linked to asparagine residues in the attached core protein, whereas this linkage occurs through serine and threonine residues in the case of KSII (Funderburgh, 2000). These linkages are not specific to individual tissues however, and therefore all asparagine linked KS chains are termed KSI and those linked through serine and threonine residues are termed KSII. In addition, a third class of KS, KSIII has been discovered based on its linkage through mannose (Krusius et al., 1986).

Within cartilage KSI can be found attached to fibromodulin, PRELP and osteoadherin (Antonsson et al., 1991; Bengtsson et al., 1995; Sommarin et al., 1998). A small number of KSI chains can also be found attached to the aggrecan core protein (Barry et al., 1995).KSII is more highly sulphated than KSI and the chains are shorter (Nieduszynski et al., 1990). As seen with CS, KS distribution is dependent on the articular cartilage zone, with much higher concentrations of the GAG seen in deeper regions of the tissue (Zanetti et al., 1985). The authors from this study demonstrated that chondrocytes found residing within the different cartilage zones had varying capabilities to synthesis KS thereby explaining the defined zonal distribution.

1.4.4.2.4 Heparin / Heparan Sulphate

Heparan sulphate (HS) is the most complex of the GAG's, composed of alternating hexuronic acid and glucosamine residues (see fig.1.5). Various sulphations along this

polymer convey the GAG's biological function (Lindahl et al., 1998). The closely related GAG heparin is considered to be an over-sulphated and intracellular HS variant. Heparin is specifically produced by mast cells and is found on the surface of the proteoglycan serglycin (Humphries and Stevens, 1992).

HS has been demonstrated to have numerous interactions with cytokines such as interleukin-6 (IL-6) (Mummery and Rider, 2000) and tumour necrosis factor α (TNFα) (Menart et al., 2002), growth factors such as members of the fibroblast growth factor (FGF) family (Ashikari-Hada et al., 2004), lipases, proteases and protease inhibitors (van Barlingen et al., 1997; Whitelock et al., 1996) as well as ECM proteins. HS has been demonstrated to associate with a number of the major ECM proteins found within articular cartilage such as fibronectin (Walker and Gallagher, 1996) and type II collagen (Keller and Furthmayr, 1986).

1.4.4.3 Collagens

Collagens within the ECM of articular cartilage provide tensile strength and stability to the tissue and protection for the encased chondrocytes. To date 27 collagens have been characterised within vertebrates. Within the collagen superfamily there are four subgroups, namely the fibrillar collagens, microfibrillar, short chain non-fibrillar and FACITs (fibril associated collagens with an interrupted triple helix). All collagens are characterised by the presence of continuous or interrupted triple helices composed of the amino acid repeat sequence Gly-X-Y where the latter two residues are often proline or hydroxyproline (Pace et al., 2003; Ricard-Blum and Ruggiero, 2005; Sandell et al., 1999). Glycine is the smallest amino acid, and the alpha (α) chains of collagens arrange themselves so that these residues are always positioned in the centre of the triple helix, allowing the larger amino acids to fall on the outside of the helix. This allows close packing of the molecule. The presence of 4-hydroxyproline is essential for the formation of intramolecular hydrogen bonds which contribute towards the thermal stability of the triple helix (Gelse et al., 2003).

The arrangement of the collagen molecules into a fibrillar structure provides collagen with the ability to resist tensile forces. Post-translational modification of collagens such as cross-linking confers greater stability and strength to the tissue. The process of collagen fibril

formation occurs extracellularly through the cleavage of pro-collagen peptides by pro-collagen metalloproteinases. The C-propeptide is considered to be involved in the initiation of fibril formation, whereas the N-propeptide is considered to have a role in regulating fibril diameter (Bateman et al., 1996).

Within cartilage the main collagen is the fibrillar triple helical type II, making up 90% of the total collagen content. The type II collagen triple helix consists of 3 α1(II) chains (Bruckner and van der Rest, 1994). Type II collagen α chains show high levels of hydroxyproline as well as glucosyl and galactosyl residues which aid in the interaction of the collagen molecule with proteoglycans within the ECM (Mayne, 1989). Alternative splicing can occur in the type II collagen pre-messenger ribonucleic acid (mRNA) which results in the generation of 2 forms of the α1(II) chain – splice variants IIA and IIB. IIB is the most predominant form in mature articular cartilage. This variant lacks exon 2 encoding for a cysteine rich domain. The splice variant IIA maintains this exon and is generally expressed in development, and is seen in pre-chondrogenic mesenchyme and perichondrium (Ryan and Sandell, 1990).

Fibrils of the FACIT type IX collagen and fibrillar type XI can normally be found associated with type II collagen (see fig.1.6). These minor collagens are thought to have roles in regulating fibril diameters of the type II component. Type IX collagen can be found covalently attached to the surface of the type II fibril and cross-linking between multiple type II and IX molecules. The presence of a CS chain on some type IX molecules suggests a role in interaction with other matrix components and also possible targeting of the molecule to the correct sites for cross-linking (Eyre et al., 1987). The NC4 (non-collagenous 4) domain of the type IX fibril may also help in maintaining matrix integrity (Morrison et al., 1996). Type XI collagen can be found within the type II/IX/XI heterofibril and is primarily concerned with regulating fibril diameter, although has also been suggested to interact with matrix proteoglycans and the chondrocytes (Eyre, 2002; Vaughan-Thomas et al., 2001; Wardale and Duance, 1993). It is generally accepted that this collagen type is required for maintaining the integrity and cohesion of the tissue. Other minor collagens such as types I, III, V, VI, X, XII and XIV have also been found in cartilage, although are normally specific to one area. For example, type VI collagen is found most concentrated

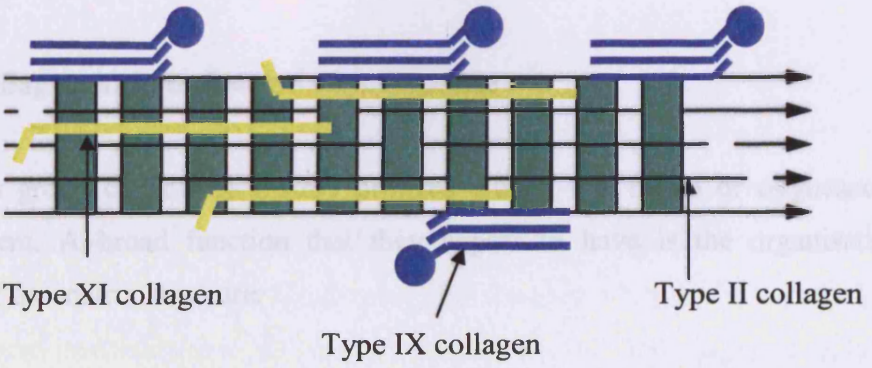


Fig.1.6 The heterofibril of type II/IX and XI collagens (adapted from Duance et al., 1999)

within the pericellular matrix surrounding the chondrocyte and acting to stabilise fibrillar structures and the linkage of matrix molecules (Chang and Poole, 1996; Hing et al., 2002; Morrison et al., 1996). Type VI collagen has been shown to interact with components of the ECM through SLRPs such as decorin and biglycan, which in turn complex with the oligomeric matrix proteins, matrilins (Wiberg et al., 2003). Matrilin-1 has previously been shown to interact directly with both aggrecan and type II collagen (Hauser et al., 1996; Paulsson and Heinegard, 1979; Winterbottom et al., 1992). Type X collagen is found associated with type II collagen and is expressed exclusively within the hypertrophic zone of the growth plate and is thought to be involved in the calcification of the surrounding matrix (Wardale and Duance, 1993).

1.4.4.4 Non-collagenous Proteins and Glycoproteins

In general this group constitutes mainly proteins with a few mono or oligosaccharides attached to them. A broad function that they appear to have is the organisation and maintenance of the matrix structure.

1.4.4.4.1 Link Protein

The LPs are a family of glycoproteins involved in the stabilisation of aggregates of proteoglycans and HA (Comper and Laurent, 1978). At least three forms of LP have been described, namely LP1, LP2 and LP3, and range in size from 45-50KDa (Baker and Caterson, 1979). LP3 is the most prominent isoform within mature articular cartilage (Roughley et al., 1982).

Studies isolating LP from rat chondrosarcoma have identified it as a 339 amino acid single peptide. Differences in the molecular weights of LP isoforms arise from glycosylation patterns. The protein core of LP3 is 37.406KDa and there is just one N-linked oligosaccharide. Disulphide bonds within the structure generate loops are separated by short sequences of 27 amino acids. It is thought that these loops are the important structures in the interaction between LP and HA, as they are known to be highly conserved within different species (Neame et al., 1986).

1.4.4.4.2 Superficial Zone Protein (SZP)

SZP, an approximately 345KDa proteoglycan, also know as proteoglycan-4 (PRG4), is homologous to the megakaryote-stimulating factor and is specifically localised in the superficial zone of cartilage. The majority of the proteoglycan is released into the synovial fluid, although some is retained within the local matrix. SZP contains large and small mucin-like O-linked oligosaccharide repeat domains flanked by cysteine-rich N and C-terminal domains and can be classified as a proteoglycan due to the CS and KS substitutions. It has growth promoting, cytoprotective, lubricating and matrix binding properties (Flannery et al., 1999a; Schumacher et al., 1994).

1.4.4.4.3 Cartilage Intermediate Layer Protein (CILP)

This 91.5KDa protein is found specifically within cartilage and is most abundantly found within the transitional zone. Its defined location suggests a role for CILP in maintaining the structural integrity of the cartilage. Synthesised by the chondrocytes it encompasses, this protein has been demonstrated to have between 3 and 5 N-glycosidically linked oligosaccharides (Lorenzo et al., 1998) and has also been shown to be up-regulated in the early stages of osteoarthritis (OA) (Bayliss et al., 1983). Like aggrecan, this protein also accumulates within cartilage with age and although exactly why this occurs is not known, it has been speculated that this is the result of decreased turnover of the protein (Lorenzo et al., 1998).

1.4.4.4.4 Cartilage Oligomeric Matrix Protein (COMP)

This pentameric glycoprotein, composed of 5 identical 110KDa monomeric subunits, is a member of the thrombospondin (TSP) gene family. Specific to this family, COMP possesses type 3 calmodulin-like repeats, which bind calcium ions with high affinity (Chen et al., 2000). Calcium will also bind to the type II epidermal growth factor (EGF)-like repeats present, helping COMP to assume a more compact unit.

It has been suggested that this glycoprotein may have roles in tissue development and homeostasis through interactions directly with cells (DiCesare et al., 1994) via an integrin-

mediated mechanism and with ECM components such as types II (Rosenberg et al., 1998) and IX collagen (Holden et al., 2001). These interactions with ECM constituents are zinc dependent and are mediated by the C-terminal of COMP. The ability of COMP to bind several matrix components at once suggests roles in fibrillogenesis or chaperoning, bringing individual fibrils together to create a heterotypic fibril (Chapman et al., 2003).

1.4.4.4.5 Annexin V / Anchorin II

Annexin V is a membrane-associated collagen binding glycoprotein with a molecular weight of 31KDa. This hydrophobic molecule has an affinity for native type II collagen fibrils and has been localised to the chondrocyte-matrix interface (Mollenhauer et al., 1984). Binding to native type II collagen is decreased after pepsinisation signifying binding to the telopeptide portion of type II collagen (Kirsch and Pfaffle, 1992). Expression of annexin V was found to be decreased on the surfaces of flattened chondrocytes and this expression has been shown to decrease further with culture time (Mollenhauer et al., 1984). Annexin V has been suggested to play a role in the calcification of cartilage. It has been shown to be expressed in both the growth plate (especially within the zone of calcification) and bone and is a constituent of matrix vesicles. It does bind to type X collagen, although to a lesser extent than type II collagen and therefore is thought to play a role in the process of attachment of the matrix vesicles to the collagenous matrix surrounding them. Within calcifying tissue annexin V also takes on the form of a calcium ion channel, which it has been hypothesised, creates a calcium rich environment inside the matrix vesicles for crystallisation (Kirsch and Pfaffle, 1992).

1.4.4.4.6 Tenascin-C

Tenascin-C is a high molecular weight glycoprotein composed of 6 subunits ranging between 190 and 300KDa in size, joined at their N-terminal by disulphide bonds. This N-terminal, which is cysteine rich, is involved in oligomerisation and contains EGF-like repeats, fibronectin type III-like repeats and a fibrinogen-like globular domain (Jinnin et al., 2004). This correlates with the ability of tenascin-C to bind to ECM proteins such as fibronectin and aggrecan as well as cell surface receptors. This glycoprotein also has the ability to interact with proteases such as the matrix metalloproteinases (MMPs) (Jones and

Jones, 2000). Tenascin-C has been visualised by rotary shadowing and has been shown to form a symmetrical structure termed a hexabrachion (Erickson and Inglesias, 1984).

During chondrogenesis, tenascin-C is selectively associated with mesenchymal condensations. With the process of endochondral ossification and the development of bone, expression becomes restricted to the epiphyseal cartilage (Mackie and Murphy, 1998). Within normal adult cartilage tenascin-C expression has been demonstrated in the territorial and interterritorial matrices of the deeper zones. Within diseased or fibrillated cartilage tenascin-C expression is up-regulated though, especially within the pericellular matrix and surface lining of the cartilage (Pfander et al., 1999). This is most probably due to a tissue repair response.

1.4.4.4.7 Fibronectin

Fibronectin is an ECM dimeric glycoprotein with 5% carbohydrate content. It is made from large, 250KDa subunits. The subunits are held together by a pair of disulphide bonds at the C-terminal of the dimer (see fig.1.7). Once in a dimeric form the polypeptide can fold itself into functional domains, which are lined up along the length of the protein. Each functional domain contains either type I, II or III repeats of between 45-90 amino acids forming separate mini-domains (Hynes, 1990).

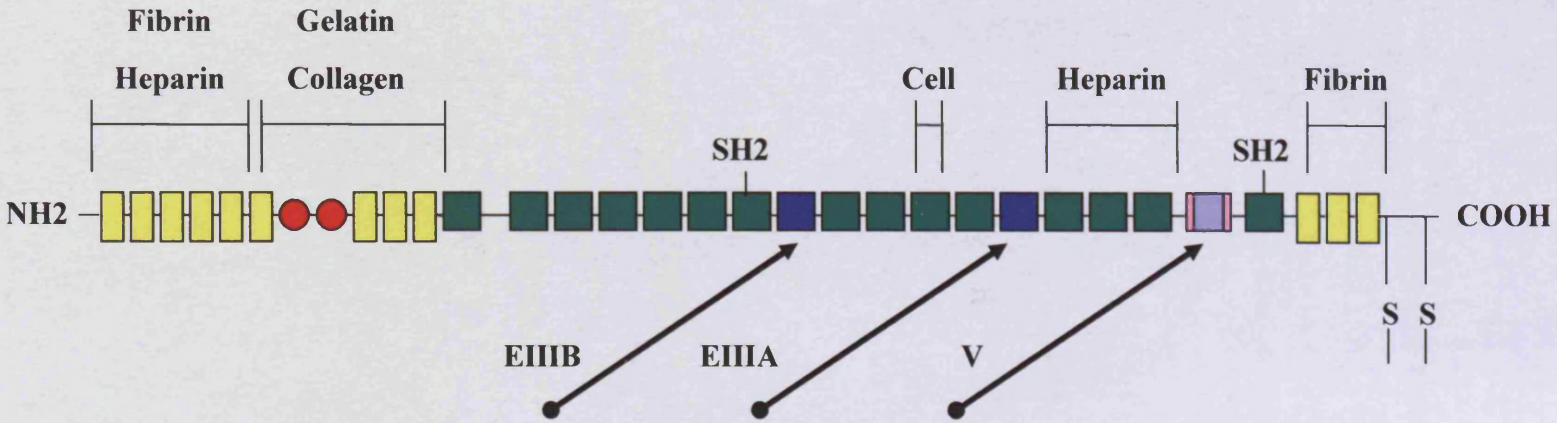
Fibronectin can be found in two forms – plasma (pFN) and cellular (cFN). pFN acts as a disulphide bonded dimer and has been shown to bind with a Kd (dissociation constant) of 1μM to the actin binding protein (ABP), gelsolin (Lind and Janmey, 1984). While cFN can be found as a dimer, it can also associate to form a high molecular weight disulphide bonded polymer or aggregate after secretion from the cell (Hynes, 1990).

Fibronectin, although encoded by a single gene product exists as many isoforms due to extensive post-translational modifications and splice variation at the pre-mRNA stage. One of these isoforms is found exclusively within cartilage and is derived from a pattern of alternative splicing (MacLeod et al., 1996). Isoforms of fibronectin expressed, change dramatically during development. Within articular cartilage a definite increase in fibronectin protein levels is seen during the process of pre-chondrocyte condensation in

limb development (Downie and Newman, 1994; Downie and Newman, 1995; Kulyk et al., 1989). Within mature cartilage studies by Brown and Jones (1999) and Hayashi et al. (1996) fibronectin biosynthesis is localised to only the surface and deep zones of cartilage.

The functions of fibronectin are diverse although definite roles in cell differentiation, adhesion and migration have been documented (Hynes, 1990). Fibronectin is able to bind to a wide variety of ECM molecules as well as to the chondrocytes via their integrin receptors. Fibronectin can bind to several integrins, in particular $\alpha5\beta1$, which is specific for fibronectin and one of the principal integrins expressed by chondrocytes. It is the arginine-glycine-aspartic acid (RGD) of the type III repeats which is essential for binding to this integrin (Loeser, 1993; Loeser et al., 1995; Salter et al., 1995; Salter et al., 1992; Sommarin et al., 1989). Cells are unable to bind to fibronectin when it is in solution, suggesting that when fibronectin binds to a substrate its conformation must change (Grinnell, 1976; Pearlstein, 1978; Schwarz and Juliano, 1984). Cell binding to fibronectin requires the presences of divalent cations (Edwards et al., 1988; Edwards et al., 1987; Grinnell, 1976; Klebe, 1974; Klebe, 1975; Klebe et al., 1977; Pearlstein, 1978) and metabolic energy (Grinnell and Hays, 1978; Grinnell and Minter, 1978).

Structural studies by Kleinman et al. (1981) demonstrated that a single region between positions 690-800 on collagen chains $\alpha1$ (I), $\alpha2$ (I), $\alpha1$ (II) and $\alpha1$ (III) is required for binding to fibronectin. Recent findings also demonstrate an interaction between the NC4 domain of type IX collagen and fibronectin (Callender, 2004). Fibronectin also binds strongly to highly sulphated HS, heparin and dextran sulphate chains. Binding of these proteoglycans strengthens the binding affinity to the α chains of the described collagens. Fragmentation studies have demonstrated 3-4 heparin-binding sites on the fibronectin molecule each with its own defined specificities and affinities for divalent cations (Gold et al., 1983; Hayashi and Yamada, 1982; Sekiguchi et al., 1982).



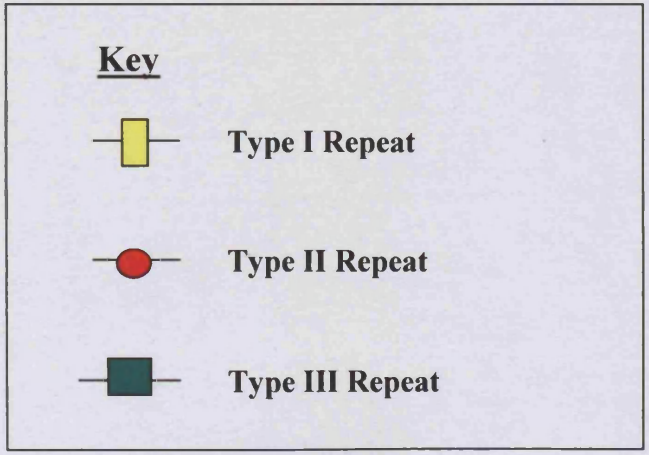


Fig.1.7 Fibronectin structure

The sequence of fibronectin is made up of type I, II and III repeats separated by introns. EIIIA and B can be included or spliced out during processing giving rise to the many isoforms of fibronectin. Splice variants can also occur within the V (variable) region.

(Adapted from Hynes, 1990)

1.5 Age Related Changes in the Morphology and Composition of Articular Cartilage

Age related changes in articular cartilage have been widely reported and primarily relate to alterations in matrix macromolecule distribution (Bobacz et al., 2004). Changes in the physical 'make-up' of articular cartilage can ultimately affect the mechanical and functional properties of the tissue leading to degenerative diseases such as OA.

Age related decreases in proteoglycan synthesis have been reported (DeGroot et al., 1999; Schafer et al., 1993; Verbruggen et al., 2000), but the question of whether this is a result of decreased cellularity of the tissue or a decrease in biosynthetic activity of the cells is currently debatable. It is widely accepted that there is a decrease in cellularity of articular cartilage with skeletal development and maturity, although conflicting reports have been made as to whether this decrease continues to progress after maturation (Huch et al., 2001; Meachim and Collins, 1962; Mitrovic et al., 1983; Vignon et al., 1976). Decline in cell density occurs primarily within the superficial zone (Kobayashi et al., 2003) and this cell death has recently been attributed to apoptosis (Kim et al., 2000; Kim et al., 2001) after years of thinking the decrease in cell number was a result of necrosis (Roy and Meachim, 1968). Analysis of aged chondrocytes has also demonstrated high level expression of pro-apoptotic genes such as Fas and caspase-8 (Todd Allen et al., 2004).

The initially high cell density seen within immature cartilage may contribute to the anabolic state of the tissue resulting in growth. Quinn et al. (1999) demonstrated that with increasing distances between cells there is decreased matrix metabolism. Deposition of matrix molecules and their turnover in bovine cartilage occurs within 8μm of the cell membrane (Quinn et al., 1999). In the case of mature cartilage, where cells are sparsely distributed, regions of the ECM will be prone to degradation.

1.6 Articular Cartilage Development

The formation of skeletal elements is under the regulation and control of transcription factors such as the homeobox (Hox) genes as well as signalling molecules and growth factors such as the bone morphogenic proteins (BMPs). The Hox genes have fundamental roles in the patterning of vertebrate embryos (Nowicki and Burke, 2000) and Hox 9-13

have definite roles in limb development and patterning (Pacifici et al., 2005). The process of joint development is divided into two phases, the first being mesenchymal condensation and the differentiation of the pluripotent cells into a cartilaginous skeleton. Within the developing limb, condensations form as a result of aggregation of uniform populations of cells (Hinchliffe and Johnson, 1980). An increase in cell-cell contact and gap junctions facilitate intracellular communication and the transport of molecules between them (Kelley and Fallon, 1978; Kelley and Fallon, 1983; Zimmermann, 1984; Zimmermann et al., 1982). The process of chondrogenesis follows directly on from the formation of condensations and it is the shape and number of these condensations that define the skeleton (Morris et al., 2002). The clustering of these cells is under the control of cell-cell adhesion molecules such as neural cell adhesion molecules (N-CAMs) (Widelitz et al., 1993) and N-cadherin (Oberlender and Tuan, 1994) as well as matrix receptors such as CD44 (Toole, 1991). The constituents of the ECM change during development with increasing levels of fibronectin, syndecan-3, versican and tenascin seen during condensation and decreases in HA. As chondrogenesis begins and the multipotent cells begin to develop into chondrocytes, levels of the characteristic type II collagen and aggrecan increase with type I collagen levels decreasing (Kosher et al., 1986a; Kosher et al., 1986b).

The second phase of joint development involves the formation of the joint structures and space. Chondrocytes within the destined joint space undergo a phenotypic change from a round to a flattened fibroblastic morphology and are interconnected by gap junctions. This non-chondrogenic region is known as the interzone, an important signalling centre within the joint (Archer et al., 2003). Cells within the interzone will express BMPs and their antagonists, for example noggin, regulating chondrocyte proliferation and differentiation (Hartmann and Tabin, 2000). Signals from the joint, such as those provided by growth and differentiation factor 5 (Gdf5), control proliferation and prevent terminal differentiation of the developing cells. The interzone itself has been shown, in a study looking at the development of rat knee joints (Ito and Kida, 2000), to be composed of 2 outer layers associated with the epiphyseal ends and a thin intermediate area. Cells within the outer zones have been shown to become later incorporated into the epiphyses, whereas those within the intermediate zone are destined to become part of the articular cartilage.

After the formation of the interzone, the cartilage anlagen will separate and undergo a process of cavitation to form the synovial cavity (Pacifici et al., 2005). The strongest evidence regarding the mechanism by which this occurs involves HA synthesis, regulated by mechanical loading. Both HA and its receptor, CD44, are expressed at the interzone and opposing cartilage surfaces (Archer et al., 1994; Dowthwaite et al., 1998; Edwards et al., 1994; Pitsillides et al., 1995). HA synthesis is up-regulated during the formation of the synovial cavity. This is coupled by the increased expression of HA synthase due to mechanical stimulation (Dowthwaite et al., 1998). Increasing concentrations of HA result in cell separation and loss of tissue integrity facilitating cavitation (Toole, 1991). In addition to the increased synthesis and accumulation of HA, there is a change in the orientation of collagen fibrils along the future joint line, together facilitating the process of cavitation (Pacifici et al., 2005). It is also important to consider the potential role of SZP is facilitating cavitation. This anti-adhesive glycoprotein may, when increased in production, aid cavitation by reducing association between the epiphyses (Pacifici et al., 2005).

1.7 The Chondrocyte Cytoskeleton

Cytoskeletal elements form a dynamic network of microfilaments, intermediate filaments (IFs) and microtubules within the chondrocyte. Collectively cytoskeletal constituents play important roles in the interactions between the chondrocyte and its ECM. They are fundamentally involved in the cellular processes of motility, division and plasticity. The cytoskeleton also contributes to the biomechanical properties of the cell and interactions as a result of mechanical loading.

1.7.1 Actin Microfilaments

Actin proteins are a highly conserved family found solely in eukaryotic cells. They have been classified into 3 isoforms, namely α, beta (β) and gamma (γ). Actin is primarily located within the cytoplasm, although it has been localised to the nucleus suggesting non-trafficking functions as well. Actin filaments are constructed from individual, uniformly orientated globular actin molecules (G-actin) forming a tight helix with a diameter of approximately 8nm. These filaments (F-actin) rarely occur in isolation but rather form cross-linked aggregates or bundles of fibrils providing strength. These microfilaments have

been implicated in processes such as cell adhesion (Turner and Burridge, 1991), migration (Heath and Holifield, 1991), ECM assembly (Hayes et al., 1999) and regulation of chondrocyte hypertrophy (Hirsch et al., 1996; Hirsch et al., 1997). Importantly actin has both active roles in movement as well as passive roles in maintaining structural integrity of the cell and providing an anchor for other cytoskeletal proteins.

Within chondrocytes Langelier et al. (2000) showed, by actin labelling, that the predominant location of actin is just beneath the cell membrane, suggesting roles in secretion and endocytosis. Focal points of actin at the cell membrane however were shown to co-localise with vinculin and therefore may also have important functions in adhesion to the ECM. Actin expression was seen throughout the different zones of the articular cartilage uniformly, in contrast to the expression of vimentin, which was concentrated within the superficial zones, especially in weight bearing regions.

1.7.1.1 Actin Binding Proteins

To date 162 ABPs have been identified and many are known to bind to the same site on the actin surface and are therefore thought to compete. These proteins have been classified into seven families and include the actin-monomer binding proteins, thymosin and profilin. Actin-monomer binding proteins associate directly with the monomer and inhibit its addition to the end of a forming filament. Profilin is located within the plasma membrane and regulates actin polymerisation in response to extracellular stimuli (Goldschmidt-Clermont et al., 1992), and thymosin has been shown to be mechanically regulated suggesting a role for actin in mechanotransduction (Blain et al., 2002; Blain et al., 2003). Other ABPs include the filament depolymerising proteins CapZ and actin depolymerising factor (ADF)/cofilin which convert F-actin to G-actin, and gelsolin which slices filaments by binding to either side of the F-actin cutting it into two pieces. Tropomyosin conversely stabilises F-actin filaments and prevents their depolymerisation, while Arp 2/3 (actin related peptide 2/3) act as a cross-linking protein aiding the formation of filament bundles (dos Remedios et al., 2003).

1.7.2 Microtubules

Microtubules are polar structures derived from α/β tubulin heterodimers and are considered highly dynamic in their ability to switch between growing and shrinking phases by the binding and hydrolysis of guanidine triphosphate (GTP) by individual tubulin subunits. The plus end of the microtubule is capable of rapid growth and shrinkage while the minus end is normally stabilised by the centrosome, which in most cells is next to the nucleus. In order to form heterodimers, tubulin needs chaperoning co-factors to be present. These co-factors also regulate the α/β tubulin ratios. There are many isotypes of both α and β tubulin subunits and each has a different stability within the cell. Microtubules are formed by the binding of polymers of tubulin dimers in a head to tail conformation. The positively charged C-terminal end of the isoform confers its ability to associate with microtubule-associate proteins (MAPs), suggesting that isoform type may be of relevance to not only the stability but also the functionality of the microtubule within the cell. Extensive post-translational modifications can also occur at the C-terminal of both tubulin subunits. These modifications alter the ability of the subunit to interact with cellular factors, suggesting importance in signal transduction pathways and cytoskeletal interactions (Nogales, 2001). Within chondrocytes microtubules are constantly renewing and have been demonstrated to play a crucial role in the process of hypertrophy (Farquharson et al., 1999) and in regulating the phenotype of the cell (Brown and Benya, 1988; Martin et al., 1999).

1.7.2.1 Microtubule-Associated Proteins and Motors

1.7.2.1.1 Kinesin

The microtubule-associated motor protein, kinesin, is a dimer (Hirose and Amos, 1999) of 2 identical 120-130KDa heavy chains. Two light chains of 60-70KDa are associated with the dimer (Kull, 2000) and are involved in the binding of kinesin to organelles but not cell motility (Howard, 1997). The heavy chains both contain an N-terminal globular motor domain with a microtubule-binding site and an adenosine triphosphatase (ATPase)-active centre, a stalk region for heavy chain dimerisation and a C-terminal tail domain for cargo binding. Kinesin is able to bind to the surface of the microtubules and use them as tracks

for the transportation of cargo (Vale, 2003; Woehlke and Schliwa, 2000). The preferred model of how kinesin performs this function is the "hand-over-hand" model where two kinesin heads bind to the microtubule, stepping along its surface by the hydrolysis of adenosine triphosphate (ATP) (Coy et al., 1999; Schnitzer and Block, 1997; Vale and Milligan, 2000). Kinesin directs its force towards the plus-ends of the microtubules for anterograde movement. Kinesin has also been documented to be involved in the movement of vimentin particles. Interactions between kinesin and IFs have been shown to occur within the tail region of kinesin's heavy chain and within a specific 62KDa region of its light chain (Liao and Gundersen, 1998).

1.7.2.1.2 Dynein

Retrograde movement of cargo towards the minus-ends of microtubules, is controlled by the dynein-dynactin motor complex (Cross, 2004). Dynein, a large enzyme complex of the cytoplasm, has two heavy chains to which light chains can bind. Dynein is a much larger and more complex molecule than kinesin. Dyneins have a molecular mass of between 1000-2000KDa and have 2-3 heads through which they bind the microtubules (Johnson and Wall, 1983; Sale et al., 1985; Vallee et al., 1988). The heads are derived from the heavy chains and contain large polypeptides involved in ATP hydrolysis (Vallee, 1993). The head of the molecule binds to the dynactin complex, which connects the complete motor complex to the microtubules. In addition to cargo transport, dynein is also thought to have roles in the movement and distribution of intracellular organelles such as elements of the Golgi apparatus (Vallee, 1993).

1.7.3 Intermediate Filaments

IFs derived from individual vimentin particles are an extensive family with over 65 different genes characterised and sub-classified into 5 families – 4 of which are cytoplasmic (I-IV) and 1 which is nuclear (lamins / V).

Vimentin particles are elongated, thin rod-shaped dimers and can passively assemble into 10μm diameter filaments with a polymeric coiled-coil configuration (Strelkov et al., 2003). Each dimer, normally homodimers, forms alignments to each other parallel to the filament

axis. All IFs exhibit 'tripartite' structures with a helical domain, the rod, and flanking this region two non-helical domains (Fuchs and Weber, 1994; Geisler and Weber, 1982). They display a tight meshwork that traverses from the cytoplasm to the nucleus, suggesting functions in mRNA transport and targeting (Langelier et al., 2000). Their location overlaps with the microtubules suggesting that these systems may interact in some way (Prahlad et al., 1998; Yoon et al., 1998). Preparations of polymerised IFs demonstrate the unique ability of these filaments to resist deformation and breakage due to mechanical loading because of their viscoelastic nature (Janmey et al., 1991; Janmey et al., 1998). Time-lapse studies have demonstrated the dynamic nature of these filaments and their ability to change shape and configuration continuously through assembly, disassembly, shortening and elongation through the cytoplasm (Martys et al., 1999; Yoon et al., 1998). IFs have no polarity, unlike the previously discussed microtubules and actin microfilaments, due to their ability to orientate in both directions along the filament (Strelkov et al., 2003). Experiments by Yoon et al. (1998) have shown the active bi-directional movement of IFs within the cytoplasm with speeds of 1-2μm/s being demonstrated by one group (Prahlad et al., 1998). Prahlad's group also described the movement of individual vimentin particles along microtubules with the majority (≈65-70%) of movement being anterograde.

Increased expression of IFs have been shown in load bearing regions of articular cartilage (Eggli et al., 1988) supporting the theory that IFs play a crucial role in the coordination of mechanical events (Lazarides, 1980). Langelier et al. (2000) showed vimentin IFs to be most concentrated in the superficial zones of articular cartilage with expression patterns being even clearer in peripheral loading bearing regions of the cartilage. Traub et al. (1995) have also suggested a role in signal transduction. Recent studies have demonstrated significant cytoskeleton disruption in osteoarthritic cartilage. Changes in both the content and distribution of vimentin, tubulin and actin have been visualised. A reduction in all three cytoskeleton components was demonstrated, with highly significant losses of vimentin and tubulin seen in the OA cartilage suggesting their involvement in OA pathogenesis (Capin-Gutierrez et al., 2004; Fioravanti et al., 2003).

1.8 Chondrocyte Cell Surface Receptors

1.8.1 CD44

CD44 is a single-pass transmembrane glycoprotein acting as a receptor on the surface of many cell types including chondrocytes (Chow et al., 1995; Knudson et al., 1996). This is the main receptor involved in the binding of HA and therefore its associated aggrecan aggregates. CD44 is of crucial importance in the formation of pericellular matrices during the process of chondrogenesis. This receptor contains a hydrophobic transmembrane domain and an intracellular domain of 70 amino acids, which has the ability to transduce signals and control receptor expression within the plasma membrane (Knudson et al., 1999). Changes within the HA rich ECM, to which CD44 is attached, results in alteration to the phosphorylation of the receptor. The intracellular domain of CD44 has binding domains allowing the attachment of ABPs, namely from both the 'ERM' (ezrin, radixin and moesin) and 'ankyrin' families. These ABPs allow the linking of the cytoskeleton to the cytoplasmic domain of CD44 and in turn the HA ECM. Chondrocytes have been shown to express both moesin and ankyrin concurrently. Chondrocytes can therefore respond to changes sensed in their extracellular environment through the association of their cytoskeleton and receptors. The association of CD44 with the discussed ABPs is just one example of this. These interactions have also been shown to alter chondrocyte metabolism (Knudson and Loeser, 2002). CD44 has also been shown to be responsible for the internalisation of HA for its catabolism. Mutations within the cytoplasmic tail of CD44 have demonstrated that this domain is required for the binding and internalisation of HA. However, only HA located within the cell's pericellular matrix can be processed in this way, perhaps contributing to the accumulation of HA and its associated G1 domain of aggrecan with age in the interterritorial matrix of articular cartilage (Embry and Knudson, 2003).

1.8.2 Integrins

Integrins are a heterodimeric (1α and 1β-subunit) family of transmembrane receptors. By definition they are glycoproteins with large globular extracellular domains, which are able to bind and interact with ECM proteins as well as other cell bound surface receptors

('outside-in' signalling). Their cytoplasmic domains are able to interact with cytoskeletal components and through alterations in signalling pathways, mediate changes in cell morphology, gene expression and matrix proteins ('inside-out' signalling) (Camper et al., 1998; Yamada, 1997). Intracellular signals can also be propagated through integrins and regulate integrin ligand-binding affinity and cell adhesion (Hynes, 1992; Schwartz et al., 1995). Through such interactions research has elucidated roles for cell surface integrins in cell survival, differentiation, migration, growth and matrix remodelling. The direct interaction of chondrocytes with their external environment allows their response to change in order to maintain the composition and mechanical properties of the cartilage. On the surface of chondrocytes the major integrin subfamilies mediating cell-matrix interactions are the β1 and αV-subunits. Integrin expression patterns change during development as well as in response to their intracellular and extracellular environments. Chondrocyte integrin expression has also been shown to alter in response to ECM damage or removal as well as by the differentiation state of the cell (Knudson and Loeser, 2002); however it is known that adult articular chondrocytes express α1β1, α2β1 (weakly), α3β1, α5β1, α6β1, α10β1, αVβ3 and αVβ5 (Loeser et al., 1995; Salter et al., 1992; Woods et al., 1994). The most prominent integrin in adult chondrocytes being α5β1 (Salter et al., 1992). This has been summarised in fig.1.8.

The α-subunits of integrins consist of a short cytoplasmic tail, a transmembrane domain and a large extracellular domain of approximately 1000 amino acids. The extracellular domain contains distinct cation binding regions, which regulate integrin mediated attachment to matrix proteins (Longhurst and Jennings, 1998). Integrins normally exist in a low-affinity state (Faull et al., 1994; Lollo et al., 1993) and cannot bind to its ligand without an energy dependent activation process involving intracellular signal transduction and mediation by the cytoplasmic tail. Within the β-subunits the terminal aspartic acid residue is necessary for the regulation of integrin activation (Hughes et al., 1996). Upon adhesion to ECM ligands, integrins will cluster within the cell membrane for their activation. Integrin signalling requires the binding of cytoskeletal elements and the reorganisation of cytoskeletal complexes for the activation of signalling molecules, which for many involves phosphorylation of tyrosine or serine and threonine residues. A study by Clancy et al. (1999) demonstrated such a signalling complex on integrin binding to

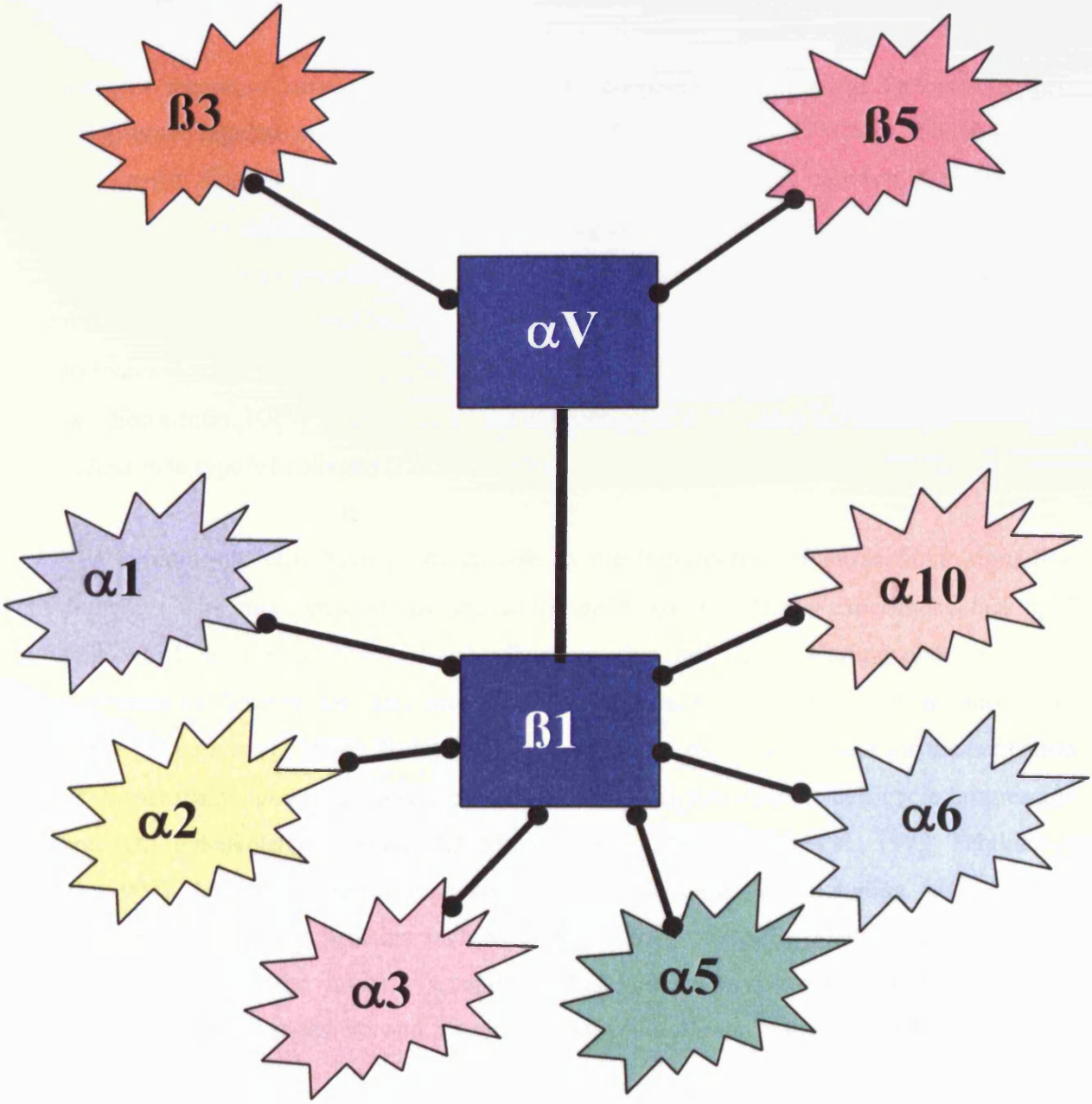


Fig.1.8 Integrin expression by articular chondrocytes

fibronectin and the resulting recruitment of F-actin, the small GTPase (guanosine triphosphatase) RhoA and focal adhesion kinase (FAK).

Integrin receptors have also been shown to cooperate with growth factors and their receptors to regulate matrix production and other functions. Growth factors, such as insulin-like growth factor-1 (IGF-1), have demonstrated potential synergy between its receptor and the β1 integrin subunit by activation of cell signalling proteins such as Shc (Shakibaei et al., 1999). Integrins generally recognise ligand amino acid sequences containing an acidic residue required for ligand binding, for example RGD. TGFβ1 and IGF-1 have been shown to increase α5β1 integrin expression and in doing so increase adhesion to type II collagen and fibronectin. IGF-1 also increased the expression of α1β1 integrin resulting in enhanced adhesion to type VI collagen (Loeser, 1997).

The cytoplasmic tails have a crucial role in the transduction of intracellular signalling pathways. The importance of this domain has been demonstrated by experiments lacking β tails which fail to localise to focal adhesions, decrease ligand binding and impair the activation of downstream signalling molecules (Hayashi et al., 1990; Marcantonio et al., 1990; O'Toole et al., 1994). Isolated β tails are also the only requirement for the activation of downstream signalling molecules such as FAK, and can regulate cell cycle progression and actin cytoskeleton assembly (Belkin and Retta, 1998; David et al., 1999; Tahiliani et al., 1997). At least 21 proteins are known to bind to these tails including ABPs such as filamin and signalling proteins such as FAK. ABPs provide the connection between the cytoskeleton and the integrin receptors. This connection provides the basis for the formation of focal adhesions and the initiation of cell adhesion, spreading and migration.

1.8.3 Syndecans

Syndecans are type I membrane proteoglycans with a single transmembrane sequence and a short cytoplasmic tail. The extracellular domain has GAGs attached which are normally HS (see fig.1.9), although some of the four members of this family possess CS or DS chains, for example syndecan-4 (Bernfield et al., 1992). These extracellular domains are heterogeneous and sequence conservation is only seen at the sites of attachment to the GAG

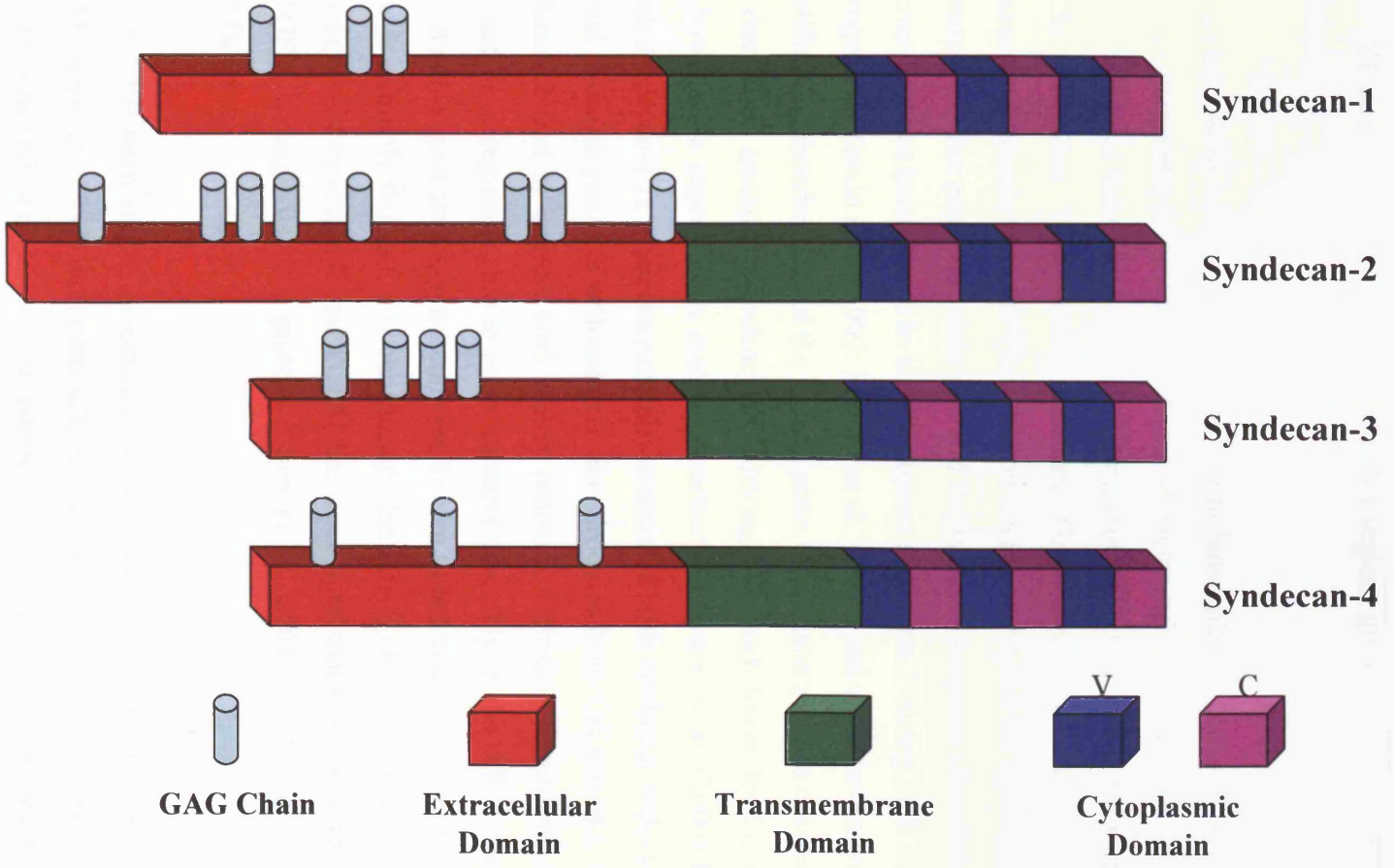


Fig.1.9 Schematic representation of the structure of syndecans (adapted from Zimmerman and David, 1999)

side chains. The syndecan core proteins contain 4 conserved tyrosine residues, which can be modified by phosphorylation. The transmembrane domain is conserved between all members and the cytoplasmic domain can be separated into three regions with two constant regions (C1 and C2) flanking a variable (V) region, giving the individual syndecan its specificity.

These glycoproteins have been demonstrated to have roles in cell proliferation, migration, adhesion, cytoskeletal organisation and growth factor signalling (Woods, 2001). Syndecans appear to carry out their functions by binding growth factors and/or their receptors as well as ECM molecules. This occurs via their GAG chains which interact with signal transducers and directly with the cytoskeleton via their cytoplasmic domain. They have also been shown to bind protease inhibitors and degradative enzymes (Bernfield et al., 1992). Chondrocytes have been shown to highly express syndecan-3 during limb development and chondrogenesis (Gould et al., 1992; Mackie et al., 1987), and while levels are maintained in the proliferative chondrocytes of the growth plate, expression profiles change to syndecan-4 in the developed articular chondrocytes. Although at much lower levels, syndecan-2 has been shown to be expressed in mature chondrocytes. Barre et al. (2000) has shown that although syndecan-1 (a syndecan normally associated with epithelial cells) is not expressed in normal chondrocytes it is with cultured chondrocytes from OA patients. Syndecan-2 has been demonstrated to interact with matrix proteins, including fibronectin (Kusano et al., 2000), and its V region is able to control matrix assembly at the cell surface (Klass et al., 2000). As discussed above, syndecans are known to act as co-receptors for growth factors and this is primarily thought to occur through their GAG chains. A recent report looking at the interaction of syndecan-2 and TGFβ1 has discovered that this syndecan can act directly with TGFβ1 through its core protein (Chen et al., 2004) regulating expression of this growth factor.

Syndecan-3 has been shown to associate with tenascin-C in chondrogenesis (Gould et al., 1992; Mackie et al., 1987) with particular emphasis to mesenchymal cell condensations. By facilitating cell-cell and cell-matrix interactions syndecan-3 promotes the formation of these condensations (Seghatoleslami and Kosher, 1996). During differentiation into chondrocytes expression decreases suggesting a role for this syndecan, and its association, in promoting differentiation and proliferation (Hoffman et al., 1988). Syndecan-4 is the

most highly expressed of the family within chondrocytes. It has been shown in a number of cell types to have a definite role in focal adhesion formation. Within fibroblasts coated on fibronectin, syndecan-4 has been shown to co-localise with vinculin in focal adhesions (Woods and Couchman, 1994) and recruit the cytoskeletal protein to the cell-binding domain of fibronectin. The important role in cell adhesion and migration of syndecan-4 is demonstrated in a study by Bloom et al. (1999) where cells plated on fibronectin mutated for the heparin-binding site fail to form filopodia and stress fibres and consequently spread slowly. It has been suggested that syndecan-4 may stimulate these processes by acting via the Rho-family of GTPases and also protein kinase C (PKC) (Keum et al., 2004; Koo et al., 2006). Syndecan-4 has also been shown to co-localise with integrins within focal adhesion complexes (Humphries et al., 2005; Woods and Couchman, 1994). It has been hypothesised that syndecans may act as organising centres within focal adhesion complexes. By binding to intracellular scaffold proteins, such as systenin, additional transmembrane receptors including integrins can be recruited to the adhesion. Clustering of syndecan-4 is also required for focal adhesion formation within cells adhering via the $\alpha 5\beta 1$ integrin (Bass and Humphries, 2002).

The interactions between the chondrocyte and its ECM through the discussed receptors have been summarised in fig.1.10.

1.9 Focal Adhesion Formation and Cell Migration

Migration of cells occurs in response to changes or signals from the extracellular environment. Any changes are sensed by transmembrane receptors initiating the activation of signalling pathways, which result in changes in cytoskeletal organisation and the formation of focal adhesion complexes. It is the actin microfilament system specifically that is thought to be the main centre for cellular migration initiation. By rearranging the microfilament organisation, cells are able to form membrane protrusions and generate intracellular forces, which ultimately culminate in translocation of the cell.

There are two main features associated with the process of cell migration, the formation of lamellipodia and filopodia (see fig.1.11). Lamellipodia have been described as veil-like

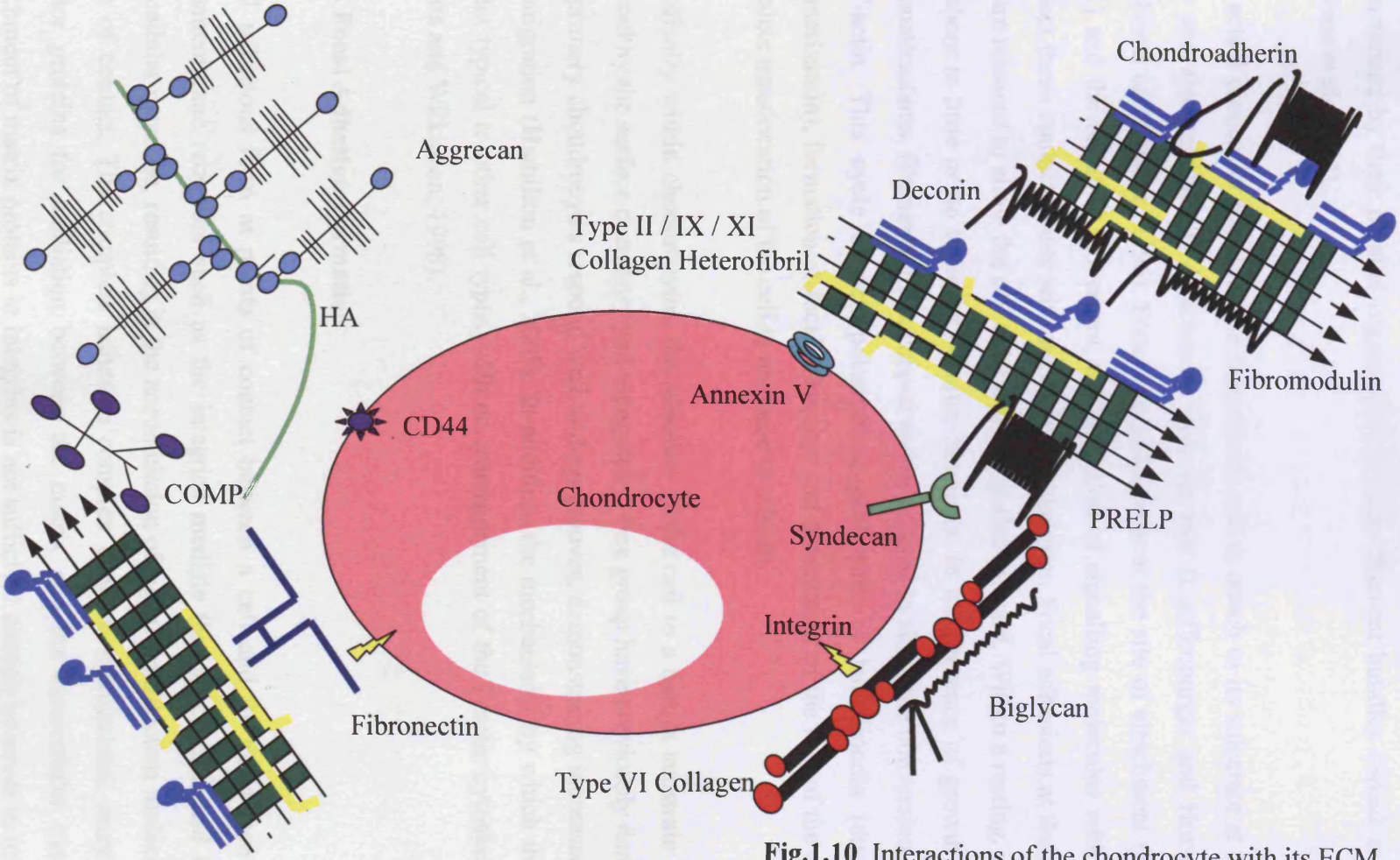


Fig.1.10 Interactions of the chondrocyte with its ECM

sheets containing highly branched actin filaments (Small, 1994). Filopodia are in contrast, long, thin structures which project beyond the leading edge of the lamellipodium and are characterised by their parallel organisation of actin filament bundles (Small et al., 1999; Svitkina et al., 2003).

The actual process of translocation requires the cell to attach to its substrate at the leading edge and also regulate its detachment from the rear (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). Focal adhesions form at the site of attachment (see section 1.9.1) and the resulting movement and activation of signalling molecules within the cell connect these contact points with the actin cytoskeleton. Focal adhesions at the rear of the cell are released to allow the cell to be actively pulled forward. Within a resting, non-motile cell there is little or no turnover of actin filaments. In the presence of growth factors or chemoattractants, filaments are uncapped and/or severed to allow the conversion of G-actin to F-actin. This cycle of cell polarisation, protrusion of lamellipodia (due to actin polymerisation), formation of focal adhesions and detachment of the rear of the cell allows complete translocation of the cell in response to stimuli.

Specifically within chondrocytes, the adhesion of the cell to a matrix substrate is directly affected by the surface chemistry and topography. One group have previously demonstrated that primary chondrocytes respond well to deep grooves, demonstrating increased levels of cell migration (Hamilton et al., 2005). In addition, the mechanisms by which they do this are not typical to other cell types, with no rearrangement of the F-actin cytoskeleton seen (Curtis and Wilkinson, 1998).

1.9.1 Focal Adhesion Formation

Focal adhesions form at points of contact between a cell and its ECM (see fig.1.12). Transmembrane receptors such as the integrins mediate this attachment and initiate an intracellular response, resulting in the accumulation of signal transduction molecules at the point of contact. The complete adhesion complex contains cytoskeletal, enzymatic and adaptor proteins for mediation between the matrix and the intracellular cytoskeleton. Attachment of matrix proteins to integrins is not sufficient enough however to initiate such

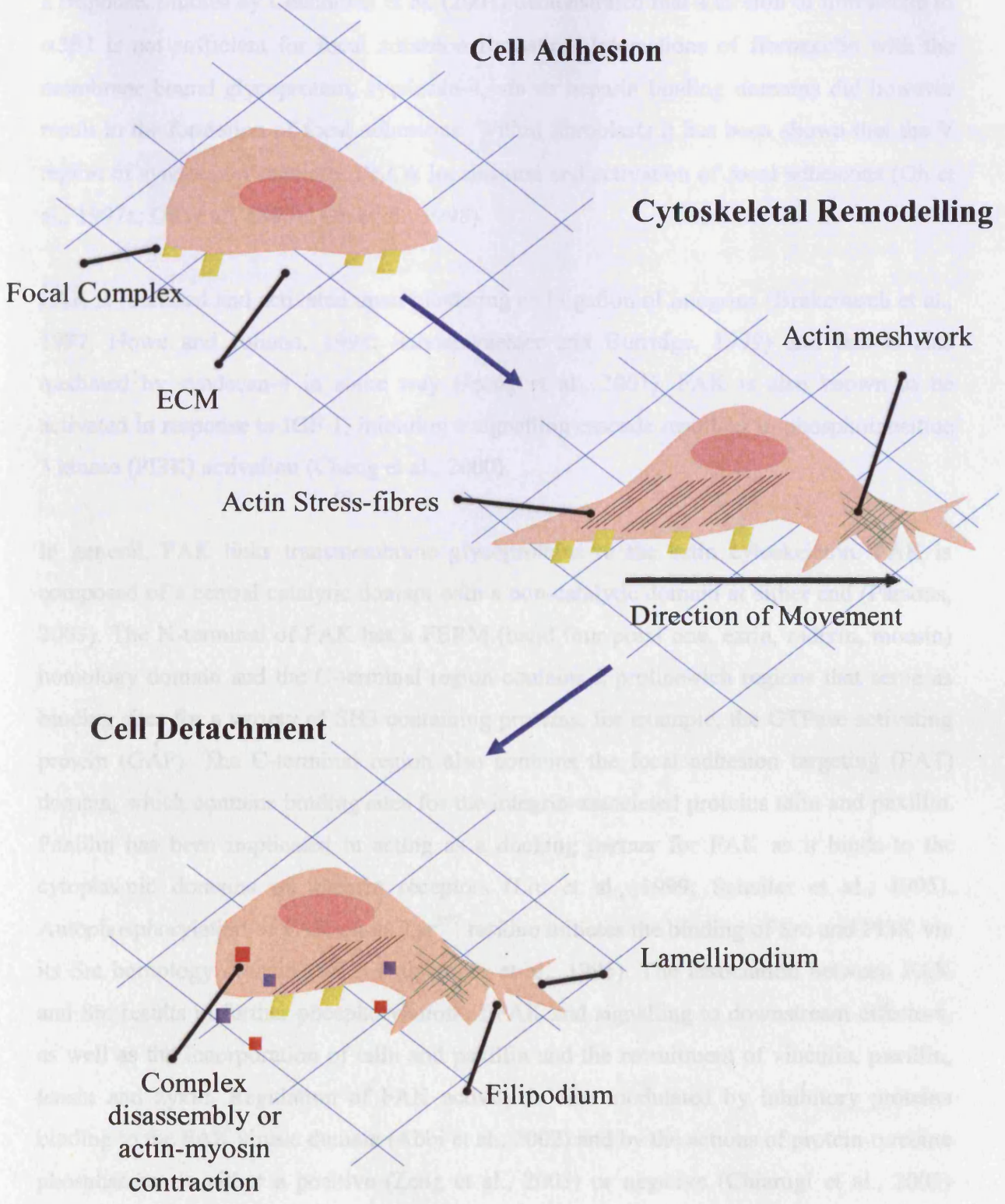


Fig.1.11 Focal complex formation and cytoskeletal remodelling during cell migration (adapted from Carragher and Frame, 2004)

a response. Studies by Couchman et al. (2001) demonstrated that adhesion of fibronectin to α5β1 is not sufficient for focal adhesion formation. Interactions of fibronectin with the membrane bound glycoprotein, syndecan-4, via its heparin binding domains did however result in the formation of focal adhesions. Within fibroblasts it has been shown that the V region of syndecan-4 mediates PKCα localisation and activation of focal adhesions (Oh et al., 1997a; Oh et al., 1997b; Oh et al., 1998).

FAK is recruited and activated upon clustering and ligation of integrins (Brakebusch et al., 1997; Howe and Juliano, 1998; Schoenwaelder and Burridge, 1999) and this is also mediated by syndecan-4 in some way (Jeong et al., 2001). FAK is also known to be activated in response to IGF-1, initiating a signalling cascade resulting in phosphoinositide 3 kinase (PI3K) activation (Cheng et al., 2000).

In general, FAK links transmembrane glycoproteins to the actin cytoskeleton. FAK is composed of a central catalytic domain with a non-catalytic domain at either end (Parsons, 2003). The N-terminal of FAK has a FERM (band four point one, ezrin, radixin, moesin) homology domain and the C-terminal region contains 2 proline-rich regions that serve as binding sites for a variety of SH3 containing proteins, for example, the GTPase activating protein (GAP). The C-terminal region also contains the focal adhesion targeting (FAT) domain, which contains binding sites for the integrin-associated proteins talin and paxillin. Paxillin has been implicated in acting as a docking partner for FAK as it binds to the cytoplasmic domains of integrin receptors (Liu et al., 1999; Schaller et al., 1995). Autophosphorylation of FAK on its Tyr^{397} residue initiates the binding of Src and PI3K via its Src homology domain (SH2) (Miyamoto et al., 1995). The association between FAK and Src results in further phosphorylation of FAK and signalling to downstream effectors, as well as the incorporation of talin and paxillin and the recruitment of vinculin, paxillin, tensin and zyxin. Regulation of FAK activity is also modulated by inhibitory proteins binding to the FAK kinase domain (Abbi et al., 2002) and by the actions of protein-tyrosine phosphatases in either a positive (Zeng et al., 2003) or negative (Chiarugi et al., 2003) fashion.

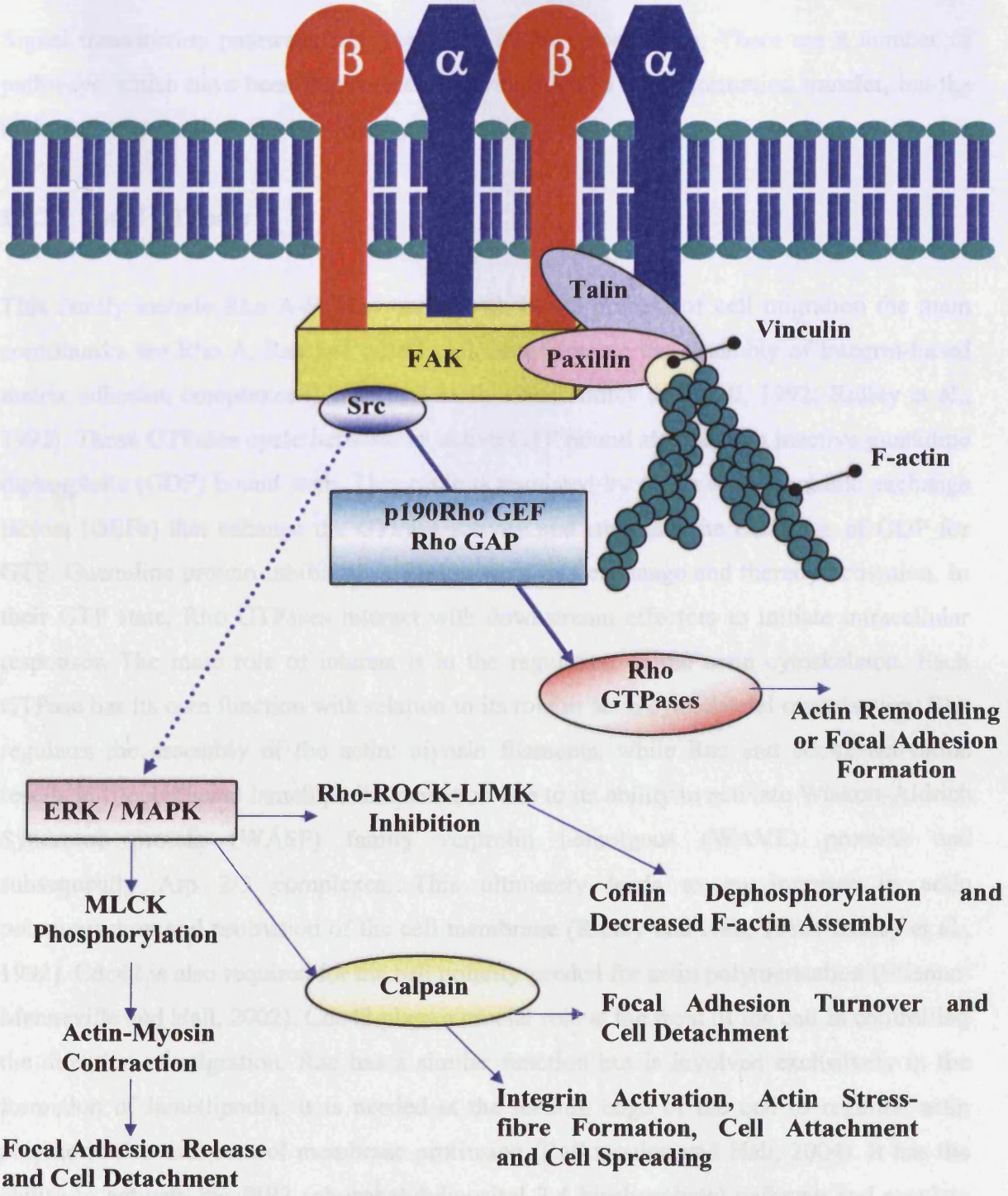


Fig.1.12 Focal adhesion signalling cascades affecting cell adhesion and spreading for migration (adapted from Carragher and Frame, 2004)

Abbreviations: ERK – Extracellular signal-related kinase, MAPK – Mitogen activated protein kinase, ROCK-LIMK – Rho activated kinase – Lin-II/Isl-1/Mec-3 domain containing protein kinase, MLCK – Myosin light chain kinase

1.9.2 Signal Transduction Mechanisms

Signal transduction pathways link the ECM to the cytoskeleton. There are a number of pathways, which have been implicated as having roles in this information transfer, but the main pathway involves the Rho family of small GTPases.

1.9.2.1 Small GTPases

This family include Rho A-H, Rac and cdc42. In the process of cell migration the main contributors are Rho A, Rac and cdc42. All three promote the assembly of integrin-based matrix adhesion complexes (Nobes and Hall, 1995; Ridley and Hall, 1992; Ridley et al., 1992). These GTPases cycle between an active GTP bound state and an inactive guanidine diphosphate (GDP) bound state. This cycle is regulated by GAPs and guanidine exchange factors (GEFs) that enhance the GTPase activity and stimulate the exchange of GDP for GTP. Guanidine protein inhibitors (GPIs) prevent this exchange and thereby activation. In their GTP state, Rho GTPases interact with downstream effectors to initiate intracellular responses. The main role of interest is in the regulation of the actin cytoskeleton. Each GTPase has its own function with relation to its role in actin cytoskeletal organisation. Rho regulates the assembly of the actin: myosin filaments, while Rac and cdc42 activation results in filopodia and lamellipodia formation due to its ability to activate Wiskott-Aldrich Syndrome protein (WASP) family verprolin homolgous (WAVE) proteins and subsequently Arp 2/3 complexes. This ultimately leads to an increase in actin polymerisation and protrusion of the cell membrane (Ridley and Hall, 1992; Ridley et al., 1992). Cdc42 is also required for the cell polarity needed for actin polymerisation (Etienne-Manneville and Hall, 2002). Cdc42 plays a crucial role at the front of the cell in controlling the direction of migration. Rac has a similar function but is involved exclusively in the formation of lamellipodia. It is needed at the leading edge of the cell to regulate actin polymerisation and control membrane protrusion (Raftopoulou and Hall, 2004). It has the ability to activate the PIP2 (phosphatidylinositol 3,4 bisphosphate) pathway and regulate adenosine diphosphate (ADP). The production of PI(3,4,5)P3 leads to an increase in the expression of GTP-Rac. Rho has crucial roles in cell adhesion, actomyosin assembly and stress fibre formation. It acts primarily via the ROCK myosin-light-chain or ROCK-LIMK pathway and diaphanous (mDIA)-profilin and regulates the contraction of the cell body and

retraction of the rear of the cell (Raftopoulou and Hall, 2004; Ridley and Hall, 1992; Ridley et al., 1992). Rho GTPases can also modulate microtubule organisation and structure (Wittmann and Waterman-Storer, 2001), reorganising the cytoskeleton towards the leading edge, directing the transport pathways there (Ma and Chisholm, 2002). These GTPases are also able to regulate each other's activities. For example, activation of Rac inhibits the GTPase Rho, which is thought to be behind the mechanism for cell detachment (Sander et al., 1999).

1.10 Proteolysis of Extracellular Matrix Components

Following joint injury a sustained increase in aggrecan metabolites can be detected within the synovial fluid as well as the damaged tissue of patients (Lohmander et al., 1999). Degradation of the large aggregating proteoglycan, aggrecan, is one of the first indications of joint injury and is thought to be primarily due to the up-regulation of aggrecanases and secondarily the MMPs (Arner et al., 1999).

1.10.1 Aggrecanases

Three aggrecanases have been isolated from articular cartilage. These are also known as the zinc metalloproteinases ADAMTS (A Disintegrin and a Metalloproteinase domain with Thrombospondin Motifs) 1, 4 and 5 (Nagase and Kashiwagi, 2003). All members of this family have an N-terminal pro-peptide domain, a metalloproteinase and a disintegrin-like domain resembling the ADAM (A Disintegrin and a Metalloproteinase) proteins, which are membrane-anchored and have a transmembrane and cytoplasmic domain in the C-terminal region (see fig.1.13). The C-terminal of ADAMTS proteins contain a number of TSP-1 motifs (Tortorella et al., 2000a). These enzymes are synthesised as pre-proteins and are targeted to the secretory pathway. All members possess a furin cleavage site just before the proteinase domain, and therefore are most probably activated intracellularly by a pro-protein convertase and secreted as active enzymes (Jones and Riley, 2005).

ADAMTS-1 has been shown to be ineffective in cleaving aggrecan. It has been suggested that this ADAMTS may be acting as a natural inhibitor by competing for the binding sites on the aggrecan molecule (Tortorella et al., 2000a). Aggrecanase 1 and 2 (ADAMTS 4 and

5 respectively) were first cloned in 1999 from the conditioned media of IL-1 stimulated bovine nasal cartilage (Abbaszade et al., 1999; Tortorella et al., 1999). The possession of TSP-1 motifs at the C-terminus allows the anchorage of the enzyme to extracellular GAG side chains. This may target the protein to distinct regions of the ECM and may serve to modulate the specificity and/or activity on the metalloproteinase domain by binding to selective sites on substrate molecules (Flannery et al., 1999b). This has been confirmed by research by Tortorella et al (2000b) illustrating that ADAMTS-4 will not bind to aggrecan if there are no glycosylation sites or if its C-terminal is lacking.

Cleavage of aggrecan primarily occurs within the highly conserved IGD region between the G1 and G2 domains. Within this region there are 2 known cleavage sites between Asn^{341}-Phe^{342} and Glu^{373}-Ala^{374}. The foremost has become known as the MMP site and the latter, the aggrecanase site (Caterson et al., 2000). Cleavage by at either site results in G1 domains remaining bound to HA and LP, accumulating in the tissue with age (Roughley, 2001). The C-terminal fragment is free to diffuse out of the tissue where it will move to the liver to be broken down (Ilic et al., 1992).

Monoclonal antibodies raised against N-terminal neo-epitopes of degraded aggrecan have allowed the quantification of these products within synovial fluid. The antibody BC-3 recognises the N-terminal ARGSV sequence of aggrecan metabolites generated by ADAMTS-4 and 5 action and BC-14 recognises the FFGVG N-terminal generated by MMPs. Development of these antibodies has elucidated that the major catabolite in cytokine-mediated cartilage degradation is the ARGSV fragment, suggesting the more efficient aggrecanases to have the predominant role in aggrecan degradation (Arner et al., 1999; Hughes et al., 1998). Cleavage at the MMP site however does seem to be up-regulated as a later event in cartilage degradation when collagenolysis has begun (Caterson et al., 2000).

ADAMTS-4 and 5 have been shown to cleave bovine aggrecan at 4 additional sites outside of the IGD in the CS rich region between G2 and G3 more readily than the site previously described, resulting in the formation of at least 8 aggrecan fragments (Tortorella et al., 2002). MMPs have also been shown to cleave preferentially in the GAG binding region rather than the IGD (Little et al., 2002). Furthermore, the inducible expression by pro-

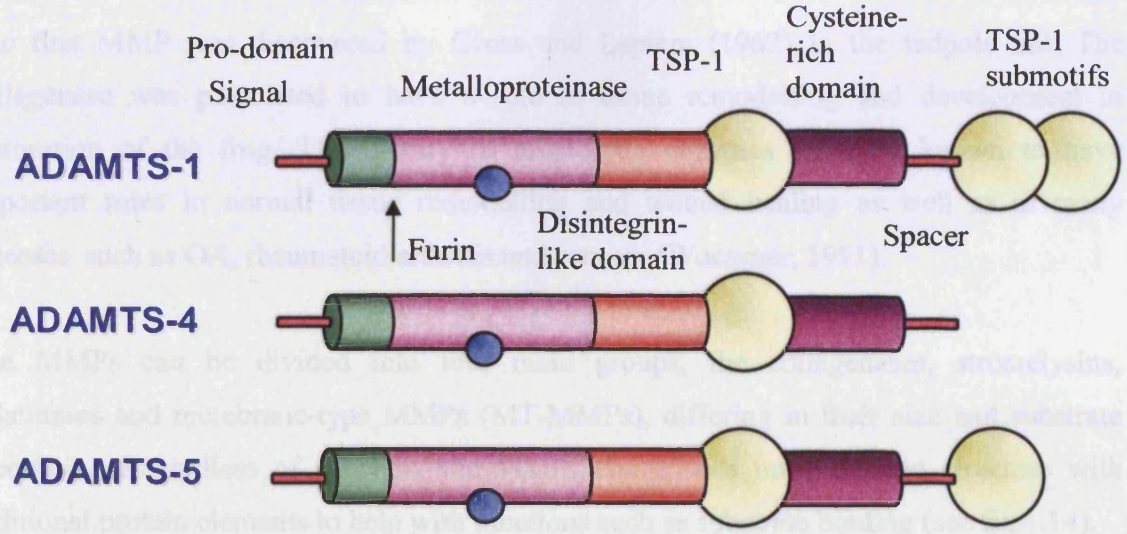


Fig.1.13 Domain structures of the ADAMTS proteolytic enzymes found within articular cartilage (adapted from Porter et al. (2005))

inflammatory cytokines of ADAMTS-4 seems to be more active in young than the constitutively expressed ADAMTS-5, which is found predominantly in older tissue (Tortorella et al., 2002). Recent reports have demonstrated that ADAMTS-5 is the main aggrecanase within murine cartilage and that this enzyme is primarily responsible for aggrecan degradation in mouse OA and inflammatory arthritis (Glasson et al., 2005; Stanton et al., 2005)

1.10.2 Matrix Metalloproteinases

The first MMP was discovered by Gross and Lapière (1962) in the tadpole tail. The collagenase was postulated to have a role in tissue remodelling and development in maturation of the frog. This family of proteolytic enzymes are now known to have important roles in normal tissue remodelling and wound healing as well as in many diseases such as OA, rheumatoid arthritis and cancers (Woessner, 1991).

The MMPs can be divided into four main groups, the collagenases, stromelysins, gelatinases and membrane-type MMPs (MT-MMPs), differing in their size and substrate specificity. Regardless of subclass, the MMPs comprise a multi-domain structure with additional protein elements to help with functions such as substrate binding (see fig.1.14).

Each MMP consists of: -

(i) a signal peptide – this signals to direct the translated product to the ER.

(ii) a pro-peptide domain (approximately 80 amino acids) which contains the conserved PRCG (V/N) PD sequence located at the site of an unpaired cysteine residue, which is lost during activation.

(iii) a catalytic domain (approximately 170 amino acids), which contains the thermolysin-type zinc binding domain and a conserved methionine residue. Structural zinc and calcium ions are also present to maintain enzyme stability and expression of enzyme activity.

(iv) a C-terminal hemopexin-like domain (approximately 210 amino acids) showing homology to vitronectin, which is a necessity for collagenases to cleave the $\alpha 1$ chains of collagen and for activation from pro-form to active in MMP 2. It has been noted that this domain is not essential in collagenases for non-collagen

substrate activity and for action by stromelysins. Mutation studies have shown this domain to be important in conveying specificity to substrate and also associations with tissue inhibitors of metalloproteinases (TIMPs) 1 and 2.

The MT-MMPs have a similar domain structure but in addition have a transmembrane domain within their C-terminal. The gelatinases also have 3 repeats of fibronectin type II domains composed of 58-59 amino acid repeats inserted into the catalytic domain which interact with collagens and gelatins. They also help to bind to their substrates and enable the enzyme to attach to other components of the ECM (Cawston, 1996; Kleiner and Stetler-Stevenson, 1993; Nagase and Woessner, 1999). MMPs are known to be able to degrade all components of the ECM and the family are made up of conserved domains and have a number of common properties.

- MMPs contain common sequences of amino acids making up to 7 distinct domains.
- They are secreted as inactive pro-enzyme forms (excluding MT-MMPs).
- Activation of the pro-enzyme is achieved by proteolysis.
- Activation results in the loss of a pro-peptide domain and a resultant fall in molecular weight.
- All MMPs have a zinc containing domain within their active site.
- All MMPs are inhibited by the naturally occurring TIMPs.
- Activity of the enzymes requires the presence of calcium ions and a neutral pH.

(Cawston, 1996)

MMPs are synthesised as pre-pro-enzymes and are secreted as inactive zymogens (Nagase and Woessner, 1999). Their activity is regulated at transcriptional and translational levels, and by their activators and inhibitors upon secretion (Sternlicht and Werb, 2001). All are synthesised in response to cytokines such as IL-1 and their activation is rate limited to proteolysis of their pro-peptide domain at the N-terminus (Saito et al., 1998).

1.10.2.1 Collagenases

There are three distinct collagenases MMPs 1 (interstitial collagenase), 8 (neutrophil collagenase) and 13 (collagenase 3). Collagenases have the ability to cleave all three of the α chains in collagens I, II and III. Cleavage occurs at a single site resulting in the formation of characteristic 1/4 and 3/4 length fragments. Unravelling of the triple helix and cleavage by the collagenases exposes sites for subsequent degradation by the gelatinases, MMPs 2 and 9, for removal of degraded fragments from the matrix (Mort and Billington, 2001). Experiments by Fosang et al. (1996) suggest MMP 13 may also be involved in aggrecan degradation.

1.10.2.2 Stromelysins

The stromelysins 1 and 2 (MMPs 3 and 10 respectively) have a broad substrate specificity. Stromelysin 1 is not widely expressed but is inducible by growth factors and cytokines. The other stromelysin (MMP 7), known as matrilysin is the only MMP to have no C-terminal region. These enzymes have the ability to cleave aggrecan, type VI collagen, LP, fibronectin, laminin, vitronectin and gelatin amongst other substrates (Cawston, 1996).

1.10.2.3 Gelatinases

MMPs 2 and 9 are often secreted in complexes associated with TIMPs 2 and 1 respectively. Activation of gelatinases usually takes place on the cell surface in association with MT-MMPs. The carboxyl terminal domain is need for activation if taking place via a cell surface receptor in this way (Kleiner and Stetler-Stevenson, 1993).

1.10.2.4 Membrane-type MMPs

These enzymes are found on the cell surface of cells. The six members of the subfamily (MT-MMPs 1-6) contain a furin recognition site, which is involved in their activation within the cell (Somerville et al., 2003). On secretion these MMPs are in their active state. There is a 10 amino acid insert between the pro-peptide and amino terminal domain. This

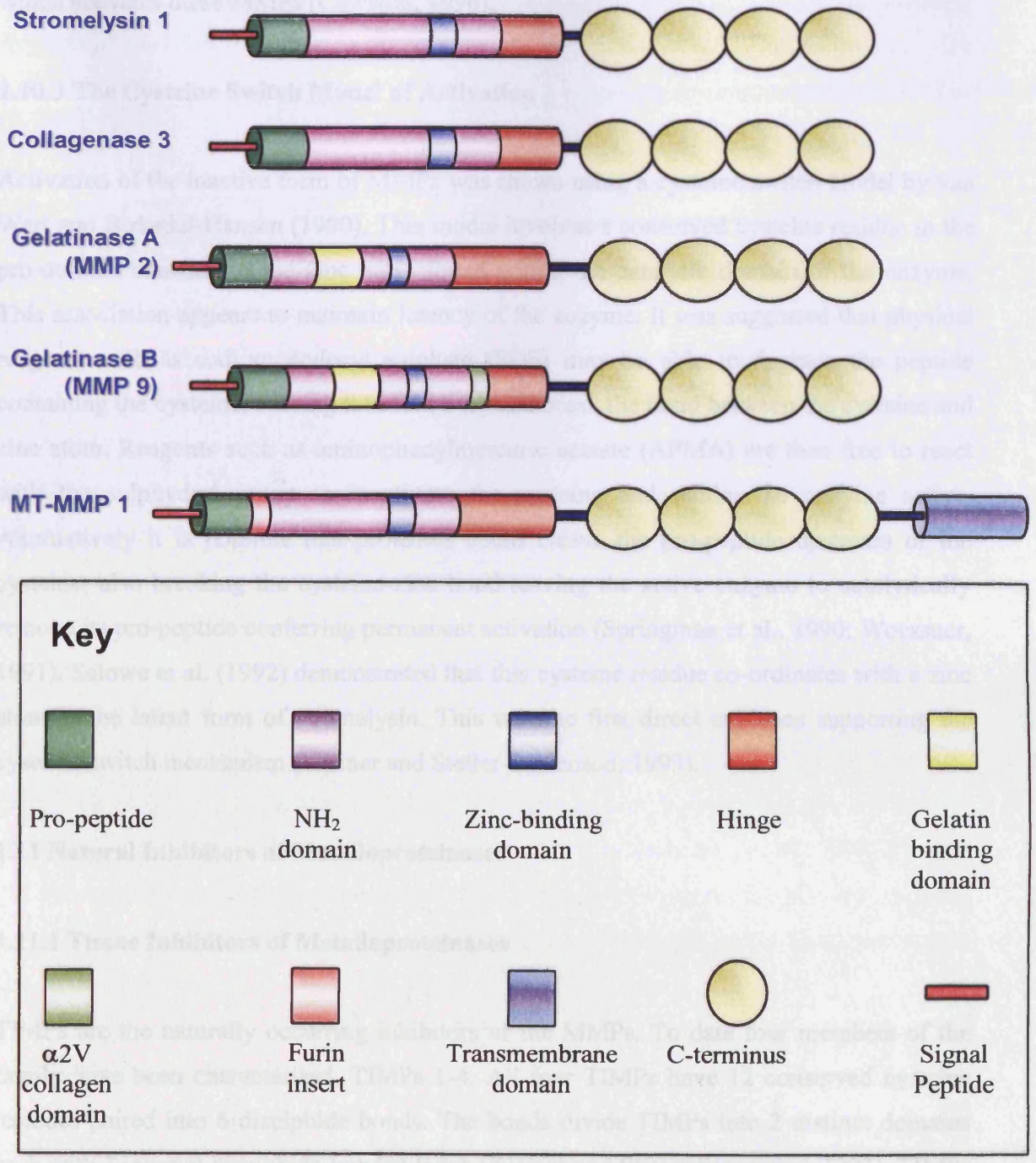


Fig.1.14 Schematic representation of the structures of the four classes of MMPs

(adapted from Somerville et al., 2003)

sequence GLSARNRQKR is recognised by a golgi associated serine proteinase furin, which activates these MMPs (Cawston, 1996).

1.10.3 The Cysteine Switch Model of Activation

Activation of the inactive form of MMPs was shown using a cysteine switch model by van Wart and Birkedal-Hansen (1990). This model involves a conserved cysteine residue in the pro-domain attached to the zinc atom found within the catalytic domain of the enzyme. This association appears to maintain latency of the enzyme. It was suggested that physical reagents such as sodium dodecyl sulphate (SDS) may be able to degrade the peptide containing the cysteine, causing it to fold back and break the bond between the cysteine and zinc atom. Reagents such as aminophenylmercuric acetate (APMA) are then free to react with the sulphydryl group to inactivate the cysteine and render the enzyme active. Alternatively it is possible that proteases could cleave the pro-peptide upstream of the cysteine, also breaking the cysteine-zinc bond leaving the active enzyme to autolytically remove its pro-peptide conferring permanent activation (Springman et al., 1990; Woessner, 1991). Salowe et al. (1992) demonstrated that this cysteine residue co-ordinates with a zinc atom in the latent form of stromelysin. This was the first direct evidence supporting the cysteine switch mechanism (Kleiner and Stetler-Stevenson, 1993).

1.11 Natural Inhibitors of Metalloproteinases

1.11.1 Tissue Inhibitors of Metalloproteinases

TIMPs are the naturally occurring inhibitors of the MMPs. To date four members of the family have been characterised, TIMPs 1-4. All four TIMPs have 12 conserved cysteine residues paired into 6 disulphide bonds. The bonds divide TIMPs into 2 distinct domains each with 3 internal disulphide-bonded loops (Kleiner and Stetler-Stevenson, 1993). TIMPs 1 and 2 have been found to be constitutively expressed and act against the MMPs in regulating degradation processes (Kashiwagi et al., 2001). It is currently thought that it is the balance in expression of MMPs and TIMPs that regulates cartilage homeostasis and it is the disruption of this balance that results in the onset of joint destruction.

TIMP-1's constitutive expression in adult and embryonic tissues suggests a role in animal development (Zafarullah et al., 1996). TIMP-1 is a 184 amino acid, 28KDa glycoprotein. Both domains of the inhibitor are thought to contribute to the MMP: TIMP interaction, the main residues conferring inhibitory activity appear to reside in the N-terminal domain. The presence of the C-terminal as well appears to tighten this binding (Cawston, 1996). TIMP-2 shows 38% sequence homology to TIMP-1 but has a much smaller size due to its lack of glycosylated residues (Woessner, 1991). Differences within the promoter region of the inhibitors suggests that TIMP-2 may protect the integrity of the ECM by MMPs as opposed to TIMP-1's role in acute protection (Zafarullah et al., 1996). The critical residues involved in MMP inhibition are located around the disulphide bond between Cys^{3} and Cys^{13} (Kleiner and Stetler-Stevenson, 1993; Nagase and Woessner, 1999). Both enzymes have been shown to share common activities *in vitro* including erythroid-potentiation, MMP inhibition and growth promotion (Wang and Soloway, 1999). They also both bind reversibly to their substrates with 1:1 stoichiometry with the active enzyme. They show a high degree of stability against high temperatures and low pH but are susceptible to reducing and alkylating agents (Cawston, 1996).

Recent work suggests a role for TIMPs in the regulation of ADAMTS expression. After secretion, TIMP-3 is bound within the matrix by negatively charged polysaccharides (Nagase and Kashiwagi, 2003). TIMP-3 has been shown to be a potent inhibitor of ADAMTS-4 and 5 with higher kD values than for MMPs (Kashiwagi et al., 2001) TIMP-1 has also been shown to inhibit the aggrecanases at very high concentrations *in vitro* but this would not occur *in vivo*. TIMP-2 has been shown to have no affinity for the aggrecanases (Hughes et al., 1998).

1.12 Articular Cartilage Injuries

Injuries to the articular surface are frequently the result of damage to other tissues within the synovial joint such as the meniscus or synovium. Trauma due to repetitive impact and sudden movements such as twisting of the joint, as seen in high impact sports, can cause damage to the joint, normally resulting in swelling and pain (Buckwalter, 2002). Due to the inability of cartilage to effectively repair itself, these injuries can in a number of cases

result in degeneration of the articular surface, resulting in conditions such as OA later in life.

A number of studies looking at the events subsequent to wounding have been performed in cartilage from both bovine (Tew et al., 2000) and chick cartilage (Walker et al., 2000). The initial events which appear to follow wounding are a zone of cell death adjacent to the wound edge, termed the 'zone of necrosis' (Stockwell, 1979), characterised by shrunken chondrocytes containing condensed nuclei as well as empty lacunae (Tew et al., 2000; Zhang et al., 2005). Work by Tew et al. (2000) demonstrated by TUNEL (terminal deoxynucleotidyl-mediated dUTP-biotin nick end-labelling) analysis and ultrastructural observations that a number of cells within this zone had died through the apoptotic pathway. The resulting area of acellularity is a limiting factor of integration in cartilage repair strategies. As a zone of mechanical weakness, it is often the area in which fissures or fractures occur, leading to subsequent degeneration of the repair tissue. Immediately behind this 'zone of necrosis' is a zone of cellular proliferation and increased matrix production. This response occurs later than the initiation of cell death and the reason for this increase in proliferation is not known. It has been hypothesised however, that chondrocytes adjacent to the 'zone of necrosis' may have been exposed to increased concentrations of 'survival factors' causing their proliferative response (Walker et al., 2000). In addition, cells within this zone of proliferation appear to up-regulate their expression of the integrin subunits, α5 and α6. This may be as a result of alterations in the matrix composition (Walker et al., 2000). These subunits, if bound to the β1-subunits would be able to form interactions with matrix molecules such as fibronectin and laminin respectively (Hall et al., 1990; Pytela et al., 1985).

In vitro studies investigating the reaction of bovine articular cartilage to experimental wounding demonstrated damage to the type II collagen fibrils, as well as loss of proteoglycans from the wound edge rapidly after wounding had occurred (Tew et al., 2000). In addition, aggrecan within the 'zone of necrosis' adjacent to the wound edge undergoes proteolytic cleavage by the invading macrophages and mesenchymal stem cells (MSCs) as well as those live chondrocytes within the cartilage which form clusters as part of the repair response (Hembry et al., 2001). Taken together, the cell death and damage to the tissue as a result of wounding, as well as that caused by proteolytic cleavage seriously

compromises the mechanical stability of the remaining tissue, a factor likely to play a huge role in the reason why regions of integration between endogenous and repair tissue are so poor.

Injuries to articular cartilage are normally classified into two broad groups, namely damage that does not cause mechanical disruption of the articular surface and those that do. The latter can be further divided into chondral (where only the cartilage has been damaged) and osteochondral (where the subchondral bone has also been penetrated) injuries. A schematic illustration of both chondral and osteochondral injuries can be seen in fig.1.15 (Buckwalter, 2002).

1.12.1 Cell and Matrix Injuries

This form of injury to the cartilage is possible to repair. Repetitive trauma to the cartilage may result in damage to the proteoglycans resulting in either an up-regulation of their degradation or a down-regulation of their synthesis. Proteoglycans within the ECM of articular cartilage are the first macromolecules to respond to impact trauma. Changes in proteoglycan and collagen degradation are altered within days of injury and remain like that for a substantial time (Patwari et al., 2001). A decrease in proteoglycan concentration faster than replenishment by new synthesis is thought to be the point at which this form of injury cannot be repaired and further impact will result in extra strain on other components of the ECM such as the collagen fibrils, resulting in degeneration of the articular cartilage (Buckwalter, 2002).

1.12.2 Chondral Injuries

This form of injury involves mechanical disruption of the articular cartilage but does not extend to the subchondral bone (see fig.1.15). Mechanical disruption of such a kind normally results in the development of fissures and chondral flaps to the articular surface (Buckwalter et al., 1994). This kind of injury mostly occurs in young and middle-aged adults who are active. The severity of this injury will be dependent on the size of the lesion, but invariably due to the avascular nature of the cartilage, no fibrin clot will be formed in a

repair response and any attempt by local chondrocytes to up-regulate matrix synthesis will only be short term and will not result in filling of the defect (Beris et al., 2005; Buckwalter, 2002). Injury to cartilage alone is sufficient to cause OA and such injuries can result in progressive degeneration of the joint (Buckwalter et al., 1994; Patwari et al., 2001).

1.12.3 Osteochondral Injuries

Injuries classified into this bracket involve damage to the articular cartilage and further penetration into the subchondral bone. Many injuries within this group occur as a result of osteochondrosis dessicans (OCD) and within the adolescent and young adult age groups. OCD is defined as a disorder in which an osteochondral fragment is separated from the articular surface. This fragment will become loose and eventually dislocate from the lesion site (Peterson et al., 2003).

Penetration of the bone results in blood flow and the formation of a fibrin clot, which will fill the defect site. Platelets within the invading blood contain the necessary growth factors and cytokines, for example, TGF-β1 and platelet derived growth factor (PDGF) to stimulate the migration of undifferentiated MSCs into the defect and their differentiation into chondrocytes to synthesis a new matrix (Buckwalter, 2002). This is followed by an inflammatory response. Repair tissue tends to be of a fibrocartilaginous (containing a mix of both type II and I collagens) nature and with time degenerates with fragmentation and fibrillation of the articular surface. Spontaneous repair responses are associated with the development of necrotic tissue adjacent to the wound edge and little remodelling of the cartilage at the centre of the defect (Shapiro et al., 1993). A loss of proteoglycans and cells is normally seen within 6-12 months. This decrease in proteoglycans, and associated damage to the collagen network, results in an increased amount of water, increasing the tissue's permeability and decreasing stiffness. In effect, this impairs the loading characteristics of healthy cartilage (Buckwalter et al., 1994). Disruption of this kind also normally leads to damage of the synovial membrane and exposure of the cartilage to the synovial fluid leading to enzymatic degradation of the cartilage (Buckwalter et al., 1994). Ultimately this results in diseases such as OA (Hunziker, 1999).

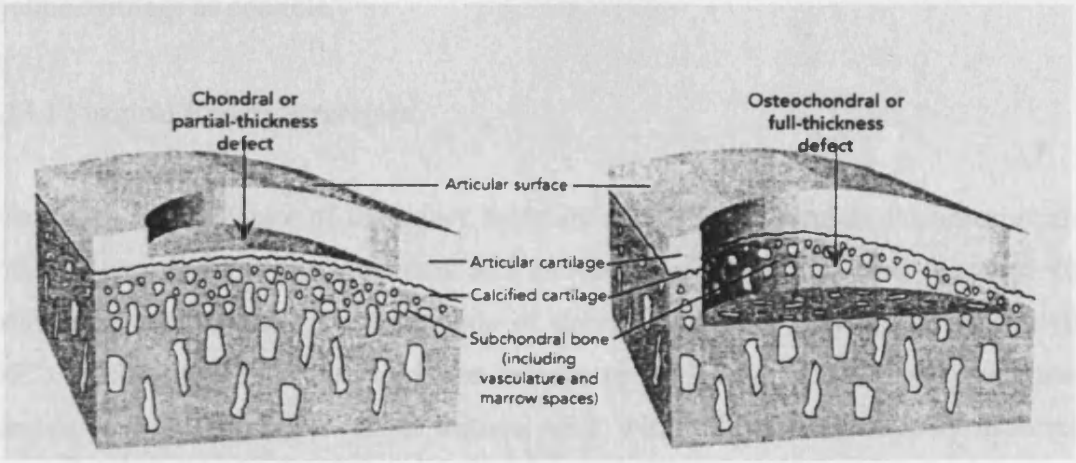


Fig.1.15 Illustrated differences between chondral and osteochondral defects within articular cartilage (reproduced from Woodfield et al., 2002)

1.13 Articular Cartilage Repair

In 1743, the British scientist William Hunter, stated, "from Hippocrates to the present age it is universally allowed that ulcerated cartilage is a troublesome thing and that, once destroyed, it is not repaired" (Hunter, 1743). This statement has largely remained true to this day due to the low intrinsic properties of cartilage to initiate a repair response following injury. This inability of self-renewal has been attributed to the avascular, alymphatic and aneural properties of articular cartilage. The low density and isolation of chondrocytes within their ECM furthers this problem as cells cannot migrate to the site of injury and up-regulate their synthesis. Today orthopaedic surgery is developing techniques to enhance this repair process with the aim of producing as near a replicate of natural, hyaline cartilage as possible.

1.13.1 Surgical Repair Strategies

Depending on the nature of the injury there are a number of surgical therapies available with the aim of relieving symptoms and increasing joint mobility and function. These include the well establishment methods of debridement and arthroscopic lavage (Bert, 1993). Such methods do not repair the damage or instigate intrinsic repair responses. A number of these techniques aim to initiate repair within the native tissue by inducing an inflammatory response and fibrin clot development by drilling through the subchondral bone into the vasculature (microfracture). The outcome of these techniques is highly variable between patients due to differences in the repair tissue formed, and the age and mobility of the patient (Akizuki et al., 1997; Steadman et al., 2003). More recently methods involve tissue transplantation. Such techniques include osteochondral transplantation (mosaicplasty), perichondral and periosteal grafts. Methods such as autologous chondrocyte transplantation (ACT) involve the insertion of isolated autologous chondrocytes into the defect to stimulate the formation of repair tissue to fill the defect. All these techniques do initiate a repair response, although repair tissue is invariably fibrocartilaginous and integrates poorly with the pre-existing tissue. Long term studies on the durability of this repair tissue remain to be done in many cases but results from Peterson et al. (2003) for the use of ACT in OCD patients suggests this tissue can last for up to 10 years. Improving the durability and stability of this repair tissue is now the goal of orthopaedic researchers and a

tissue resembling natural hyaline articular cartilage with good integration would surely provide these answers.

1.13.1.1 Autologous Chondrocyte Transplantation

Treatment of cartilage defects using the isolation and re-implantation of autologous chondrocytes was first demonstrated in a study by Grande et al. (1989) using rabbit chondrocytes isolated from healthy cartilage, and their subsequent transplantation into focal patellar defects within the animal. The study was declared successful, however integration was reported to be poor and the repair tissue brittle leading ultimately to its degeneration (Brittberg et al., 1996).

The first reported use in humans was in 1994 when 16 patients with femoral condyle defects were treated. The procedure, as summarised in fig.1.16, was used initially for full thickness defects ranging from 1.6-6.5 cm^2. Healthy chondrocytes were isolated from biopsies (300-500mg of tissue) taken from minimal weight bearing areas of healthy cartilage by arthroscopy. The cells were cultured for 14-21 days in autologous serum to increase cell number by 20-50 fold. The relationship between the number of implanted chondrocytes and biosynthetic activity is linear (Chen et al., 1997). In a second procedure the lesion is debrided back to healthy cartilage before the expanded chondrocytes are injected at a concentration of 2.5-5 million cells per 50-100µl into the defect covered with a sutured periosteal flap isolated from the proximal medial tibia (Brittberg et al., 1994).

Histology taken from biopsies of the repair tissue generated at various points after surgery show any attempts at integration were vertical not lateral. In general, a fibrocartilaginous tissue was seen with some of the periosteum remaining incorporated into the repair tissue. Studies investigating the molecular mechanisms behind the ACT technique have demonstrated the low expression of type II collagen mRNA, as well as transcriptional factors Sox-9 and aggrecan (Grigolo et al., 2005), thus explaining the fibrocartilaginous nature of the repair tissue. Studies looking at methods to encourage the re-expression of the chondrocytic phenotype after monolayer expansion of the cells have focussed on the development of a defined chondrogenic medium. A recent report by Goldberg et al. (2005) suggests that culturing monolayer expanded chondrocytes in media containing ITS (insulin,

transferrin and selenium) and TGFβ1 enhances the re-expression of the chondrocytic phenotype as characterised by the production of type II collagen. The fibrocartilaginous nature of ACT repair tissue raises the question as to the source of the cells generating the repair tissue. Are the cells contributing to the repair tissue the autologous chondrocytes injected into the defect, the periosteal cells or stem cells entering the defect from the damaged subchondral bone (Redman et al., 2005)? A recent study by Dell'Accio et al. (2003) used a goat model to look at the contribution of fluorescently labelled transplanted cells. This group demonstrated that the implanted chondrocytes do participate in the formation of repair tissue.

For the patient, a reduction in locking, swelling and pain was seen with this surgical technique, but failures were due to lack of integration and insufficient filling of the defect (Peterson et al., 2002). Clinical findings by Minas (1998) 12-24 months post-ACT suggest, 72% of patients had improved function and quality of life. Micheli (2001) reported overall improvement in the knee condition and functional outcome in 84% of patients.

Concerns with the method currently being addressed are (i) the phenotype of the cells– dedifferentiated cells being introduced into the defect lose their plasticity after numerous passages in culture; (ii) unequal distribution of the cells leading to an uneven repair tissue. This uneven distribution may be due to weight bearing and 'clumping' of the cells within one area of the defect; (iii) leakage of cells - weight bearing of the joint may result in leakage of the cells from under the sutured periosteum, which is acting as a cover, and supply of natural growth factors to stimulate their redifferentiation.

One newly developed technique to address some of these issues is matrix-induced chondrocyte implantation (MACI) (Bachmann et al., 2004). This technique involves the implantation of chondrocytes using a collagen based scaffold. This scaffold can be used instead of a periosteal graft (Behrens et al., 1999).

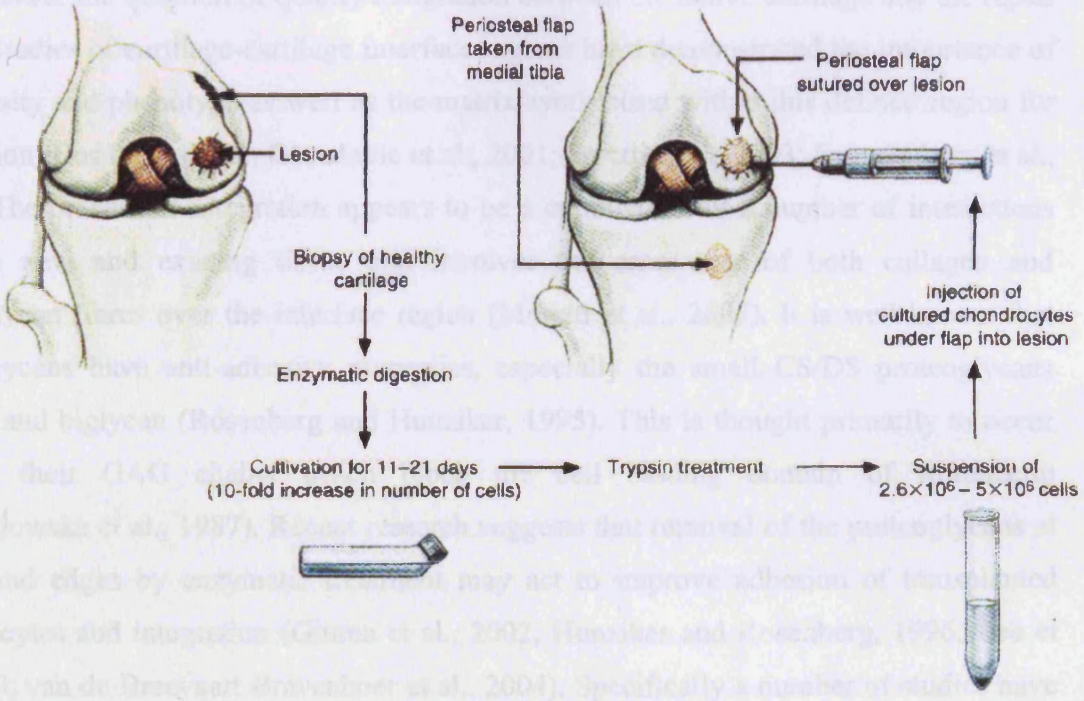


Fig.1.16 Overview of the process of ACT for treatment of full thickness defects (reproduced from Brittberg et al., 1994)

1.13.2 Adhesion and Integration

Surgical techniques designed to initiate a cartilage repair response *in situ* have the main goals of relieving symptoms of the patient and increasing the function and mobility of the joint. The repair tissue generated is invariably fibrocartilaginous and does not have the correct biomechanical characteristics to withstand loading and preventing excessive shearing forces, which leads to ultimate degeneration of the repair tissue and surrounding tissues (Ghadially et al., 1977; Shapiro et al., 1993). Unfortunately these techniques do not really answer the question of quality integration between the native cartilage and the repair tissue. Studies of cartilage-cartilage interface regions have demonstrated the importance of cell density and phenotype as well as the matrix synthesised within this defined region for integration (Bos et al., 2002; Obradovic et al., 2001; Peretti et al., 2003; Spangenberg et al., 2002). The process of integration appears to be a culmination of a number of interactions between new and existing tissue and involves the cross-over of both collagen and proteoglycan fibres over the interface region (Moretti et al., 2005). It is well known that proteoglycans have anti-adhesive properties, especially the small CS/DS proteoglycans decorin and biglycan (Rosenberg and Hunziker, 1995). This is thought primarily to occur through their GAG chains which block the cell binding domain of fibronectin (Lewandowska et al., 1987). Recent research suggests that removal of the proteoglycans at the wound edges by enzymatic treatment may act to improve adhesion of transplanted chondrocytes and integration (Giurea et al., 2002; Hunziker and Rosenberg, 1996; Lee et al., 2000; van de Breevaart Bravenboer et al., 2004). Specifically a number of studies have used the enzyme chondroitinase ABC to pre-treat the wound edge (Hunziker and Kapfinger, 1998; Lee et al., 2000). This enzyme, at pH 8.0, specifically targets the CS GAG chains attached to the aggrecan core protein as well as the SLRPs (Yamagata et al., 1968). The removal of proteoglycans at the wound edge may then expose pro-adhesive matrix macromolecules such as fibronectin or collagen. The presence of SLRPs also tightly regulates the development of collagen fibrils and is known to inhibit fibrillogenesis (Roughley, 2001). Removal of these macromolecules by digestive enzymes may encourage the development of stronger cross-links within the interface region and enhance collagen synthesis. Interestingly, the role of GAGs in inhibiting integration would also explain why newly formed cartilage integrates more successfully with adjacent bone. Bone contains, relative to cartilage, very little GAG content and exhibits a higher adhesive strength than

native cartilage (Tognana et al., 2005). In addition, this group have also shown the negative effects of GAG on GAG synthesis and deposition within constructs (Freed et al., 1998).

The integration seen within mature articular cartilage is directly correlated to collagen cross-linking and remodelling. During cartilage maturation collagen content as well as the amount of non-reducible collagen cross-links increases (Verzijl et al., 2000). This in turn increases the mechanical stability of the cartilage. DiMicco and Sah (2001) state that it is the deposition of newly synthesised collagen and the process of lysyl-oxidase mediated cross-linking that defines the adhesive strength between opposing cartilage explants. The importance of these cross-links was demonstrated by Ahsan et al. (1999) when explants were exposed to the lysyl oxidase inhibitor β-aminopropionitrile (BAPN), which inhibits cross-linking and consequently integration. Recent research demonstrated that pre-treatment of cartilage with BAPN allows the accumulation of hydroxyproline residues on collagen molecules, and after removal of the inhibitor, the rapid development of cross-links. The authors hypothesise that the use of BAPN in this manner at a cartilage-cartilage interface would allow the rapid formation of cross-links for greater integrative strength (McGowan and Sah, 2005).

Chondrocytes in mature cartilage are trapped within their matrix and are unable to move through the tissue to the site of an injury. Removal of limited GAGs may also increase the permeability of the tissue, temporarily improving chances for local cell migration. Treated matrix also has less water so it is easier for newly synthesised matrix to infiltrate the tissue. Interestingly, in addition to the effects of GAG chains in inhibiting chondrocyte adhesion to the wound edge, a recent report has highlighted the effects of the micro-geography of the wound edge on chondrocyte adhesion. The authors demonstrated that the tightness of attachment was dependent on the smoothness of the cartilage to which the cells were to adhere (Zhang et al., 2005). In addition, *in vivo,* poor integration has been attributed to the presence of PRG4 in synovial fluid. PRG4, also known as lubricin or SZP is a lubricant found in the synovial fluid as well as the articular cartilage surface zone and is synthesised by both synoviocytes and superficial zone chondrocytes. Recently it has been shown that integration within *in vivo* models of cartilage repair is likely to be hindered by the presence of PRG4 (Englert et al., 2005, Schaefer et al., 2004).

Work by Shapiro et al. (1993) demonstrated that tissue at the defect site becomes necrotic and there is no suggestion of resorption or remodelling. Injury or surgical intervention results in cell death at wound edges resulting in an acellular wound edge next to a zone of proliferating cells (Hunter and Levenston, 2004; Tew et al., 2000). Integration needs the presence of proliferating cells as well as deposition of ECM not just the latter alone. The proliferating cells will also be affected by the age of the tissue as older tissue has less active cells and are not capable of repairing damage to the cartilage. Removal of the ECM is known to trigger deoxyribonucleic acid (DNA) synthesis and cell proliferation (Giurea et al., 2002). Combining these factors with the introduction of proliferating cells and mitogenic factors into the interface region may be the key to initiating hyaline repair responses.

Of course an important factor in the process of repair and integration is the actual adhesion of cells to the injured tissue. Studies have demonstrated that native cartilage will adhere to both native (Reindel et al., 1995) and engineered cartilage (Silverman et al., 2000). The levels of adherence are poor however, and with use of the joint, fissures inevitably develop. The boundary between the definitions of integration and adhesion are slim but it is important to consider the factors involved in the attachment of chondrocytes to their extracellular matrices. It is the initial adhesion of transplanted cells within a cell-based method of repair, which may retain them within a defect. The adhesion of chondrocytes to articular cartilage is time dependent and with increasing seeding durations the level of adhesion and the stability of the interaction increases (Lee et al., 2000). The authors hypothesised that this increase in adherence may be due to the development of chondrocyte receptors for HA, collagen and/or fibronectin, and the interaction of these newly formed receptors with their ligands. This is likely to be integrin mediated. The level of adhesion may be affected by ligand density, which will invariably differ between interterritorial and territorial regions and also with depth from the articular surface (Hayashi et al., 1996; Pfander et al., 1999). As previously discussed in section 1.13.2, proteoglycans such as the SLRPs, especially decorin and biglycan, as well as aggrecan are anti-adhesive molecules and inhibit cell adhesion. In the case of decorin it is able to modulate the adhesiveness of cells to fibronectin through interactions of its GAG chain and protein core with the cell adhesive domain of fibronectin (Bidanset et al., 1992; Winnemoller et al., 1991). However, adhesion is not solely the answer to good integration. A study by Silverman et al. (2000)

showed that when chondrocytes were attached to articular cartilage using fibrin glue then cells were retained within a defect and adhered well to the surface, but still a clear line between repair and endogenous tissue was seen and integration was poor. Although numerous reports have been published emphasising the enhancement of tissue integration by seeding chondrocytes between cartilage interfaces, it is obvious that a balance between adhesion and migration of chondrocytes into injured cartilage, and the subsequent generation of a biomechanically stable repair tissue is required for good integration (Bos et al., 2002; Obradovic et al., 2001).

1.14 Mesenchymal Stem Cells

This pluripotent cell lineage arises from the bone marrow and can be cultured *in vitro* to induce osteogenic, chondrogenic or adipogenic differentiation (Barry, 2003). Around 1 in every 10^5 cells isolated from bone marrow are stem cells and these are mixed with endothelial and stromal cells (Tuan et al., 2003). Protocols for MSC usage in cartilage repair have been previously described (Wakitani et al., 1994; Wakitani and Yamamoto, 2002) as well as in a number of other connective tissues such as bone and tendon. The advantages of using MSCs as opposed to mature chondrocytes is their higher proliferative capacity and also that no biopsy needs to be taken from healthy cartilage in order to isolate the cells (Tuan et al., 2003). This reduces the risk of further degeneration in healthy cartilage areas. A large number of cells can further be obtained from bone marrow aspirations as opposed to isolation from tissue as the cells within cartilage are so sparse (Cancedda et al., 2003). There is also the possibility of introducing viral vectors into these pluripotent cells in a gene therapy attempt to improve the characteristics of the cell. However further research is required as the experiments by Wakitani et al. (1994; 2002) show no integration between the differentiated cells and the pre-existing tissue when using rabbit MSCs in full thickness cartilage defects.

1.15 Growth Factors

Growth factors are soluble proteins of small molecular weight and are rapidly absorbed and degraded *in vivo* (Hickey et al., 2003). Their use in therapeutics therefore needs to consider delivery methods in order to be effective. Growth factors can be used in conjunction with

pre-existing technology to enhance or stabilise chondrocyte proliferation *in vitro*. Within spontaneous repair processes growth factors are seen to be differentially expressed and play an active role in the process of regeneration. A study by Bos et al. (2001) using injured articular cartilage demonstrated an up-regulation of the growth factors TGFβ1, IGF-1/2 and FGF after injury and their subsequent return to normal expression on restoration of proteoglycan levels. Knowledge of this suggests that the expression of growth factors indicates an autocrine and/or paracrine response after injury to restore the pericellular matrix (Bos et al., 2001). Manipulation of growth factor expression levels therefore in conjunction with current repair strategies may be of use in finding the answer to the process of enhancing effective cartilage repair.

Within this study, the major growth factors considered are IGF-1 and TGFβ1. The following sections give a detailed description of the structure and mechanisms of action by these growth factors.

1.15.1 Insulin-like Growth Factor 1

IGF-1, originally known as somatomedin C, is a 70 amino acid polypeptide with a molecular weight of 7.5KDa, first isolated from the liver (Humbel, 1990). IGF-1 comprises a 4-domain structure, with 2 domains demonstrating homology to the A and B chains of insulin. A third domain is similar to the pro-peptide of pro-insulin, but the fourth is unique to IGF-1. The mature protein is folded and stabilised by 3 intra-chain disulphide bridges held between 6 cysteine residues (Rotwein et al., 1987). IGF-1 is expressed from a gene consisting of 6 exons (exons 1-6) and spans more than 80 kilobases (Kb) of genomic DNA (Dickson et al., 1991; Kajimoto and Rotwein, 1991; Rotwein et al., 1986; Shimatsu and Rotwein, 1987). The growth factor is produced by most cells of the body and is therefore thought to work on its target tissues through autocrine, paracrine and endocrine mechanisms. Within cartilage, IGF-1 is accepted as being the major stimulatory factor found in the synovial fluid. Here it is found at concentrations of approximately 50ng/ml and is synthesised by chondrocytes and stored in the ECM at concentrations of approximately 10ng/ml. In OA patients this concentration is normally seen to increase 5 fold (Schneiderman et al., 1995).

IGF-1 has been defined as anabolic in its actions and specifically, within articular cartilage, has strongly been implicated in the maintenance of matrix homeostasis (Luyten et al., 1988; McQuillan et al., 1986). Its anabolic effects are demonstrated through its ability to increase both proteoglycan and collagen synthesis *in vitro*, and inhibit the rate of matrix degradation by regulating the transcription of degradative enzymes such as the MMPs and up-regulating the expression of TIMPs (Hui et al., 2001a; Hui et al., 2001b). IGF-1 has been demonstrated to reverse the effects of IL-1 and TNFα on the degradation of proteoglycans (Fosang et al., 1991; Tyler, 1989). IGF-1 increases cellular proliferation and differentiation with an increase in DNA synthesis (Trippel, 1997). In addition to its mitogenic effects, IGF-1 has been suggested to act as a chemoattractant. Chang et al. (2003) showed that in chondrocytes from newborn calves, IGF-1 can act as a chemoattractant enhancing the migration of these cells. A study by Gentilini et al. (2000) using hepatic stellate cells suggested that the chemotactic and mitogenic effects of IGF-1 are regulated through the PI3K pathway discussed later in this section.

The receptor for IGF-1 is heterotetrameric with 2 α and 2 β-subunits held together covalently. The IGF-1 receptor (IGF-1R) is synthesised as a single precursor polypeptide with a molecular weight of approximately 180,000. This chain is cleaved, yielding 2 polypeptide chains with apparent molecular weights of 135,000 (α chain) and 95,000 (β chain) (Jacobs et al., 1983). The extracellular α-subunit contains a cysteine-rich domain that is involved in the IGF-1 ligand binding to its receptor. The β-subunits anchor the receptor to the membrane and contains within its cytoplasmic region tyrosine kinase activity which is enhanced on IGF-1 binding to the α-subunit (LeRoith et al., 1995). Binding of the ligand to the α-subunit promotes autophosphorylation of the ligand at Tyr^{950}, Tyr^{1131} and Tyr^{1136} (Hongo et al., 1996). This leads to activation of the tyrosine kinase in the cytoplasmic β-subunit, recruiting ATP and initiating autophosphorylation of the receptor (Kato et al., 1994). This phosphorylation also extends to other cellular substrates such as insulin receptor substrate 1 (IRS-1), and Shc. These substrates can then function as docking proteins for adaptor proteins such as growth factor receptor bound protein 2 (Grb2).

IRS-1, a 160-190KDa phosphoprotein, is the most characterised of these exogenous substrates (Sun et al., 1991; White, 1994; White et al., 1985). This protein contains at least 20 potential tyrosine phosphorylation sites and is predicted to bind to the SH2 domains of

the regulatory subunit of PI3K (see fig.1.17). Yamamoto et al. (1992) demonstrated that the phosphorylated IGF receptor can also interact directly with PI3K's regulatory subunit without IRS-1. Activation of IRS-1 is a signal to many SH2 containing proteins including Grb2, which is involved in Ras regulation (White, 1994). Activation of PI3K's regulatory subunit and Grb2 results in activation of both the PI3K and MAPK pathways. In addition to this role, IRS-1 contains over 30 phosphorylation sites for serine/threonine kinases (Sun et al., 1991). Although the role of IRS-1 in IGF-1 signalling has not yet been well characterised it is known to have a crucial role in the control of cell cycle progression in response to IGF-1 (Chuang et al., 1993).

Independent of the IRS-1 protein, other docking proteins such as Shc have been implicated in IGF-1 signalling pathways. Shc is also tyrosine phosphorylated by the β receptor subunit and is thought to be involved in Ras signalling downstream (Rozakis-Adcock et al., 1992). Activation of Ras can lead to the downstream activation of the MAPK signalling cascade ultimately leading to MEK and RSK (ribosomal S6 kinase) translocation to the nucleus where transcription factors such as c-jun, c-fos and c-myc will be activated (Blenis, 1993).

Expression and activity of IGF-1 is controlled primarily through the IGF-binding proteins (IGFBPs). These binding proteins are a group of 6 highly versatile proteins which can differentially regulate IGF signalling in addition to acting independently of the growth factor (Shimasaki and Ling, 1991). Independently IGFBPs are involved in the regulation of processes such as growth, differentiation and apoptosis. IGFBPs are located within the ECM and exert their effects by binding to cell surface receptors. IGFBP3 and 5 have also been shown to be internalised by endocytotic pathways (Lee et al., 2004; Singh et al., 2004). Together IGFBPs act to prolong the half life of IGFs-up to 12 fold (Baxter and Dai, 1994)- while inhibiting their availability and activity. Understanding of the mechanism of action of these binding proteins is still slim and is thought to be cell-type specific.

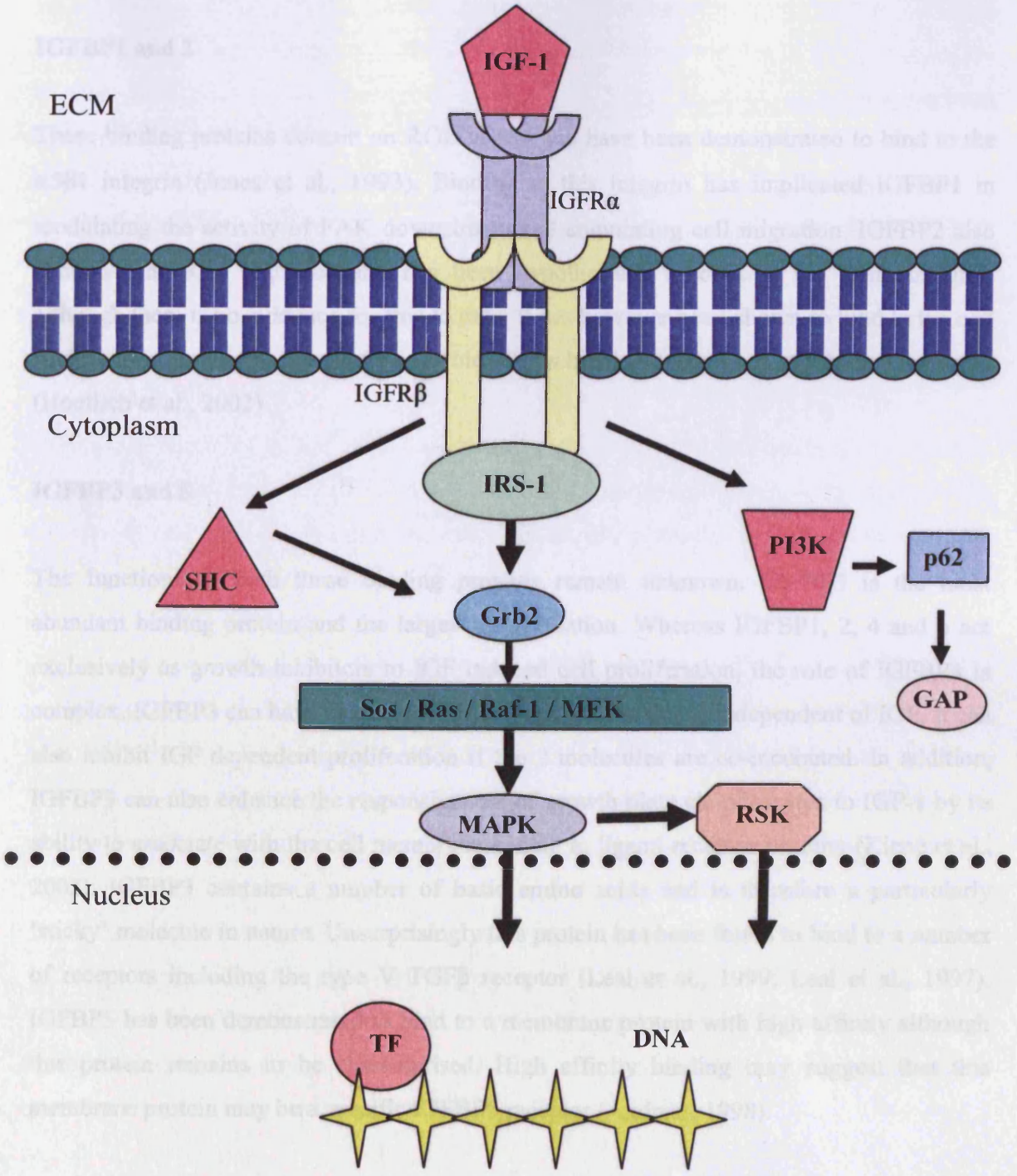


Fig.1.17 Downstream signalling pathways activated by IGF-1
Abbreviations: TF–transcription factor, MEK-mitogen activated extracellular signal-related protein kinase
(adapted from Benito et al., 1996)

1.15.1.1 Insulin-like Growth Factor Binding Proteins

IGFBP1 and 2

These binding proteins contain an RGD repeat and have been demonstrated to bind to the α5β1 integrin (Jones et al., 1993). Binding to this integrin has implicated IGFBP1 in modulating the activity of FAK downstream and stimulating cell migration. IGFBP2 also contains an RGD sequence and has been hypothesised to bind to the same integrin, although there is no evidence for this to date. It has however been shown to bind to the cell surface in rat brain but the nature and role of this binding protein has still to be elucidated (Hoeflich et al., 2002).

IGFBP3 and 5

The functions of both these binding proteins remain unknown. IGFBP3 is the most abundant binding protein and the largest in circulation. Whereas IGFBP1, 2, 4 and 6 act exclusively as growth inhibitors to IGF induced cell proliferation, the role of IGFBP3 is complex. IGFBP3 can have an anti-proliferative effect but this is independent of IGF. It can also inhibit IGF dependent proliferation if the 2 molecules are co-incubated. In addition, IGFBP3 can also enhance the responsiveness of growth plate chondrocytes to IGF-1 by its ability to associate with the cell membrane, aiding in ligand-receptor binding (Kiepe et al., 2002). IGFBP3 contains a number of basic amino acids and is therefore a particularly 'sticky' molecule in nature. Unsurprisingly this protein has been found to bind to a number of receptors including the type V TGFβ receptor (Leal et al., 1999; Leal et al., 1997). IGFBP5 has been demonstrated to bind to a membrane protein with high affinity although this protein remains to be characterised. High affinity binding may suggest that this membrane protein may be a specific IGFBP5 receptor (Andress, 1998).

Evidence from the structural analysis of both these IGFBPs suggests that may also translocate to the nucleus. Nuclear localisation signals (NLS) within the C-terminal domains of IGFBP3 and 5 support this theory (Radulescu, 1994). Internalisation of IGFBP3 is dependent on binding to either caveolae (Lee et al., 2004; Singh et al., 2004) or the transferrin receptor via the metal binding domain within the C-terminus of IGFBP3 (Singh

et al., 2004). The mechanisms of these interactions remain unknown. Numerous studies have localised IGFBP3 to the nucleus and its transportation from the cytosol possibly by association with importin-β. Importin-β is a protein with known roles in the transportation of other proteins from the cytosol into the nucleus. The translocation of IGFBP5 has been shown to be controlled by importin-β (Schedlich et al., 2000). The functions of these binding proteins on translocation to the nucleus are not well characterised but appear to involve regulation of transcription and therefore could lead to IGFBP dependent cellular effects such as apoptosis (Ricort, 2004).

1.15.2 Transforming Growth Factor Beta

TGFβs are members of the TGF superfamily, which includes the BMPs, Gdfs, activins, inhibins and Mullerian inhibitory factor (MIF). In total there are over 100 proteins within this family, all with at least one region of homology, normally occurring within the C-terminus (Burt and Law, 1994).

TGFβ1 was first identified from human platelets as a homodimeric peptide with a molecular weight of 25KDa (Assoian et al., 1983). In addition, a heterodimer of TGFβ1 and TGFβ2 has been demonstrated (Cheifetz et al., 1988). The cloned, human sequence suggests that the monomer form of TGFβ1 (112 amino acids) is encoded at the C-terminus of a larger 390 amino acid precursor molecule (Derynck et al., 1986). The three mammalian isoforms (TGFβ1-3) of the growth factor are secreted from virtually all cell types, including the chondrocyte, as the precursor form, preventing its attachment to the TGFβ receptor (TGFβR) and rendering the molecule inactive (Wakefield et al., 1987). This latent precursor is the major point of control for TGFβ activity. A fourth isoform of TGFβ, TGFβ4 is found only in avian tissues and is thought to have the same role as TGFβ1 within mammalian cells (Jakowlew et al., 1991; Pan and Halper, 2003).

The small latent complex (SLC) to which TGFβ is attached on secretion consists of an N-terminal latency associated peptide (LAP) non-covalently associated with the mature peptide (Munger et al., 1997). In some cases this SLC is associated with the latent TGFβ binding protein forming the large latent complex (Young and Murphy-Ullrich, 2004). LAP association with the mature peptide is critical for latency and also for expression of the

mature domain. Mutations within the LAP-mature domain binding region result in diminished mature domain secretion (Sha et al., 1991). The mechanism by which LAP is able to confer latency is largely unknown, however a recent paper by Young and Murphy-Ullrich (2004) elucidated the sequence RKPK as the binding site of LAP to TGFβ and a molecular basis for dissociation and activation of the mature TGFβ through TSP-1.

Nine membrane receptors for TGFβ have been identified to date and they all bind to TGFβ with different affinities (Fanger et al., 1986). They are classified as either type I (7 identified) or type II (2 identified) transmembrane serine/threonine receptors which form a functional complex of two type I and two type II. When not bound to a ligand the receptors exist as homodimers at the cell surface (Itoh et al., 2001).

Ligand binding results in the formation of these receptor complexes within the membrane. The type II receptors are known to induce the phosphorylation of their type I counterparts within the GS domain, an area rich in glycine and serine residues. The activation of the type I receptor kinase results in the downstream phosphorylation of receptor regulated Smads (R-Smads 1, 2, 3, 5 and 8) at 2 serine residues within their C-terminus (see fig.1.18). Auxiliary proteins such as Smad anchor for receptor activation (SARA) provide access of the R-Smads to the phosphorylated receptor (Shi and Massague, 2003). It is believed that phosphorylated R-Smads form complexes with co-Smad 4 which translocate and accumulate in the cell's nucleus and control gene expression in a cell type and ligand specific manner by interacting with various transcription factors, co-activators and co-repressors (Zhu and Burgess, 2001). The TGFβR will remain activated for 3-4 hours after initial phosphorylation and continuous receptor activation by ligand binding will maintain the accumulating Smad complexes within the nucleus, regulating gene expression (Inman et al., 2002). This mechanism has recently been shown to actually involve continuous shuttling of the Smad proteins between the nucleus and cytoplasm via an interaction of the Smad MH2 (MAD homology 2) domain binding to the nuclear pore complex (Shi and Massague, 2003) and is importin independent (Chen et al., 2005).

1.15.2.1 Regulation of Smad Function

The localisation and activity of Smads is controlled by their binding to adaptor molecules such as chaperones (Wurthner et al., 2001), microtubules (Dong et al., 2000) or SARA (Tsukazaki et al., 1998). Inhibitory Smads (I-Smads), 6 and 7, can interfere with receptor regulated Smad signalling by controlling access to the receptor. Kaiser et al. (2004) were the first to demonstrate the expression of both these I-Smads in normal and OA chondrocytes. Cytoplasmic localisation of the I-Smads within chondrocytes suggests that these inhibitors are permanently activated *in vivo*. Inactive I-Smads are generally thought to be localised within the nucleus. Their expression is induced by TGFβ (Hata et al., 1998; Hayashi et al., 1997) (Imamura et al., 1997; Nakao et al., 1997). I-Smads can bind to TGFβRs blocking R-Smad phosphorylation (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997). Smad 6 can also compete directly with Smad 4 for binding to phosphorylated Smad 1 (Hata et al., 1998).

Smad signalling is also controlled by a number of other pathways independent of type I receptor phosphorylation. R-Smads are targeted by the Erk MAPK pathways with or without the stimulation of Ras. Activation of this pathway results in inhibition of Smad translocation to the nucleus and signalling (Kretzschmar et al., 1999). Smads also contain phosphorylation sites for calcium/calmodulin dependent protein kinase II (CamKII) (Wicks et al., 2000) and PKC (Yakymovych et al., 2001) inhibiting TGFβ induced Smad transcriptional activity. TGFβ1 activity is also controlled via a number of non-Smad pathways such as Erk, c-jun N-terminal kinase (JNK), MAPK and PI3K as well as Rho GTPase family (Derynck and Zhang, 2003). Activation of the PI3K pathway requires a functional type II receptor (Yi et al., 2005).

The effects of matrix metabolism by TGFβ1 in chondrocytes are controversial. Conflicting reports have demonstrated both increases and decreases in proteoglycan synthesis with chondrocytes in monolayer culture as well as enhanced differentiation and proliferation versus inhibition of growth (Trippel, 1995). These differing effects are dependent on the chondrocyte's cell cycle stage. TGFβ will enhance proliferation of those cells within S phase, whereas those dividing slowly within G1 will be inhibited (Trippel, 1995; Vivien et al., 1992; Vivien et al., 1990).

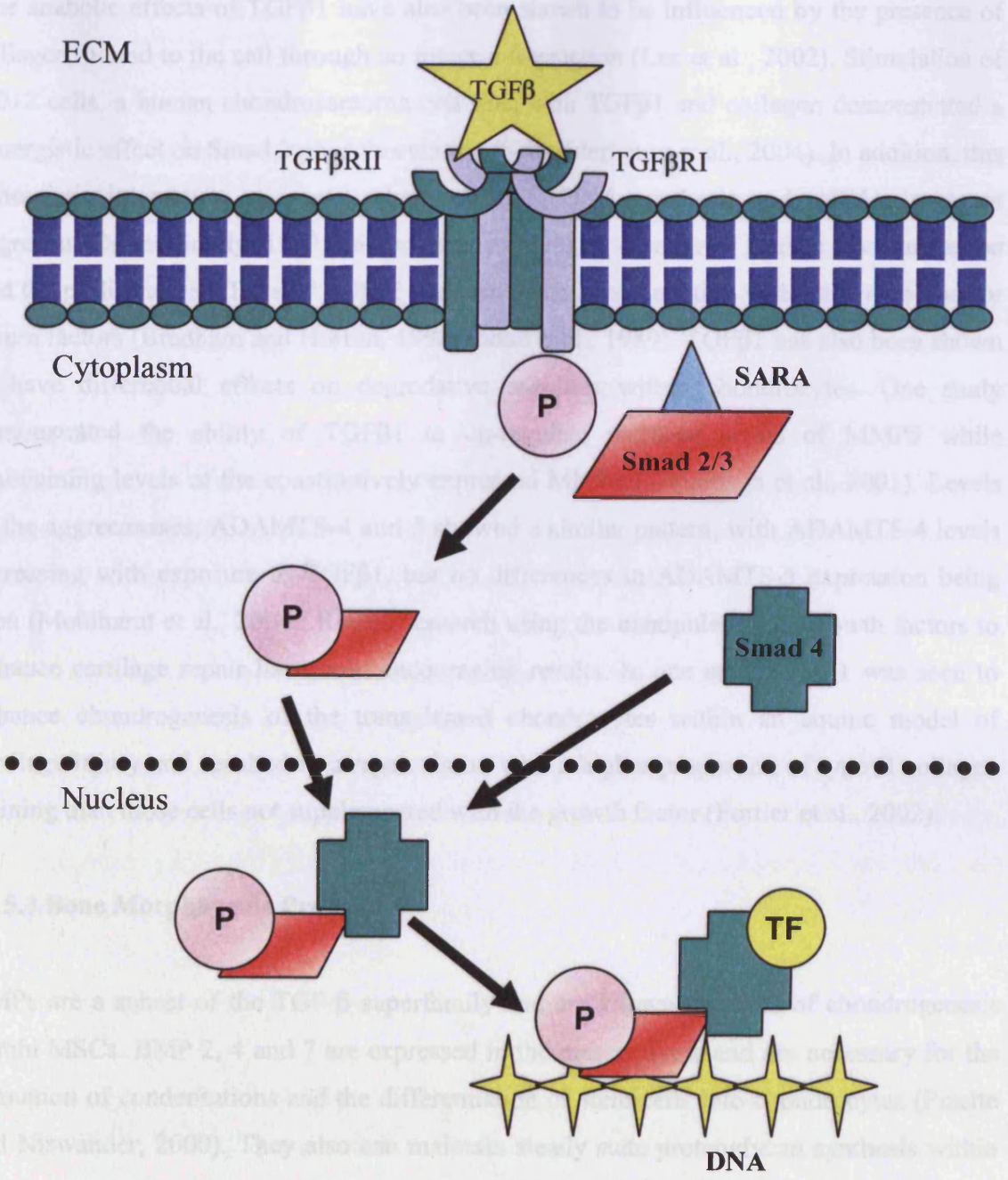


Fig.1.18 Smad activation by TGFβ
(adapted from ten Dijke and Hill, 2004)

The anabolic effects of TGFβ1 have also been shown to be influenced by the presence of collagen, bound to the cell through an integrin interaction (Lee et al., 2002). Stimulation of JJ012 cells, a human chondrosarcoma cell line, with TGFβ1 and collagen demonstrated a synergistic effect on Smad 2 phosphorylation (Schneiderbauer et al., 2004). In addition, this synergistic interaction was seen when analysing DNA synthesis and mRNA levels of aggrecan (Qi and Scully, 1997; Qi and Scully, 1998). A number of studies have suggested that the proliferative effects of TGFβ1 are seen when in conjunction with other cytokines or serum factors (Bradham and Horton, 1998; Inoue et al., 1989). TGFβ1 has also been shown to have differential effects on degradative enzymes within chondrocytes. One study demonstrated the ability of TGFβ1 to up-regulate message levels of MMP9 while maintaining levels of the constitutively expressed MMP2 (Thompson et al., 2001). Levels of the aggrecanases, ADAMTS-4 and 5 showed a similar pattern, with ADAMTS-4 levels increasing with exposure to TGFβ1, but no differences in ADAMTS-5 expression being seen (Moulharat et al., 2004). Recent research using the manipulation of growth factors to enhance cartilage repair have seen encouraging results. In one study, IGF-1 was seen to enhance chondrogenesis of the transplanted chondrocytes within an equine model of cartilage injury and resulted in a repair tissue with a higher proportion of type II collagen staining than those cells not supplemented with the growth factor (Fortier et al., 2002).

1.15.3 Bone Morphogenic Proteins

BMPs are a subset of the TGF-β superfamily and are known inducers of chondrogenesis within MSCs. BMP 2, 4 and 7 are expressed in the mesenchyme and are necessary for the formation of condensations and the differentiation of stem cells into chondrocytes (Pizette and Niswander, 2000). They also can maintain steady state proteoglycan synthesis within developed chondrocytes (Rosier and O'Keefe, 1998). Similar to TGFβ1 (see section 1.15.2) BMPs transduce their signals through serine/threonine kinase receptors, resulting in the activation of Smads (Kawabata et al., 1998; ten Dijke et al., 1994).

1.15.4 Fibroblast Growth Factor

At least 22 members of the FGF family have been identified to date (Eswarakumar et al., 2005). During development FGFs play crucial roles in regulating cell proliferation, differentiation and migration. Within the adult, FGFs play a part in both tissue repair and wound healing. FGF2, also known as basic FGF, exhibits anabolic effects which can be enhanced synergistically in conjunction with IGF-1, stimulating TGFβ1 release (Hickey et al., 2003). The response to FGFs is age and concentration dependent. At low concentrations in young cartilage, FGF2 is mitogenic and increases proteoglycan synthesis. However at high concentrations these responses become catabolic. Within adult cartilage FGF2 is anabolic and in a dose dependent manner (Sah et al., 1994). Over-expression studies in chondrocytes suggest that this growth factor may decrease type II collagen production and induce dedifferentiation of chondrocytes (Schmal et al., 2005).

FGFs mediate their responses by binding to their tyrosine kinase receptors. Four FGF receptors have been characterised (FGFR1-4). Point mutations occurring within FGFR1-3 have been linked to a number of skeletal dysplasias, with obvious impairment to skeletal, digital and cranial development (reviewed in Webster and Donoghue, 1997). Specifically, point mutations within FGFR3 have been linked to achondroplasia, hypochondroplasia and thanatophoric dysplasia type I and II (Deng et al., 1996; Eswarakumar et al., 2005).

1.15.5 Hepatocyte Growth Factor

Hepatocyte growth factor (HGF), also known as scatter factor, is a regulator of cartilage development in embryogenesis and is up-regulated after injury (Hickey et al., 2003; Takebayashi et al., 1995). Conflicting reports have been documented regarding the effects of HGF on chondrocyte matrix turnover (Bau et al., 2004; Hickey et al., 2003). A detailed investigation into the effects of HGF on chondrocyte catabolic and anabolic gene expression suggests that this growth factor is not a major regulator in cartilage matrix turnover (Bau et al., 2004). Studies suggest that HGF does stimulate motility and proliferation within chondrocytes however (Hickey et al., 2003). This growth factor may be important in the induction of chondrogenesis as it is expressed prior to the formation of condensations (Takebayashi et al., 1995).

1.15.6 Connective Tissue Growth Factor

Connective tissue growth factor (CTGF) is 1 of 6 members of the CCN (cyr61, ctgf, nov) family. Within cartilage, CTGF is predominantly expressed within the chondrocytes of the hypertrophic and calcifying cartilage zones (Nakanishi et al., 1997). This growth factor has regulatory roles in the process of endochondral ossification, including angiogenesis (Takigawa, 2000). CTGF has also been demonstrated to play roles in cell proliferation, migration and wound healing (Oemar and Luscher, 1997). CTGF has been found to be up-regulated in OA chondrocytes. Clustering chondrocytes at the damaged articular surface in OA sufferers highly express CTGF in addition to those within the hypertrophic zone (Omoto et al., 2004).

CTGF is thought to be a downstream mediator of TGFβ1 due to the induction of CTGF expression in response to TGFβ1 stimulation (Nakanishi et al., 1997), and also because of the presence of a TGFβ1 response element within the CTGF promoter sequence (Grotendorst et al., 1996).

1.15.7 Platelet Derived Growth Factor

PDGF is a wound healing growth factor and is secreted by chondrocytes at the site of injuries (Hickey et al., 2003). This growth factor is a powerful mitogen and chemoattractant for all cells derived from MSCs including chondrocytes. A recent study has demonstrated that exposing resting zone chondrocytes to PDGF encourages cell proliferation and proteoglycan production whilst inhibiting their progression into endochondral ossification (Schmidt et al., 2006). PDGF is involved in matrix homeostasis as it can increase proteoglycan synthesis alone, without an associated increase in DNA synthesis (Schafer et al., 1993), or interact with IL-1 to induce matrix degradation (Hickey et al., 2003).

1.15.8 Ageing and Sensitivity to Growth Factors

With age the ability of chondrocytes within articular cartilage to synthesise and maintain their ECM becomes diminished, a factor illustrated by the increased prevalence of degenerative joint diseases such as OA. The biosynthetic abilities of chondrocytes are in

part regulated by their response to anabolic growth factors such as IGF-1 and TGFβ1. Studies have demonstrated that the response of chondrocytes to growth factors such as these becomes diminished with age and OA. Despite higher concentrations of IGF-1 and its associated receptor within osteoarthritic joints, chondrocytes are less responsive to the anabolic growth factor (Dore et al., 1994; Middleton et al., 1996; Tavera et al., 1996). The reason for this decrease in responsiveness has been partly attributed to the increased number of IGFBPs found within older and OA cells (Dore et al., 1994; Martin et al., 1997). These binding proteins may associate with the cell surface and compete with IGF-1 for its receptor. Messai et al. (2000) have also suggested that chondrocytes may exhibit decreased sensitivity to IGF-1 with age due to an altered signalling pathway. The authors addressed the activation of adenylcyclase for cAMP production required for the PKC pathway. They demonstrated that aged chondrocytes produced lower levels of cAMP, ultimately affecting the ability of the chondrocytes to produce matrix macromolecules. The role of cAMP in stimulating DNA synthesis and matrix production is well documented (Leipold et al., 1992; Stack and Brandt, 1980).

The responsiveness to other growth factors such as TGFβ1 and PDGF have also been investigated (Guerne et al., 1994). In this study the only growth factor able to stimulate a proliferative response in old chondrocytes was TGFβ1. These findings are supported in a number of publications. A study by Hickery et al. (2003) illustrated the ability of TGFβ1 to increase total GAG synthesis in immature but not mature cartilage. The findings from this study are supported by Blaney Davidson et al. (2005) who demonstrated decreased expression of TGFβRI and II in old when compared to young mice. These results also correlated with decreased Smad 2 phosphorylation. In addition, the authors also observed an increase in the I-Smads 6 and 7, further supporting a decreased sensitivity to TGFβ1 in old chondrocytes. Within humans a similar observation has been made, with anabolic effects of TGFβ1 only observed in donors of less than 40 years of age (Barbero et al., 2004).

1.16 Background

In summary, once injured articular cartilage will not spontaneously repair. This phenomenon has been attributed to its avascular nature and the entrapment of chondrocytes within an extensive ECM. Surgical attempts at repairing cartilage have seen the development of fibrocartilaginous repair tissue that integrates poorly with native cartilage. This study aims to address the issue of integration for cartilage repair. As discussed in section 1.13.2, integration is dependent on the interactions of newly synthesised matrix with the pre-existing cartilage as well as the migration of chondrocytes into wounded cartilage in order to induce a synthetic response.

Experiments outlined in the following chapters address factors influencing the migration and adhesion of chondrocytes, their integration into a wounded cartilage matrix as well as their biosynthetic responses with growth factor stimulation.

1.17 Hypothesis

Integration between injured articular cartilage surfaces can be enhanced by the addition and stimulation of isolated chondrocytes to the interface regions.

1.18 Aims

The overall objective of this project is to define novel strategies for enhancing the integration of newly synthesised repair tissue and pre-existing cartilage in articular cartilage repair. The aims to be addressed to achieve the main objective are outlined below:

- Compare the migratory capacities of chondrocytes isolated from young and skeletally mature articular cartilage.

- Establish an *in vitro* model for investigating articular cartilage repair.

- Investigate the migration and integration of chondrocytes isolated from young and skeletally mature cartilage into wounded articular cartilage.

- Elucidate the effects of the growth factors IGF-1 and TGFβ1 on matrix macromolecule biosynthesis using chondrocytes isolated from young and skeletally mature articular cartilage.

- Evaluate the effects of over-expressing IGF-1 and TGFβ1 on the migration of articular chondrocytes.

Chapter 2

Investigating the Migration of Articular Chondrocytes

2.1 Background

In situ it is generally accepted that chondrocytes are a non-motile cell type. Entrapped within the extensive ECM that they synthesise, their static nature is thought to be one of the major reasons why articular cartilage does not spontaneously repair.

Frenkel et al. (1996) were the first group to demonstrate directed migration of chondrocytes. They revealed the effects of nitric oxide on the chondrocyte's cytoskeleton and how this directly affected the migration of these cells. Despite the fact that chondrocytes have been shown to possess the components required for cell movement, their migratory capacities have not been investigated in detail. A more recent study has shown that chondrocyte migration is an active process and involves the formation of pseudopodia and lamellipodia at the leading edge with ruffled borders seen at the posterior of the moving cell (Chao et al., 2000). The development of cell-based methods of articular cartilage repair, such as ACT, require more detailed knowledge of the migratory abilities of chondrocytes once removed from their constraining ECM.

Studies that have been carried out to date do not consider in detail the fragility of the chondrocyte phenotype and the well known fact that once plated and passaged, chondrocytes will dedifferentiate into fibroblast-like cells (Kuettner et al., 1982; Kuroda, 1964). A recent report on the effects of passage number on the adhesion, migration and cytoskeletal organisation of articular chondrocytes has highlighted the changes in F-actin arrangements between primary and passaged cells (Hamilton et al., 2005). This is associated with the loss of the chondrocyte's characterising spherical shape. The authors in this study question how passaging of chondrocytes and the rearrangement of the cytoskeleton will lead to internal changes in signal transduction pathways and gene expression profiles. A study by Chang et al. (2003) has recently addressed this issue of phenotype and documented that chondrocytes isolated from newborn calves do have the capacity to migrate on fibronectin. To date no groups have looked at the ability of chondrocytes from skeletally mature articular cartilage to migrate. This is of particular importance now as cell-based methods of repair generally use autologous chondrocytes isolated from adult articular cartilage.

Groups studying the migratory capacities of chondrocytes have focussed on their ability to move on different ECM substrates and also their response to different growth factors

in enhancing this migratory process. The glycoprotein, fibronectin (see section 1.4.4.4.7) is generally considered to be pro-migratory and the previously discussed study by Chang et al. (2003) demonstrated that chondrocytes can bind to this ECM molecule and migrate on its surface. The chemotactic and haptotactic effects of fibronectin as well as type I and II collagen on chondrocyte migration have been shown by Shimizu et al. (1997). The group furthered this work by demonstrating that this migration was mediated through protein tyrosine phosphorylation initiated by the cross-linking or clustering of β1 integrins. Other studies have used time-lapse video-microscopy to show that chondrocytes can bind to the surface of collagen fibrils and pull themselves along the fibres like tracks. Work by Maniwa et al. (2001) using monolayer expanded chondrocytes also demonstrated migration by time-lapse microscopy on HA. No investigations have been documented on the effects of aggrecan, a major component of the articular cartilage ECM, on chondrocyte migration. As previously discussed in section 1.13.2, proteoglycans including aggrecan and the SLRPs are generally considered to be anti-adhesive due to the presence of their negatively charged GAG side chains (Culp et al., 1979; Lewandowska et al., 1987; Merle et al., 1997; Winnemoller et al., 1991; Winnemoller et al., 1992; Yamagata et al., 1989) and are therefore also thought to be anti-migratory (Merle et al., 1999).

A small amount of research has also been documented looking at the chemotactic effect of growth factors including IGF-1 (Chang et al., 2003) and FGF2 (Maniwa et al., 2001) to enhance the migration of chondrocytes *in vitro*. The chondrocytes used in these experiments had been isolated from young animals though and it is generally accepted that chondrocyte responsiveness to growth factors decreases with age (Hickery et al., 2003; Martin and Buckwalter, 2000; Martin et al., 1997). No groups have looked at the response of chondrocytes to growth factors with age in respect to migration. The chondrocytes from the Martin and Buckwalter study (Martin and Buckwalter, 2000) had also been expanded in monolayer culture, altering the native integrin pattern on the surface of the cell as seen in freshly isolated chondrocytes.

The objective of this study was to compare the migratory capacities of both chondrocytes derived from young and skeletally mature animals. The cells were freshly isolated from the tissue and not expanded in monolayer so as to preserve their chondrocytic phenotype and cause as little alteration to their integrin expression pattern as possible. The chondrocytes were seeded onto different ECM macromolecules

including type II collagen, fibronectin and aggrecan to investigate the effects of these substrates on the migration of chondrocytes from both age groups. The chemotactic effects of IGF-1 and TGFβ1 on chondrocyte migration were also investigated. The effects of the CS side chains, linked to the aggrecan core protein, on chondrocyte migration are also addressed within this chapter.

Aims

1. Compare and contrast the migratory capacities of chondrocytes isolated from both young and skeletally mature bovine articular cartilage on a variety of ECM macromolecules using an *in vitro* model for cellular migration.
2. Investigate the chemotactic effect of IGF-1 and TGFβ1 on chondrocytes migrating on a variety of ECM macromolecules.
3. To investigate the role of the GAG, CS, in modulating chondrocyte migration on monomeric aggrecan.

2.2 Materials

All materials were obtained from Sigma Chemical Company, UK unless otherwise stated within the protocol.

2.3 Methods

2.3.1 Harvesting of Bovine Articular Cartilage Explants

Feet from both 7 day old (young) and 18 month old (skeletally mature) cattle were washed and skinned before being sprayed with 70% ethanol (Fisher Scientific UK Ltd., UK) and dissected. Upon opening of the metacarpophalangeal or metatarsophalangeal joint, full depth slices of articular cartilage were removed from the medial and lateral condyles of the joint using a sharp, size 10 scalpel blade (Swann Morton Ltd., UK) (see fig.2.1). Harvested explants of cartilage were serially washed in Hanks balanced salt solution (HBSS) with 400U/ml penicillin, 400μg/ml streptomycin, 10μg/ml Fungizone and 200μg/ml Gentamicin twice and 100U/ml penicillin, 100μg/ml streptomycin, 2.5μg/ml Fungizone and 50μg/ml Gentamicin once (Invitrogen Ltd., UK). Washed explants were maintained overnight in Dulbecco's modified Eagle's medium (DMEM)/Glutamax™ 1, 10% foetal bovine serum (FBS), 100U/ml penicillin and 100μg/ml streptomycin (Invitrogen Ltd., UK).

2.3.2 Isolation of Chondrocytes from Bovine Articular Cartilage Explants

Pronase (15U/ml) was made up in DMEM/Glutamax™ 1, 10% FBS, 100U/ml penicillin, 100μg/ml streptomycin and filter sterilised using a 0.2μm pore size serum Acrodisc® syringe filter (Pall Corporation, USA). Explants, isolated as previously described, were digested in pronase for 30 minutes at 37°C with gentle agitation. Digested explants were transferred to filter sterilised type II collagenase (11U/ml) and digested in the above conditions. Isolated cells were removed at 30 minute intervals and spun down at 1000 RPM (revolutions per minute) for 10 minutes. The collagenase supernatant was removed and pipetted back onto the explants and the pelleted cells were resuspended in DMEM:Ham's F-12 (1:1)/Glutamax™ 1, 1 X ITS (insulin, transferrin and selenium) supplement, 100U/ml penicillin, 100μg/ml streptomycin (Invitrogen Ltd., UK) for counting. This was the basal media used for subsequent experiments.

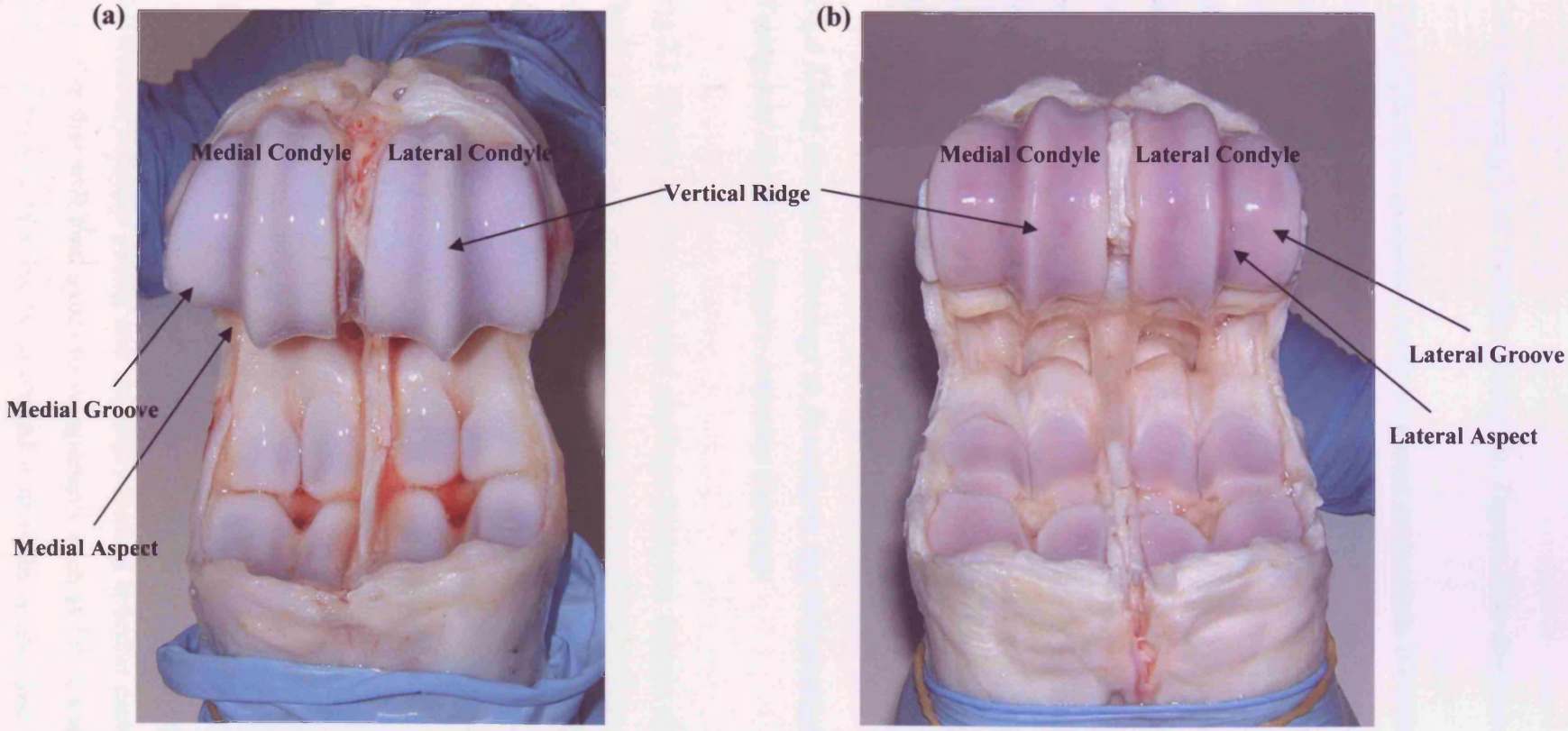


Fig.2.1 Comparing the anatomy of (a) 7 day and (b) 18 month old bovine metacarpophalangeal/metatarsophalangeal joints

A cell count was performed using a haemocytometer and the trypan blue exclusion assay as a measure of cell viability.

2.3.3 Assessing Cell Viability Using the Trypan Blue Exclusion Assay

The trypan blue exclusion assay was used to ascertain the quality of the cells extracted before they were used for experimentation. Trypan blue is an acidic dye and can only be taken up by cells which have a compromised cell membrane and, are therefore, considered to be dead. Within the cell, trypan blue can bind to intracellular proteins and therefore turns the cell blue. An aliquot of isolated cells (suspended in basal media), at an appropriate dilution, was incubated with a working concentration of 0.08% trypan blue for 5 minutes before counting. Three aliquots of cells were prepared and an average 3 counts were taken from each aliquot in order to obtain an accurate measure of chondrocyte viability. Cells were counted using a haemocytometer. Total and dead cell counts (those cells which stained blue after incubation with trypan blue) were calculated in order to obtain a percentage cell death figure.

2.3.4 Using General Histology to Investigate the Morphological Differences between Young and Skeletally Mature Articular Cartilage

Fig.2.1 illustrates that articular cartilage becomes thinner during development. Full thickness cartilage isolated from young bovine (7 day old) metacarpophalangeal and metatarsophalangeal joints is on average 4-5mm thick, whereas that obtained from skeletally mature joints (18 month old) tends only to be, at maximum, 1mm in depth. Here general histological methods have been employed to investigate the differences between the age groups of cartilage at a microscopic level.

2.3.4.1 Haematoxylin and Eosin Staining

Haematoxylin and eosin staining was used to look at the arrangement of chondrocytes within the different zones of articular cartilage and also demonstrate the profound differences between young and skeletally mature articular cartilage. Haematoxylin is a basic dye that will bind to acidic components such as DNA and therefore will stain cell nuclei. Typically this dye is combined with eosin, a bromine derivative of fluorescein which will stain both cell nuclei and the cytoplasm (Masson, 1929).

Cryosections of 10μm thickness from explants taken from both young and skeletally mature bovine metacarpophalangeal/metatarsophalangeal joints were stained. Slides were washed in running water for 2 minutes to hydrate them before staining in Mayer's haematoxylin for 30 seconds. Slides were then transferred back to the running water and washed until the sections turned blue (a period of approximately 5 minutes). The sections were then stained in 1% aqueous eosin for 4 minutes before again washing in running tap water. Slides were then dehydrated in a series of increasing alcohol concentrations (Fisher Scientific UK Ltd., UK) before fixing in xylene (Fisher Scientific UK Ltd., UK) for 4 minutes. Sections were then mounted under a coverslip using DPX mountant (Agar Scientific Ltd., UK). Sections were visualised using an upright light microscope (Leitz DMRB, Leica, Germany) at x 40 magnification and photomicrographs recorded using the Image Grabber PCI software, Version 2.5 (Neotech Ltd., UK).

2.3.4.2 Masson's Trichrome Staining

Masson's trichrome staining procedure was used for the localisation of collagen within the ECM. In this method cell nuclei are stained blue/black, collagen and polysaccharides green and any cytoplasmic granules red (Flint, 1975). As described above, 10μm cryosections of cartilage were used. Sections were hydrated in running tap water for 2 minutes before staining with Celestine blue for 5 minutes. Slides were then washed in running tap water until sections appeared clear. Sections were stained in Mayer's haematoxylin for a further 5 minutes before again washing in running tap water until the sections appeared to turn blue. A further staining step in acid fuchsin was used for 5 minutes to stain any cytoplasmic granules present. The sections were again washed in running water for 30 seconds before differentiating in 1% phosphomolybdic acid for 5 minutes. The slides were transferred directly to light green stain for 2 minutes before washing in 1% acetic acid for an extra 2 minutes. The sections were briefly washed in running tap water before dehydrating in increasing concentrations of alcohol, and fixing in xylene. Slides were allowed to air dry before mounting under a coverslip using DPX.

2.3.4.3 Safranin O Staining

Safranin O is a basic dye which in the context of cartilage is used to stain for the presence of GAG chains (Rosenberg, 1971). In this method it has been combined with a Mayer's haematoxylin stain in order to visualise cell nuclei at the same time. As for the haematoxylin and eosin staining and Masson's trichrome staining, 10μm cryosections of full depth cartilage were used. The sections were hydrated in running tap water for 2 minutes, before staining as previously described with Mayer's haematoxylin. Slides were then washed in running water until sections appeared blue, before staining with 1% fast green for 1 minute. Sections were subsequently rinsed in 1% acetic acid before staining in 0.1% Safranin O for 4 minutes. The slides were then rinsed in 1% acetic acid before the sections were dehydrated in alcohol and mounted under a coverslip using DPX mountant. Sections were visualised as per the method described for haematoxylin and eosin staining.

2.3.5 Measuring Cell Migration Using Boyden Chambers

2.3.5.1 Generating a Chondroitinase ABC Treated Aggrecan Substrate

Monomeric, A1D1 fraction, aggrecan purified from bovine nasal cartilage (courtesy of Professor Bruce Caterson, Cardiff University, UK) was digested with chondroitinase ABC at a concentration of 0.001U/10μg of sGAG, as assessed by Dimethylmethylene blue assay (DMMB), for 24 hours at 37°C on a roller. Loss of the CS side chains was assessed by both the DMMB colorimetric assay and slot blotting.

2.3.5.1.1 Assessment of Sulphated Glycosaminoglycan Content Using the Dimethylmethylene Blue Assay

This plate assay uses the ability of the metachromatic dye, dimethylmethylene blue to detect sGAGs through a colour change from blue to pink which can be read at 540nm (Farndale et al., 1986).

DMMB Reagent

The 1,9-dimethylmethylene blue dye (32mg) was dissolved overnight on a roller at room temperature in 20ml of 100% ethanol. The vial containing the reagent was wrapped in foil to prevent exposure to light. The dissolved dye was added to 1.5 litres of distilled water, 59ml of 1M sodium hydroxide (Fisher Scientific UK Ltd., UK) and 7ml of 98% (v/v) formic acid (Fisher Scientific UK Ltd., UK), mixed and made up to 2 litres with distilled water. The complete reagent was stirred for 2 hours at room temperature. Absorbance readings were taken at 525 and 595nm using a multi-well plate reader (Labsystems Multiskan MS, Thermo Electron Corporation, UK) with respective absorbencies of 0.3 and 1.4 recorded if the correct amount of dye had successfully been dissolved.

The DMMB Plate Assay

A standard curve was calculated from dilutions of a 0.5mg/ml stock of CS C (C-6-S) derived from shark cartilage, within the range of 0-40µg/ml using distilled water. Standards and samples (40µl/well) were added to the 96 well plate (Greiner Bio-One Ltd., UK) in triplicate followed by 200µl of DMMB reagent before being read immediately at 525nm. Complete digestion of the A1D1 aggrecan was assessed by the loss of the sGAG chains.

2.3.5.2 Detection of Chondroitinase ABC Generated Neo-epitopes by Slot Blotting

Slot blotting principally involves the transfer of proteins within a sample onto transfer membrane (Towbin et al., 1979). A primary antibody raised against the specific antigen required (the chondroitinase ABC generated C-0-S and C-4-S stubs in this case) will attach specifically to this immobilised antigen upon incubation. An appropriate species specific secondary antibody conjugated with a reporter enzyme such as horseradish peroxidase (HRP) is subsequently used and the location of the reporter enzyme tracked using chemiluminescence detection reagents.

Native, non-digested and chondroitinase ABC digested A1D1 aggrecan samples were loaded onto a slot blot system. Using this method for blotting, samples are loaded directly onto Immobilon-P polyvinylidene fluoride (PVDF) transfer membrane

(Millipore Ltd., UK) pre-soaked in transfer buffer (1x Laemmli buffer [see appendix 5.1.3], 20% (v/v) methanol) sandwiched between the slots and filter paper and held under vacuum. Samples were pulled into the membrane by the vacuum over a period of 10 minutes. On completion of transfer, the membrane was washed 3 times over 30 minutes in blocking buffer (1 x Tris-buffered saline (TBS) [see appendix 5.1.4], 0.05% (v/v) Tween 20™) with 3% (w/v) skimmed milk powder (CWS Ltd., UK) to block any remaining binding sites before incubation with the primary antibody.

2.3.5.2.1 Primary Antibodies – 1B5 and 2B6

The murine monoclonal antibodies, 2B6 and 1B5, have been developed to specifically recognise chondroitinase ABC generated CS-stub epitopes of the CS GAG chains projecting out from the aggrecan core protein (see fig.2.2). 2B6 recognises C-4-S stubs, specifically an unsaturated glucuronic acid residue at the non-reducing end of the chain adjacent to a 4-sulphated N-acetylgalactosamine residue of the CS unsaturated disaccharide (Caterson et al., 1985). 1B5 is designed to recognise unsulphated chondroitin-stubs. This antibody is raised against an unsaturated uronic acid residue linked to a N-acetylgalactosamine residue (Couchman et al., 1984).

Membranes were exposed to either 2B6 (1:100) or 1B5 (1:1000) for 2 hours at room temperature on a roller. Primary antibodies were diluted in blocking buffer. Primary antibody negative controls were exposed to just blocking buffer for the incubation period. On removal of the primary antibody, the membrane was washed in blocking buffer 3 times over 30 minutes. All membranes including the negative controls were exposed to the secondary antibody (sheep anti-mouse) conjugated to HRP for 2 hours with agitation at room temperature. This antibody was diluted to 1:10,000 in blocking buffer. The membranes were again washed in blocking buffer for 30 minutes with 3 changes before detection.

2.3.5.2.2 Detection of Antibody Binding Using Enhanced Chemiluminescence (ECL™)

Excess buffer was removed before ECL™ reagent (GE Healthcare UK Ltd., UK) was added to the membranes. The ECL™ method allows detection of immobilised antigens

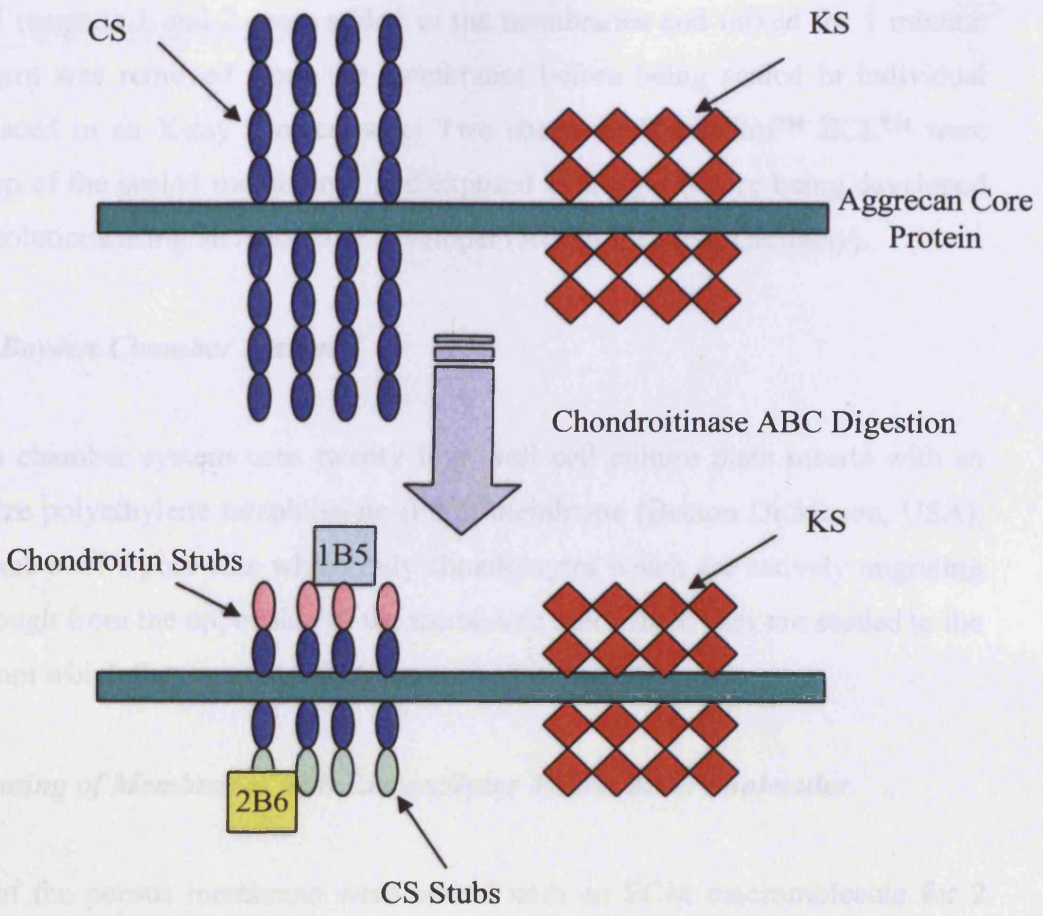


Fig.2.2 Schematic representation of the chondroitinase ABC generated CS-stubs recognised by monoclonal antibodies 2B6 and 1B5 (adapted from Caterson, 1999)

using HRP conjugated antibodies. The reagent works by providing the cyclic diacylhydrazide, luminol, which in the presence of HRP and chemical enhancers such as phenols is oxidised (see fig.2.3). This reaction provides light emission which has an approximate half life of 60 minutes and can be detected by Hyperfilm™ ECL™, a blue light sensitive autoradiography film (GE Healthcare UK Ltd., UK). An equal volume of both ECL™ reagents 1 and 2 were added to the membranes and mixed for 1 minute. Excess reagent was removed from the membranes before being sealed in individual bags and placed in an X-ray film cassette. Two sheets of Hyperfilm™ ECL™ were placed on top of the sealed membranes and exposed overnight before being developed in standard solutions using an automatic developer (AGFA Gevaert, Germany).

2.3.5.3 The Boyden Chamber System

The Boyden chamber system uses twenty four well cell culture plate inserts with an 8μm pore size polyethylene terephthalate (PET) membrane (Becton Dickinson, USA). The membrane is of a pore size where only chondrocytes which are actively migrating can pass through from the upper side of the membrane onto which they are seeded to the underside from which they are counted (summarised in fig.2.4).

2.3.5.3.1 Coating of Membranes with Extracellular Matrix Macromolecules

Both sides of the porous membrane were coated with an ECM macromolecule for 2 hours (individual matrix molecules, their concentrations and diluents are outlined in table 2.1 below). Bovine plasma fibronectin was purchased from Sigma Chemicals, UK, type II collagen was purified from bovine cartilage (courtesy of Professor Vic Duance, School of Biosciences, Cardiff University, UK) and A1D1 aggrecan was purified from bovine nasal cartilage (courtesy of Professor Bruce Caterson, School of Biosciences, Cardiff University, UK). Subsequent to coating, remaining binding sites were blocked with filter sterilised 0.1% (w/v) bovine serum albumin (BSA) diluted in 1x phosphate buffered saline (PBS) (pH 7.4) for 1 hour and washed with three 5 minute washes in PBS (pH 7.4) before being stored at 4°C.

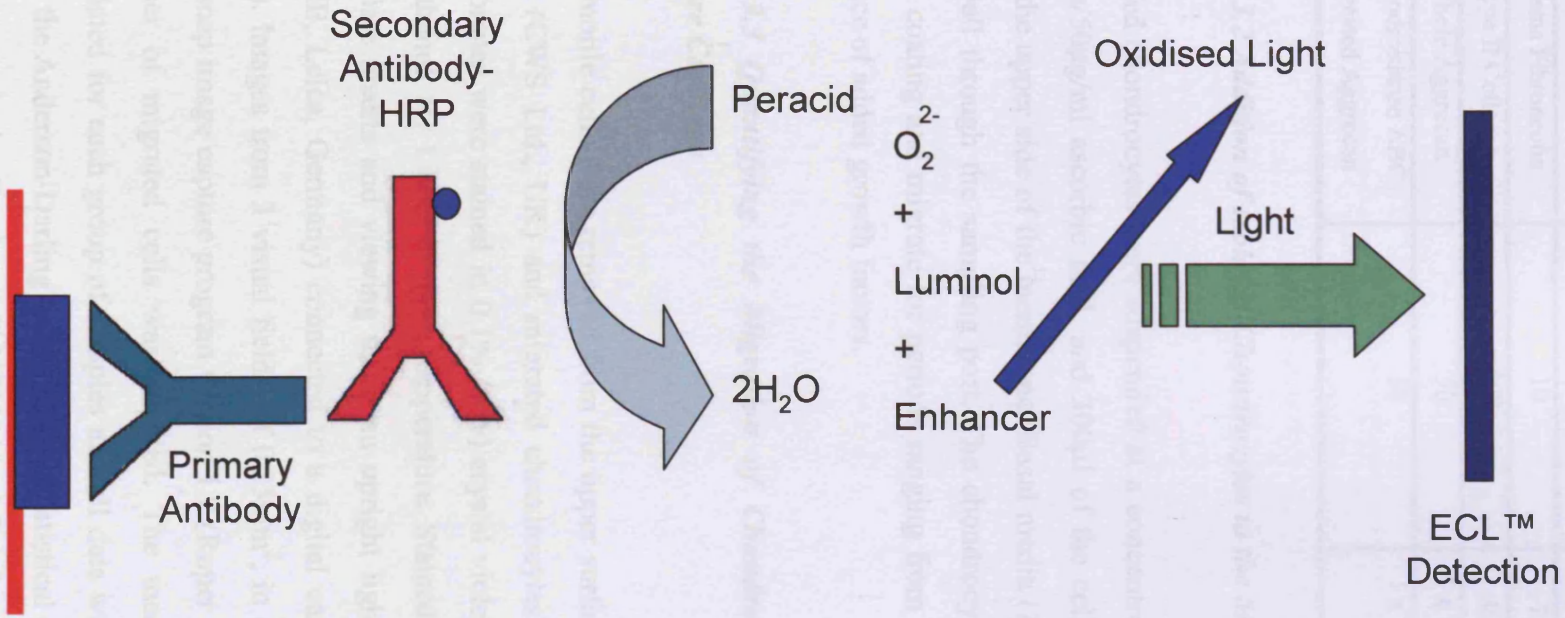


Fig.2.3 Summary of Western blotting method for the detection of specific protein bands (adapted from Amersham Biosciences, 2002)

Table 2.1 Working concentrations of ECM coating macromolecules for Boyden chambers and their respective diluents.

ECM Coating Macromolecule	Concentration (μg/ml)	Diluent	Reference for Concentration Used
Plasma Fibronectin	10	1 x PBS (pH 7.4)	(Shimizu et al., 1997)
Type II Collagen	500	0.5M Acetic Acid	(Luo et al., 1997)
Whole Aggrecan	50	1 x Tris-acetate buffer	(Johnson et al., 2005)
Chondroitinase ABC Digested Aggrecan	50	1 x Tris-acetate buffer	-

2.3.5.3.2 Addition of Isolated Chondrocytes to the Membranes

Isolated chondrocytes were suspended at a concentration of 6.7 x 10^5 cells/ml in basal media/50μg/ml ascorbic acid, and 300μl of the cell suspension was pipetted directly onto the upper side of the membrane. Basal media (1.4 ml) was added to the bottom of the well through the sampling port. The chondrocytes were allowed to adhere to the ECM coating and migrate for periods ranging from 1 to 24 hours in the presence and absence of added growth factors.

2.3.5.3.3 Quantifying the Migration of Chondrocytes Isolated from Young and Mature Cartilage

Non-motile cells were removed from the upper surface of the membrane using a cotton swab (CWS Ltd., UK) and migrated chondrocytes attached to the underside of the membranes were stained in 0.1% (w/v) crystal violet dye in 0.1M Borax (pH 9.0) and 2% ethanol, for 1 hour at room temperature. Stained cells were visualised by inverting the plate inserts and viewing using an upright light microscope (Laborlux 12, Leitz DRMB, Leica, Germany) connected to a digital camera (Coolsnap RS Photometrics, USA). Images from 3 visual fields, of 0.25mm^2, in each well were recorded using the Coolsnap image capture program version 1.2 (Roper Scientific Inc., USA). The average number of migrated cells was counted. The mean ± standard error (SEM) was calculated for each group of samples and all data was assessed for normal distribution using the Anderson-Darling test ($P>0.05$). Statistical significance was calculated using a one-way analysis of variance (ANOVA) and Bonferroni post-hoc test where $P<0.05$.

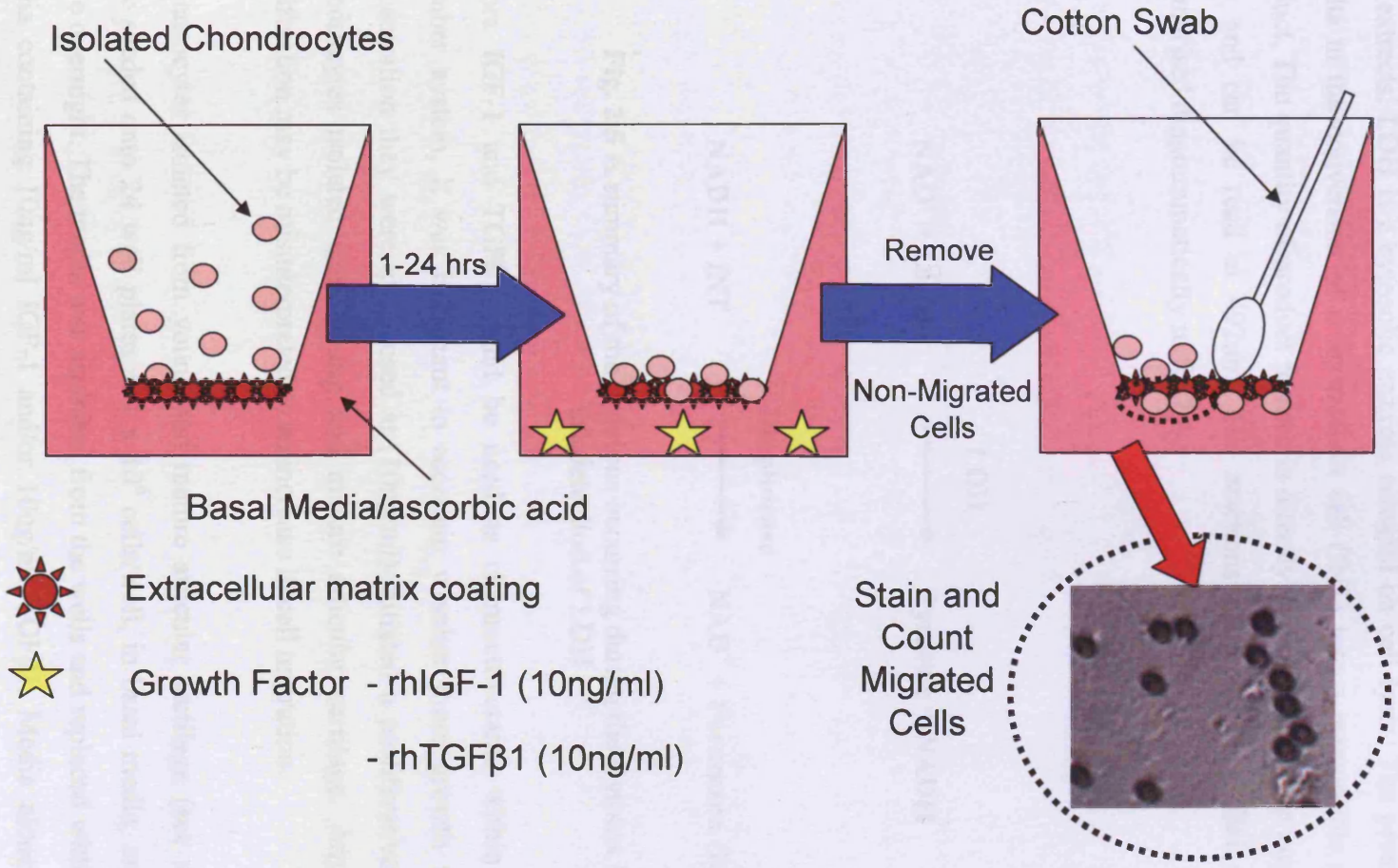


Fig.2.4 Schematic representation of the Boyden chamber system for quantifying chondrocyte migration

2.3.6 Measuring Cellular Proliferation Using the Cytotox 96® Assay

The Cytotox 96® non-radioactive assay (Promega, UK) allows colorimetric, quantitative measurement of lactate dehydrogenase (LDH) within cell culture media and cell extracts. LDH is a cytosolic enzyme released on cell lysis. The presence of LDH results in the conversion of a tetrazolium salt (INT) into a measurable red formazan product. The quantity of product formed is directly proportional to the number of lysed cells and can be read at 492nm. The reactions occurring for LDH detection are summarised diagrammatically in fig.2.5.

$$NAD^+ + \text{Lactate} \xrightarrow{\text{LDH}} \text{Pyruvate} + \textbf{NADH}$$

$$\textbf{NADH} + \text{INT} \xrightarrow{\text{Diaphorase}} NAD^+ + \textbf{Formazan (Red Product)}$$

Fig. 2.5 A summary of the reactions occurring during the Cytotox 96® assay in the detection of LDH

Before IGF-1 and TGFβ1 could be used as chemoattractants within the Boyden chamber system, it was important to ascertain whether these growth factors-at the concentration they were to be used at (10ng/ml)-initiated a proliferative response on chondrocytes isolated from young and mature articular cartilage. Any increase in proliferation may be misinterpreted as an increase in cell migration.

Chondrocytes isolated from young and mature articular cartilage (see section 2.3.2) were seeded onto 24 well plates at 1 x 10^6 cells/well, in basal media, and allowed to settle overnight. The media was aspirated from the wells and replaced with 1ml of basal media containing 10ng/ml IGF-1 and/or 10ng/ml TGFβ1. Media alone served as a control. The chondrocytes were left in the presence of the growth factors at 37°C for 24 hours, before the media was removed and the cells lysed in 0.9% Triton® X 100 (Fisher Scientific UK Ltd., UK).

The provided assay buffer (12ml) was thawed and added to the lyophilised substrate mix. Cell lysates were pipetted in duplicate into a 96 well microtitre plate. The reconstituted substrate mix (50µl) was added to each well and the plate incubated in the dark for 10 minutes at room temperature. After the incubation period, 50µl of stop solution (1M acetic acid) was added to each well and the absorbance read at 492nm.

The mean ± the SEM were calculated for each treatment group. Data was assessed for normality, and statistical significance calculated using the methods outlined in section 2.3.5.3.3.

2.3.7 Assessing the Chemotactic Effects of IGF-1 and TGFβ1 on Chondrocytes Migrating on Extracellular Matrix Macromolecules

As previously described in section 2.3.5.3.1, Boyden chamber membranes were coated with ECM macromolecules for both age groups of chondrocytes to migrate on. In this experiment the membranes were coated with fibronectin, type II collagen, native aggrecan (all at the concentrations stated in table 2.1) or 0.1% (w/v) BSA, which served as a control. Isolated chondrocytes were seeded onto the upper side of the membrane as described in section 2.3.5.3.2, and allowed to adhere to the substrate for 48 hours. Basal media/50µg/ml ascorbic acid (1.4ml) was added through the portal to the bottom of the well. Growth factors were supplemented to this at a concentration of 10ng/ml after the 48 hour attachment period. BSA was added to the control wells. Treatment groups of IGF-1, TGFβ1 and IGF-1 and TGFβ1 were set up in triplicate. After the addition of growth factors to the bottom of the well the chondrocytes were allowed to migrate over a period of 24 hours before non-motile cells were removed as described in section 2.3.5.3.3 and the migrated cells stained with crystal violet and counted. Statistical analysis was performed as stated in section 2.3.5.3.3.

2.3.8 Assessing the Chemotactic Effects of IGF-1 and TGFβ1 on Chondrocytes Migrating on Chondroitinase ABC Treated Aggrecan

Boyden chamber membranes were coated with chondroitinase ABC treated aggrecan as previously described in section 2.3.5.3.1, and isolated chondrocytes from both age groups of cartilage were allowed to adhere to the substrate on the upper side of the membrane for 24 hours. Basal media was added to the bottom of the well as stated in

section 2.3.5.3.2. Growth factors were supplemented to the media in the bottom of the well as described in section 2.3.7 after the initial attachment period, and BSA served as a control. Statistical analysis was performed as previously described in section 2.3.5.3.3.

2.4 Results and Discussion

2.4.1 Comparing Articular Cartilage Isolated From Young and Mature Bovine Metacarpophalangeal/Metatarsophalangeal Joints Using General Histology

2.4.1.1 Morphological Differences between Young and Mature Articular Cartilage

When comparing chondrocytes isolated from different age groups of articular cartilage, it is important to consider the environment that they have come from and note differences in their morphology and distribution within the tissue. Here haematoxylin and eosin staining has been used to stain both the nuclei of the chondrocytes and their surrounding ECM to look at their zonal distribution and the changes that occur during maturation of the tissue. Articular cartilage isolated from skeletally mature animals is notably thinner than that dissected off of a young, immature joint. This difference is neatly seen when comparing the gross morphology of the joints as shown in fig.2.1. Microscopically, the differences seen are much more detailed (see fig.2.6). The number of chondrocytes found within mature cartilage was found to be greatly reduced when compared to the young tissue, with a higher ECM volume: cell ratio, meaning that the cells are more isolated from each other within the mature cartilage. More extensive zonal organisation was also seen with maturation. Within the superficial zone of both age groups the chondrocytes can be seen to be flattened and disc-like, arranged parallel to the articular surface. Deeper into the cartilage, the young tissue demonstrates random organisation of the cells compared to the more columnar organisation seen in the mature cartilage.

2.4.1.2 Proteoglycan Distribution

The ECM component of articular cartilage is synthesised by the resident chondrocytes, and the noted changes in cell morphology and cellular distribution within the tissue is also accompanied by a change in the composition of the ECM. These changes ultimately affect the biomechanical as well as structural aspects of the tissue.

As fig.2.7 illustrates, Safranin O staining for proteoglycans can be found extending throughout the full depth of the tissue, within both the young (see fig.2.7a) and skeletally mature (see fig.2.7b) cartilage. These findings support those previously

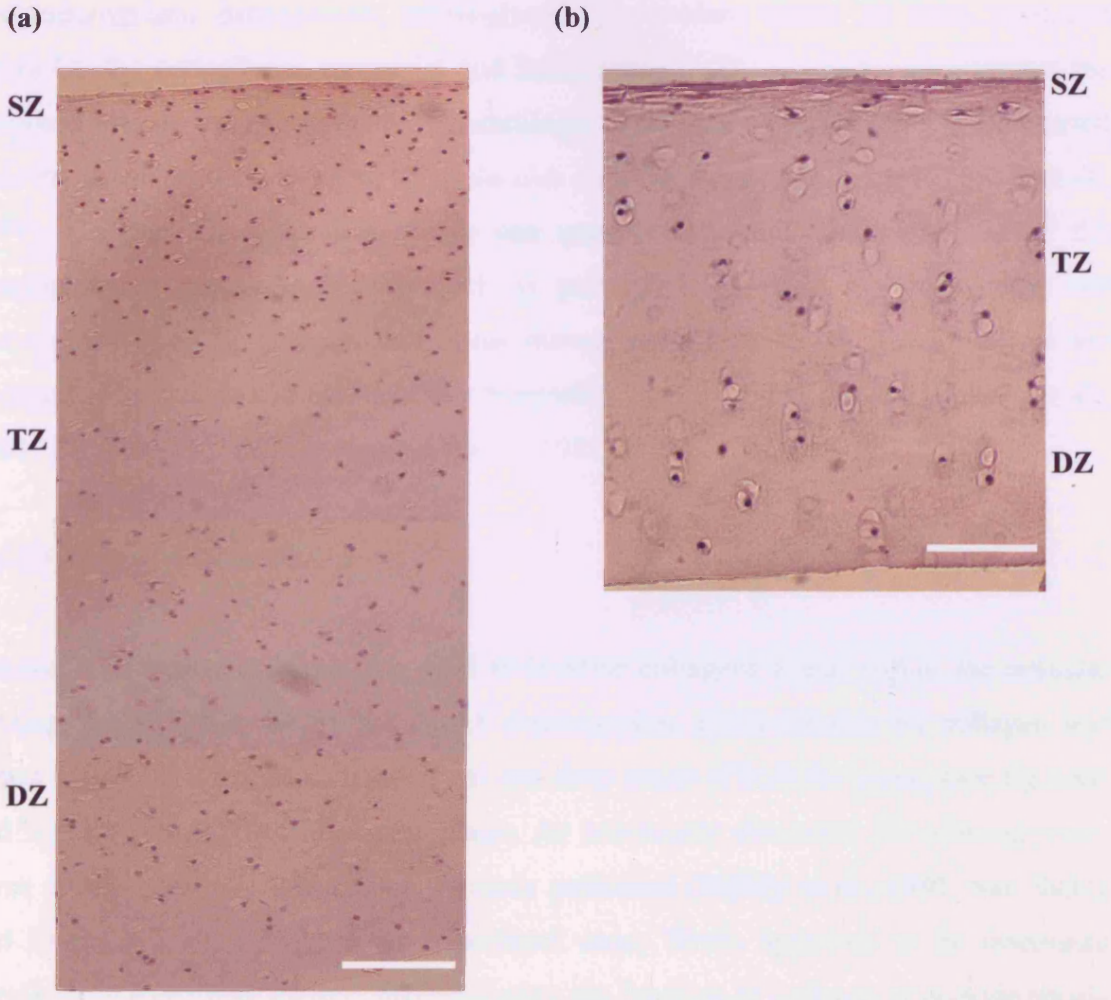


Fig.2.6 Haematoxylin and eosin staining of full depth (a) young (7 day old) and (b) skeletally mature (18 month old) bovine articular cartilage.
SZ = superficial zone, TZ = transitional zone and DZ = deep zone.
Bar = 50μm

documented (Buckwalter and Mankin, 1998; van Sickle and Evander, 1997). As these photomicrographs demonstrate, proteoglycans are evident within all three territorial zones i.e. the pericellular, territorial and interterritorial. These results complement the proposed role of proteoglycans within cartilage; to withstand compressive force exerted onto the tissue by entrapping water molecules and forming a hydrated gel (Guilak et al., 1999). General histological methods can only give a gross indication as to the distribution of matrix molecules such as proteoglycans. Such a method does not demonstrate specific changes that occur during maturation of the tissue such as the decrease in proteoglycan size and the increasing ratio of C-6-S:C-4-S (Hickery et al., 2003; Plaas et al., 1988; Thonar and Sweet, 1981).

2.4.1.3 Collagen Distribution

Masson's trichrome staining was used to localise collagens found within the articular cartilage ECM (Flint, 1975). As fig.2.8 demonstrates, green staining for collagen was found within the superficial, transitional and deep zones of both the young (see fig.2.8a) and mature (see 2.8b) articular cartilage. As previously discussed for proteoglycans, these data corroborate with that previously published (Jeffery et al., 1991; van Sickle and Evander, 1997). Within the superficial zone, fibrils appeared to be orientated parallel to the articular surface, demonstrating the function of collagen to provide tensile strength alongside the compressive resistance provided by the proteoglycans. The orientation of fibrils within the superficial zone also lends itself to provide a functional barrier to the transport of molecules from the synovial fluid into the cartilage (Huber et al., 2000). The percentage of collagen within the tissue is seen to increase with age, until maturation is reached, at which time levels plateau out (Brama et al., 2000; Thonar and Sweet, 1981). The organisation and orientation of collagen within the ECM is also altered (Bland and Ashhurst, 1996; Luder, 1998). This information is well documented in papers but is an important consideration when comparing the activities of cells isolated from both age groups of cartilage.

2.4.2 Confirming the Generation of a Chondroitinase ABC Treated Aggrecan

One aim of this investigation was to investigate the influence CS side chains found projecting from the aggrecan core protein have on the migration of chondrocytes. To achieve this aim it was necessary to generate a substrate that could be

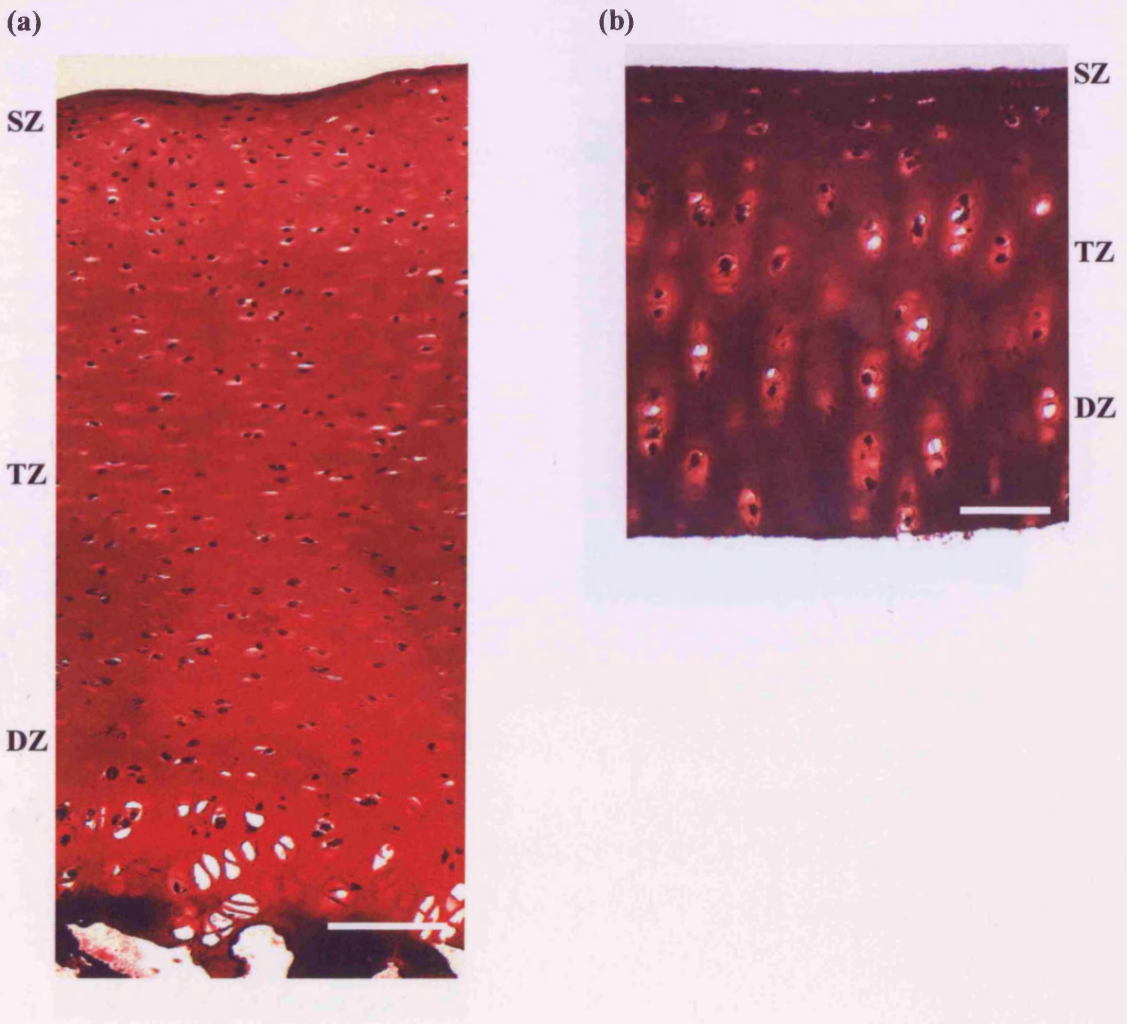


Fig.2.7 Safranin O staining, illustrating proteoglycan distribution within (a) young and (b) skeletally mature bovine articular cartilage.
SZ = superficial zone, TZ = transitional zone and DZ = deep zone.
Bar = 50μm

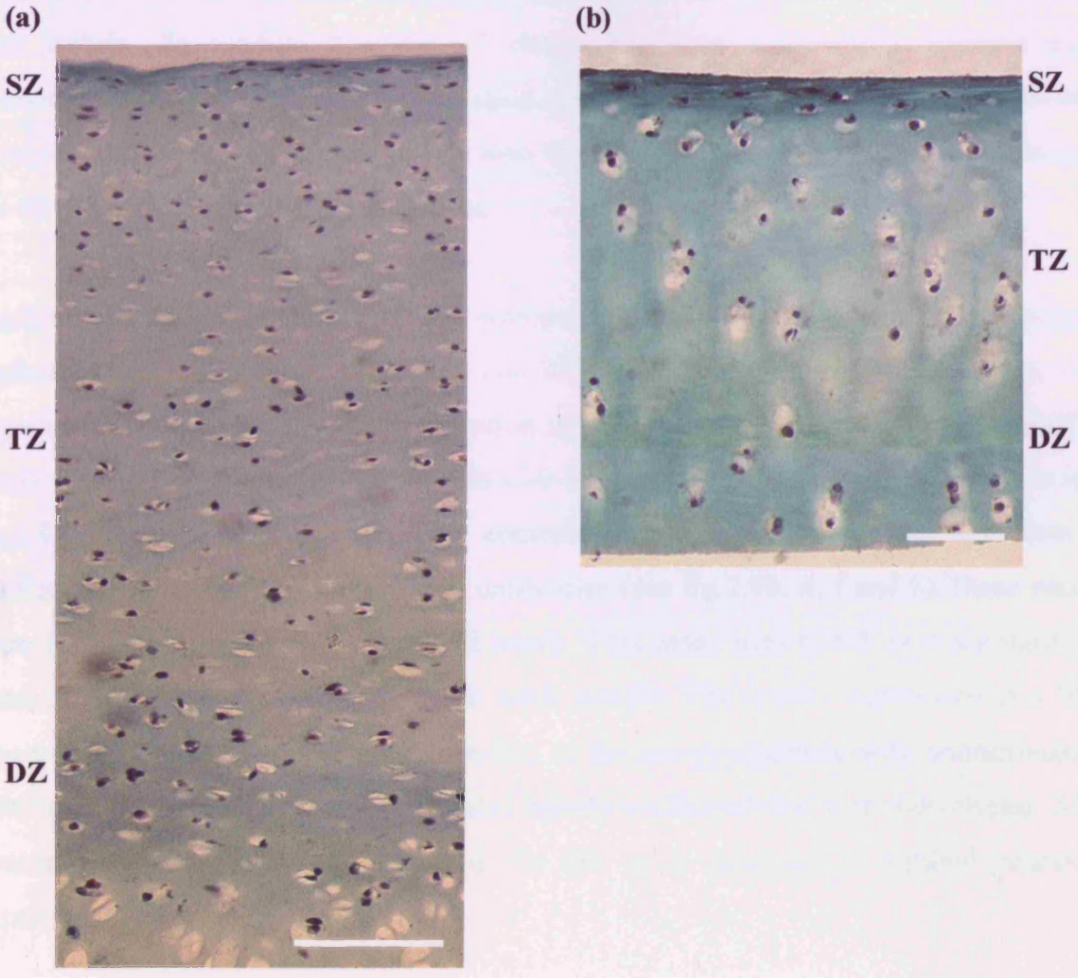


Fig.2.8 Masson's trichrome staining, illustrating collagen distribution within (a) young and (b) skeletally mature bovine articular cartilage.
SZ = superficial zone, TZ = transitional zone and DZ = deep zone.
Bar = 50μm

coated onto the PET membranes of the Boyden chamber system allowing the direct comparison of chondrocyte migration on native aggrecan (containing multiple GAG side-chains) and also aggrecan which had been treated with the enzyme chondroitinase ABC to remove the CS side-chains, leaving only CS-stubs attached to the remaining core protein. To confirm that the CS chains had been successfully digested using chondroitinase ABC, slot blotting was used to demonstrate the presence of CS-stub neo-epitopes generated after digestion and also the DMMB assay to illustrate a decrease in the sGAG concentration after treatment.

Fig.2.9a and c demonstrate that the epitopes recognised by the murine monoclonal antibodies 1B5 (raised against C-0-S) and 2B6 (C-4-S) are not present in native, non-digested aggrecan. However after digestion with chondroitinase ABC for a period of 24 hours distinct bands are visible for both C-0-S stubs (see fig.2.9e) and C-4-S stubs (see fig.2.9g). Primary antibody negative controls were run for both these slot blots to confirm the specificity of the primary antibodies (see fig.2.9b, d, f and h).These results were further confirmed by the DMMB assay. This assay uses C-6-S as a standard and estimates sGAG concentrations within each sample. The results demonstrated a 96% loss in sGAG concentration after digestion of the native aggrecan with chondroitinase ABC (see fig.2.10). Taken together these results confirmed that a chondroitinase ABC treated aggrecan had been generated for use as a substrate in further migration experiments.

2.4.3 Comparing the Migration of Chondrocytes Isolated from Young and Mature Articular Cartilage on Different ECM Macromolecules

Quantitative assessment of the migration of chondrocytes was obtained using a Boyden chamber system. See section 2.3.5.3 for full method. Briefly PET membranes were coated with purified ECM molecules, plasma fibronectin, type II collagen, whole A1D1 aggrecan or chondroitinase ABC treated aggrecan and chondrocytes were allowed to migrate on these substrates for periods of 1-24 hours. Chondrocytes, obtained from both 7 day old and 18 month old bovine metacarpophalangeal or metatarsophalangeal joints were seen to migrate on all four matrix molecules (see fig.2.11a and b). This is the first known report demonstrating the ability of chondrocytes isolated from skeletally mature bovine articular cartilage to migrate. No significant difference in the number of

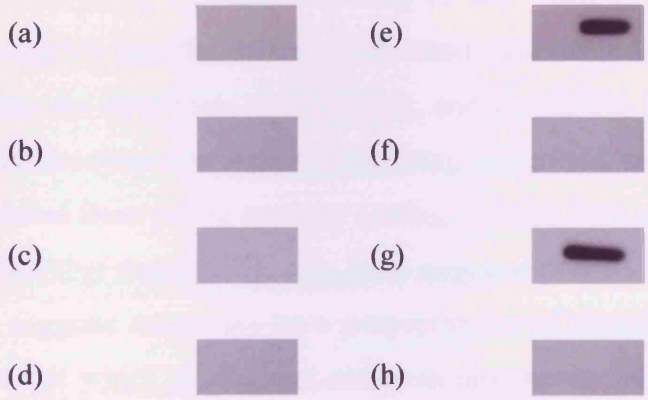


Fig.2.9 Slot blot analyses using 2B6 and 1B5 to confirm the presence of chondroitinase ABC generated neo-epitopes against C-4-S and C-0-S stubs respectively. (a) native and (e) chondroitinase ABC treated aggrecan probed with 1B5, (b) native and (f) chondroitinase ABC treated aggrecan 1B5 primary negatives, (c) native and (g) chondroitinase ABC treated aggrecan probed with 2B6, (d) native and (h) chondroitinase ABC treated aggrecan 2B6 primary negatives.

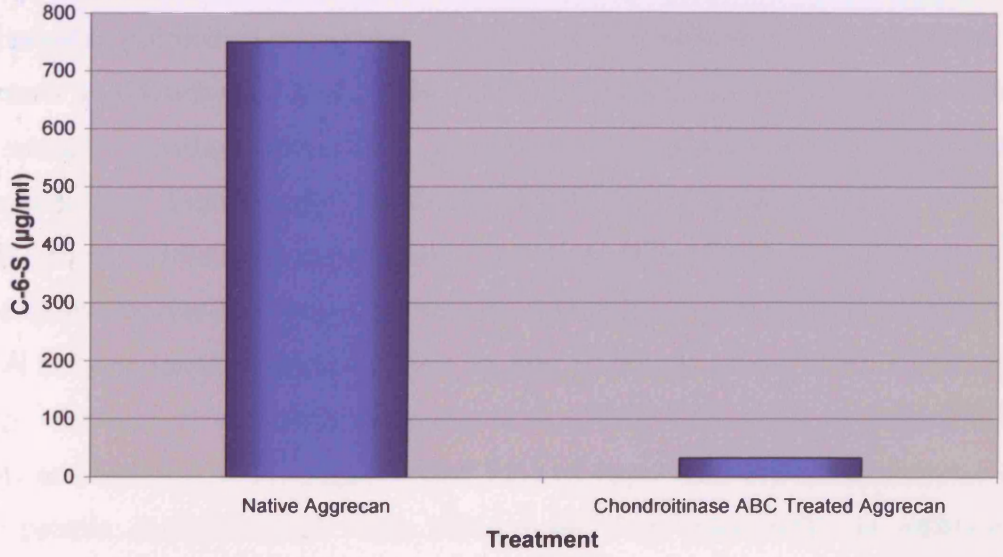


Fig.2.10 Graph illustrating the results of the DMMB assay illustrating significant loss of sGAGs after treatment of native aggrecan with chondroitinase ABC.

chondrocytes isolated from young cartilage migrating on any substrate between time points was seen. This suggests that the chondrocytes which had the ability to migrate, appeared to do so within the first hour of incubation, and no further increase in the number of migrating chondrocytes was seen by incubating them for a further 23 hours. Of the chondrocytes isolated from young articular cartilage, which were seeded onto the upper most side of the chamber membranes, only 0.9% migrated to the lower side of the membrane. This result suggests there may be a subpopulation of chondrocytes within full depth articular cartilage which possess the ability to migrate on matrix substrates. Interestingly, a similar trend was seen with the chondrocytes isolated from the mature articular cartilage (see fig.2.11b). Comparable numbers of chondrocytes isolated from mature cartilage were migrating to the young, and as previously discussed, no differences between the number of chondrocytes migrating between time points was shown either. As with the chondrocytes isolated from young cartilage, 0.9% of the chondrocytes isolated from mature cartilage were migrating from the upper to the lower side of the membrane. This suggests that the subpopulation of chondrocytes previously discussed is maintained throughout development and maturation of the cartilage. Recent research by (Dowthwaite et al., 2004) has demonstrated that within the superficial zone of articular cartilage there is a progenitor cell population, with stem cell-like characteristics. Interestingly, an immunohistochemical study by Kim and Spector (2000), demonstrated the expression of the contractile protein, α smooth muscle actin (SMA), in articular chondrocytes. Previous studies have demonstrated the expression of SMA in bone marrow stromal MSCs as well (Bonanno et al., 1994; Charbord et al., 1985; Charbord et al., 1990; Galmiche et al., 1993). The study by Kim and Spector (Kim and Spector, 2000) suggests that 75% of superficial zone chondrocytes express this protein compared with only 10% of the deep zone cells. In addition, these chondrocytes were still producing the chondrocytic marker, type II collagen (Kinner and Spector, 2001). The role of SMA within chondrocytes is not known, although may be a mechanism through which these cells are able to remodel their ECM *in vivo*. It would be interesting to investigate whether the chondrocytes migrating within this model are in fact these chondroprogenitors and also express SMA. In a canine model of articular cartilage repair it was demonstrated that many of the reparative cells did in fact express SMA, with a higher percentage of SMA positive cells within the cartilage/fibrocartilage repair tissue compared to the fibrous component (Wang et al., 2000). These results suggest a possible role for SMA positive chondrocytes in cartilage repair.

No significant differences in the number of chondrocytes isolated from either age group of cartilage were seen migrating between fibronectin and type II collagen substrates (see fig.2.11a and b). A consistent trend of increased migration, for both age groups, was seen on the chondroitinase ABC treated aggrecan when compared to the native monomeric aggrecan substrate. In the case of the young chondrocytes, this was a significant difference at 24 hours [$P<0.05$]. As previously discussed, in section 1.13.2, proteoglycans have been shown to be anti-adhesive in a number of cell types including fibroblasts (Winnemoller et al., 1991). Specifically this is thought to be due to the presence of their GAG chains (Culp et al., 1979; Lewandowska et al., 1987; Merle et al., 1997; Winnemoller et al., 1992; Yamagata et al., 1989). The GAG chain component of the proteoglycan has also been shown directly to have an anti-migratory effect in fibroblasts (Merle et al., 1997). The data from this study supports these findings; however, this is the first time these interactions have been discussed with reference to chondrocytes. The nature of this anti-adhesive/anti-migratory interaction is unknown. The method by which chondrocytes interact with proteoglycans remains questionable. It is not thought to occur via integrin receptors found on the chondrocyte cell surface. Interaction studies have confirmed that the GAG, CS can bind directly to CD44, L-selectins, P-selectins, galectins and annexin 6, another member of the lectin family (Fujimoto et al., 2001; Kawashima et al., 2000; Moiseeva et al., 2003; Takagi et al., 2002). Previous studies have demonstrated the presence of CD44 and galectin 3 on the surface of chondrocytes, however, it is not known whether annexin 6 is present (Guevremont et al., 2004; Hua et al., 1993). In the case of chondrocytes it has been previously believed that CS chains inhibit chondrocyte migration through blocking the binding domain of fibronectin, which is pro-adhesive, or by competing for cell interactions with other matrix components such as collagen fibril surfaces. This study indicates that this inhibitory effect is still apparent even when no other matrix components are present and therefore indicates a direct interaction of the cell with the CS chain in chondrocytes. No direct interactions between chondrocytes and CS have been studied to date. Aside from this, a study by Cao et al. (1998), identified the G1 domain of aggrecan as the domain inhibiting chondrocyte attachment. Fluorescence-activated cell sorter (FACS) analysis by this group suggests this effect is due to high affinity binding of the G1 domain to the cell surface of chondrocytes. Interestingly it is known that *in vivo* the G1 domain of aggrecan accumulates with age in articular cartilage, as degradation of this domain decreases while synthesis of aggrecan is maintained. The accumulation of this anti-adhesive domain may be a contributory factor

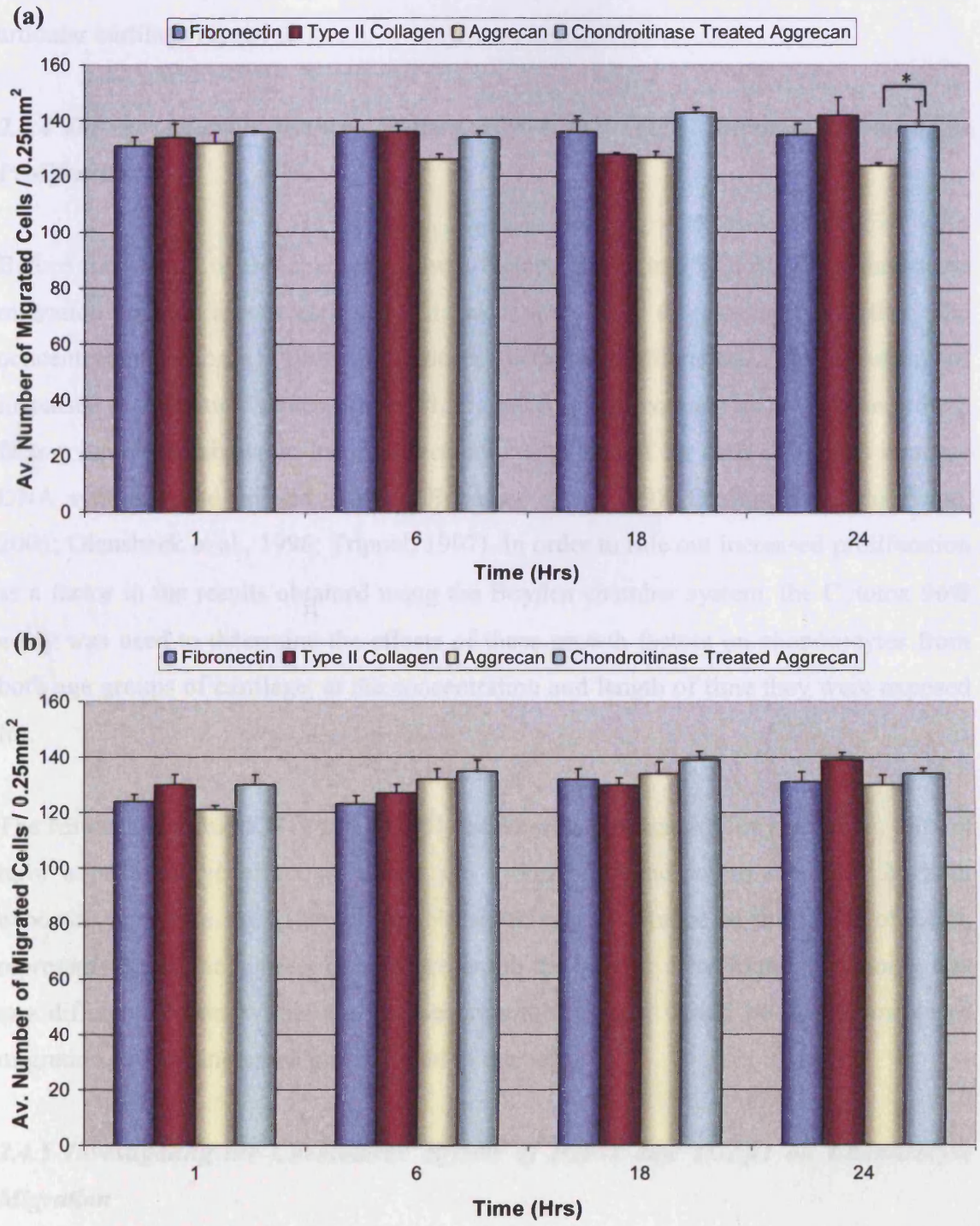


Fig. 2.11 The migration of chondrocytes using a Boyden chamber system isolated from (a) young and (b) mature articular cartilage on fibronectin, type II collagen, native aggrecan and chondroitinase ABC treated aggrecan over periods of 1-24 hours. Results expressed as the average number of migrated cells with SEM bars.
*$P<0.05$

in the lack of transplanted cell adhesion and integration seen with cell-based methods of articular cartilage repair.

2.4.4 Do the Anabolic Growth Factors, IGF-1 and TGFβ1 Increase Chondrocyte Proliferation?

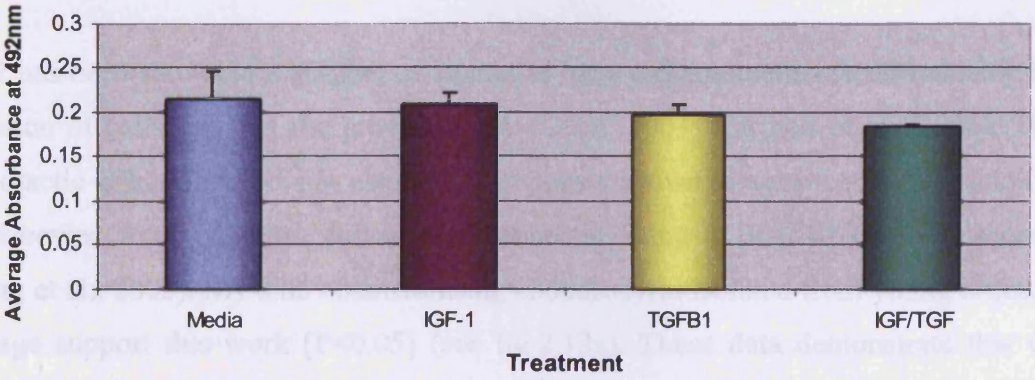
Before the effects of the anabolic growth factors, IGF-1 and TGFβ1, on chondrocyte migration could be investigated, it was important to ascertain whether the concentrations chosen (10ng/ml) induced cellular proliferation. The question of increased proliferation versus increased migration was of concern as both these growth factors have been shown to increase metabolic activities of the cells as well as increase DNA synthesis and mitotic activity (Bonassar et al., 1997; Darling and Athanasiou, 2005; Glansbeek et al., 1998; Trippel, 1997). In order to rule out increased proliferation as a factor in the results obtained using the Boyden chamber system, the Cytotox 96® assay was used to determine the effects of these growth factors on chondrocytes from both age groups of cartilage, at the concentration and length of time they were exposed for.

The results show that IGF-1 and TGFβ1, whether used singularly or combined, did not have a proliferative effect on either age group of chondrocytes over the 24 hour exposure time (see fig.2.12a and b). No significant difference in the levels of LDH, representative of the number of cells present in the lysates, were found confirming that any differences seen within the Boyden chamber system would be due to increased migration and not increased proliferation of the cells.

2.4.5 Investigating the Chemotactic Effects of IGF-1 and TGFβ1 on Chondrocyte Migration

The chemotactic effects of the anabolic growth factors, IGF-1 and TGFβ1, were investigated using chondrocytes seeded onto type II collagen, fibronectin, A1D1 aggrecan and BSA as a control. In contrast to the method previously described in section 2.3.5.3.2, the chondrocytes were allowed to adhere to the substrate for 48 hours before the addition of growth factors to the bottom of the chamber. IGF-1 and TGFβ1 were allowed to act as chemoattractants for 24 hours before the migrated cells were counted. Differences in the initial adherence time of the chondrocytes to the substrate on

(a)



(b)

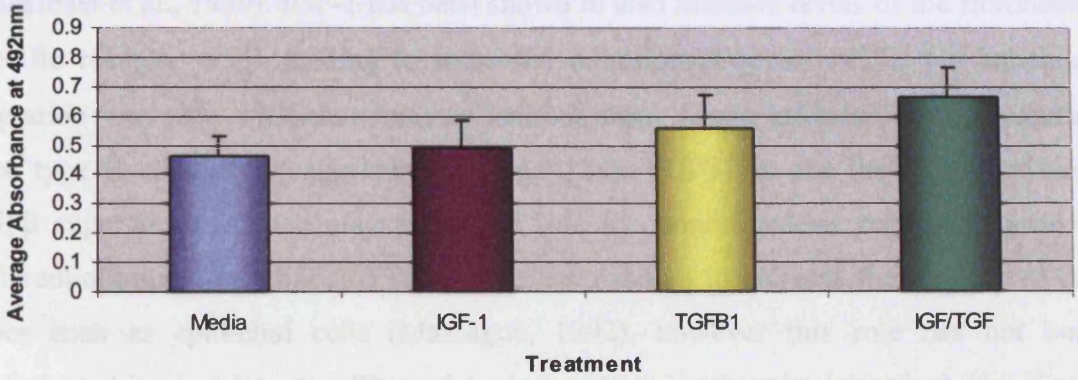


Fig.2.12 The effects of IGF-1 and TGFβ1 on the proliferation of chondrocytes isolated from (a) young and (b) mature articular cartilage as measured by the Cytotox®96 assay

the upper side of the membrane have been taken into account in the analysis of these data, but previously work discussed in section 2.4.3 demonstrated that the number of chondrocytes migrating does not appear to significantly alter after 1 hour of adhesion to the substrate surface.

IGF-1 has been shown in a number of studies to have a chemotactic effect, inducing the migration of cells towards the growth factor (Duan, 2003; Hartnell et al., 2004). This chemotactic effect of IGF-1 has also been previously shown to enhance the migration of chondrocytes from newborn calves on fibronectin using a Boyden chamber system (Chang et al., 2003). My data obtained using chondrocytes isolated from young articular cartilage support this work [$P<0.05$] (see fig.2.13a). These data demonstrate that the chemotactic effect is substrate dependent and requires the chondrocytes to be seeded onto fibronectin. As interactions between fibronectin and chondrocytes is β1 integrin dependent it is interesting to link research suggesting potential synergy between the IGF-1R and the β1 integrin subunit by activation of cell signalling proteins such as Shc (Shakibaei et al., 1999). IGF-1 has been shown to also increase levels of the fibronectin specific receptor α5β1 leading to increased adhesion (Loeser, 1997). No enhanced migration was seen with chondrocytes isolated from young articular cartilage seeded onto type II collagen or aggrecan (see fig.2.13a). TGFβ1 is one member of a large TGFβ superfamily, which plays a crucial role in chondrogenesis regulating growth, differentiation and metabolism. TGFβ1 has been shown to increase the motility of cell types such as epithelial cells (Massague, 1992), however this role has not been investigated in chondrocytes. These data demonstrate a substrate dependent, significant increase in the migration of chondrocytes isolated from young articular cartilage in response to TGFβ1 when coated onto fibronectin [$P<0.005$] (see fig.2.13a). As previously stated for IGF-1, TGFβ1 has also been shown to increase α5β1 expression and in doing so increase adhesion to fibronectin (Loeser, 1997). Loeser's study also illustrated that TGFβ1 can increase adhesion of chondrocytes to type II collagen. In addition, type II collagen has been demonstrated to modulate the effect of TGFβ1 on chondrocytes. Type II collagen has been shown to down-regulate α2 integrin expression in chondrocytes (Lee et al., 2002). It is the binding of chondrocytes to type II collagen that modulates TGFβ1 activity. Increased level of type II collagen gene expression parallels Smad phosphorylation and leads to TGFβ1 activation (Schneiderbauer et al., 2004).

A further experiment was carried out to look at the possible interaction of these two growth factors in potentiating any response that they may have on their own. Although there are no data in the literature to support interactions of these growth factors in stimulating migration of chondrocytes, there is evidence that these growth factors will interact in articular cartilage. Studies have demonstrated that combinations of IGF-1 and TGFβ1 stimulate the proliferation and differentiation of chondrocytes (Hill et al., 1992; Rosselot et al., 1994; Tsukazaki et al., 1994; Yaeger et al., 1997) and that TGFβ1 can increase the expression of IGF-1Rs by chondrocytes (Tsukazaki et al., 1994). A recent study demonstrated that this interaction occurs *in situ* during periosteal chondrogenesis (Fukumoto et al., 2003). The interaction of these growth factors was confirmed by the significant increase in the migration of chondrocytes from young cartilage seeded onto fibronectin when IGF-1 and TGFβ1 were used in combination compared to IGF-1 alone [$P<0.05$] (see fig.2.13a). As discussed in section 1.15.8, it is well documented that chondrocytes demonstrate decreased sensitivity to growth factors with age (Dore et al., 1994; Guerne et al., 1994; Middleton et al., 1996; Tavera et al., 1996). In support of the literature no significant increase in the migration of chondrocytes isolated from mature articular cartilage was seen in response to either growth factor (see fig.2.13b). A significant increase in the migration of chondrocytes from mature cartilage was seen when IGF-1 and TGFβ1 were used in combination and the cells were seeded onto fibronectin [$P<0.05$] (see fig.2.13b).

Photomicrographs taken of the migrated cells in all the experiments using the Boyden chamber system demonstrate that in both age groups the chondrocytic morphology has been maintained and that it is unlikely that migration was due to dedifferentiation of the cells (see fig.2.14).

2.4.6 Can the Presence of IGF-1 and TGFβ1 Increase Chondrocyte Migration on Chondroitinase ABC Treated Aggrecan?

IGF-1 and TGFβ1 were added to the bottom of Boyden chambers, as described in section 2.3.7, after seeding of the chondrocytes onto a chondroitinase ABC treated aggrecan substrate 24 hours previous. Chondrocytes were allowed to migrate on the substrate, and the growth factors allowed to act as chemoattractants for 24 hours after seeding of the cells.

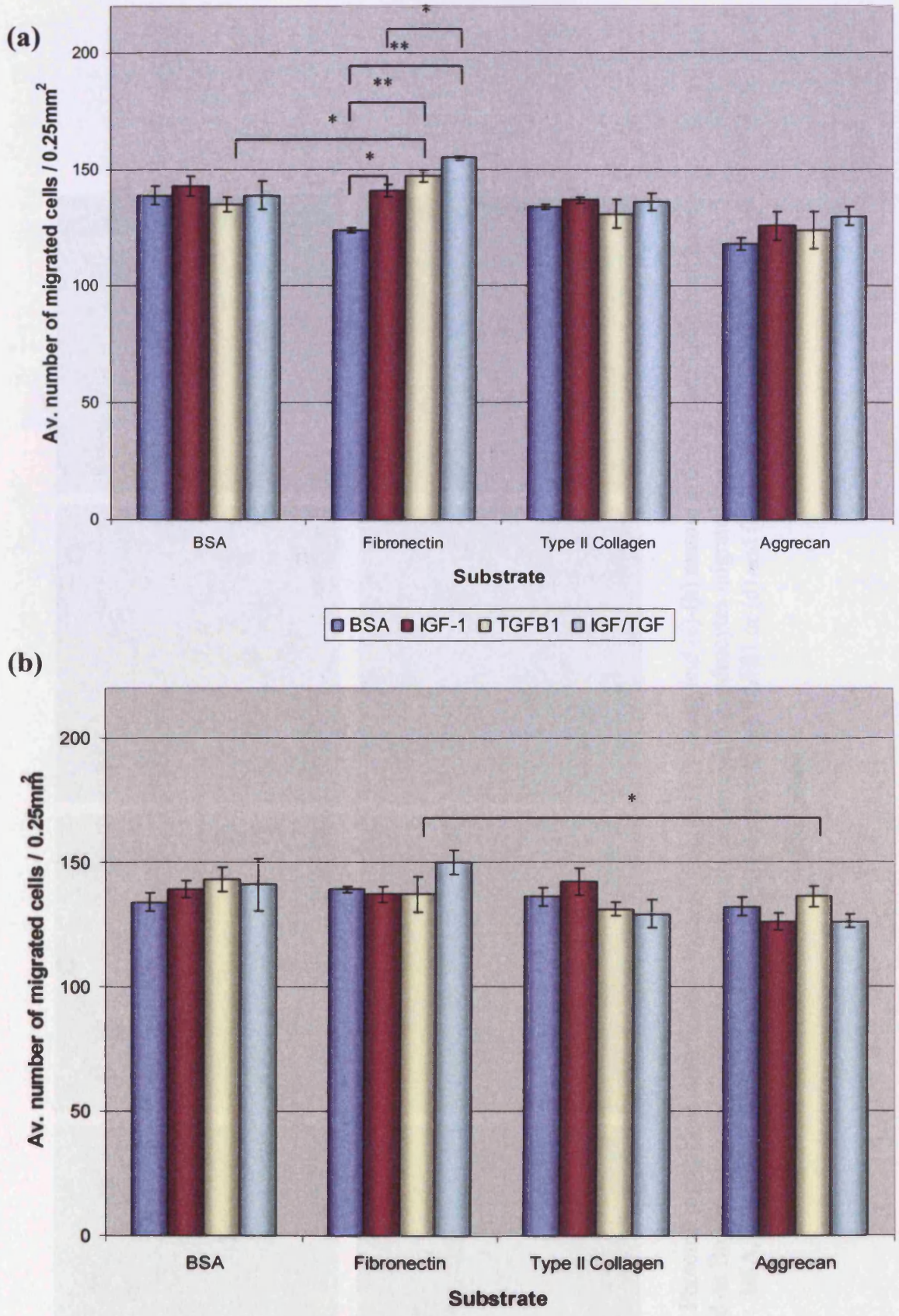


Fig.2.13 Comparing the migration of chondrocytes isolated from (a) young and (b) skeletally mature articular cartilage on ECM molecules with IGF-1 and TGFβ1 as chemoattractants. Results are expressed as the number of migrated cells within a 0.25mm^2 area. N=3

* P<0.05

** P<0.005

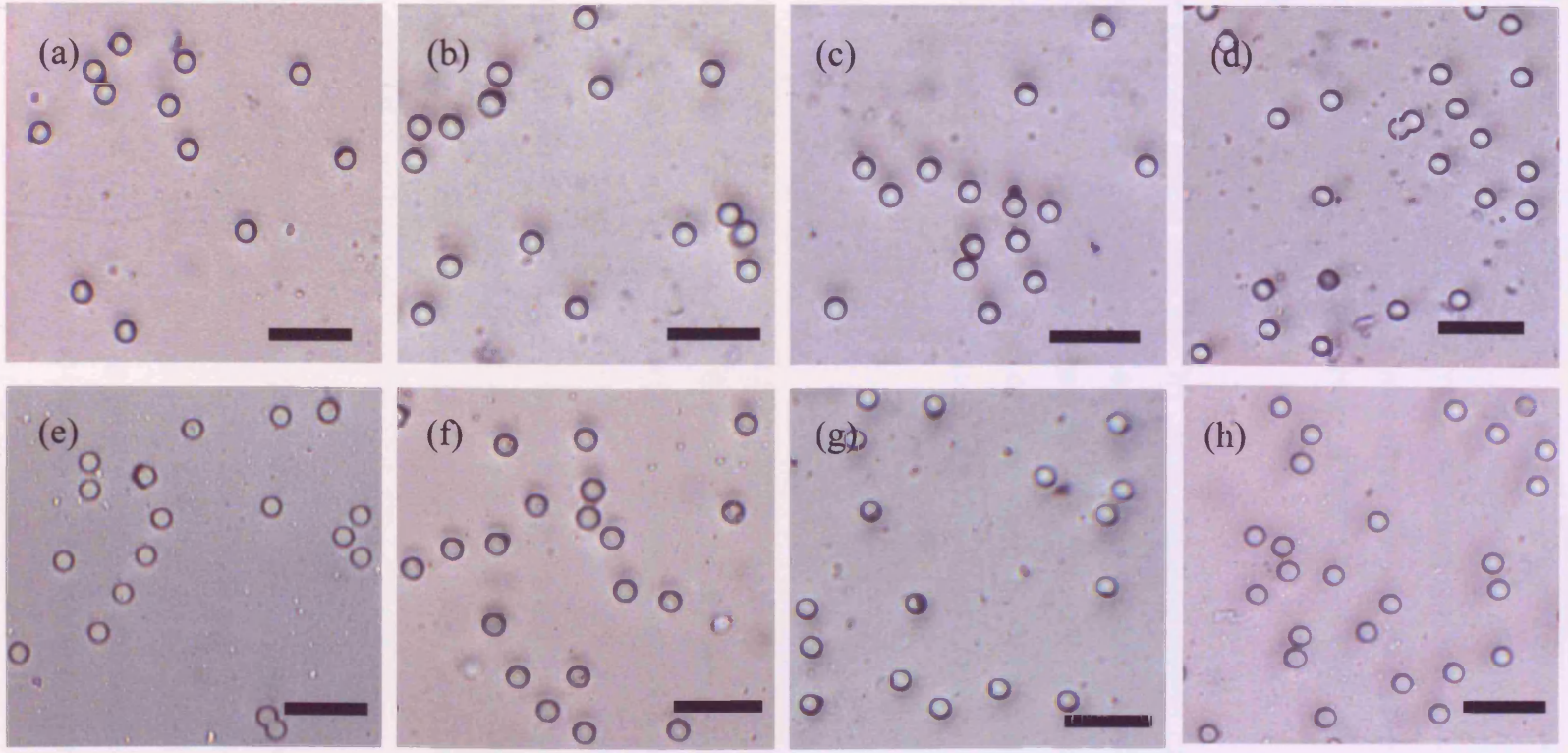


Fig.2.14 Photomicrographs of chondrocytes isolated from (a)-(d) young and (e)-(h) mature articular cartilage which have migrated on Boyden chamber membranes coated with fibronectin. Chondrocytes migrated in the presence of (a) and (e) BSA, or the chemoattractants (b) and (f) IGF-1, (c) and (g) TGFβ1 or (d) and (h) IGF-1 and TGFβ1.

Bar=50μm

Data discussed in section 2.4.3 has demonstrated the inhibitory effect of native aggrecan on chondrocyte migration and the beneficial effects seen on removal of CS chains from the core protein. This study investigates whether the addition of growth factors as chemoattractants can further enhance the migration of chondrocytes on chondroitinase ABC treated aggrecan. Neither IGF-1 nor TGFβ1 had a stimulatory effect on chondrocyte migration of cells extracted from young or skeletally mature articular cartilage (see fig.2.15a and b). This confirms the substrate dependent effects of these growth factors on enhancing chondrocyte migration through a chemotactic mechanism, and poses the question, 'what role does the substrate on which the chondrocyte is migrating play in eliciting the effect of IGF-1 and TGFβ1 in potentiating chondrocyte migration?'

No significant effects were seen by combining these growth factors on the migration of young or mature chondrocytes on chondroitinase ABC treated native aggrecan (fig. 2.15a and b), confirming those results discussed in section 2.4.5 and further demonstrating the importance of fibronectin in enhancing chondrocyte migration through the chemotactic mechanisms of IGF-1 and TGFβ1.

2.5 Conclusions

The aim of the work described in this chapter was to investigate the migratory capacities of chondrocytes freshly isolated from young and skeletally mature articular cartilage. In contrast to previous studies investigating the migration of chondrocytes, these cells were not previously expanded in monolayer culture prior to experimentation in order to preserve the chondrocytic phenotype. Using a Boyden chamber model of cell migration, both chondrocytes isolated from young and mature articular cartilage were shown to be able to migrate when removed from their constraining ECM. In addition, the numbers of migrating cells were comparable between the 2 age groups.

The matrix macromolecule that the cells are attached to proved to be crucial in both their migratory capacity and also in their response to supplemented growth factors. No differential effects were seen in the number of migrating cells between a fibronectin and type II collagen coated substrate; however when comparing native and chondroitinase ABC treated aggrecan a constant trend of increased migration was seen on the latter

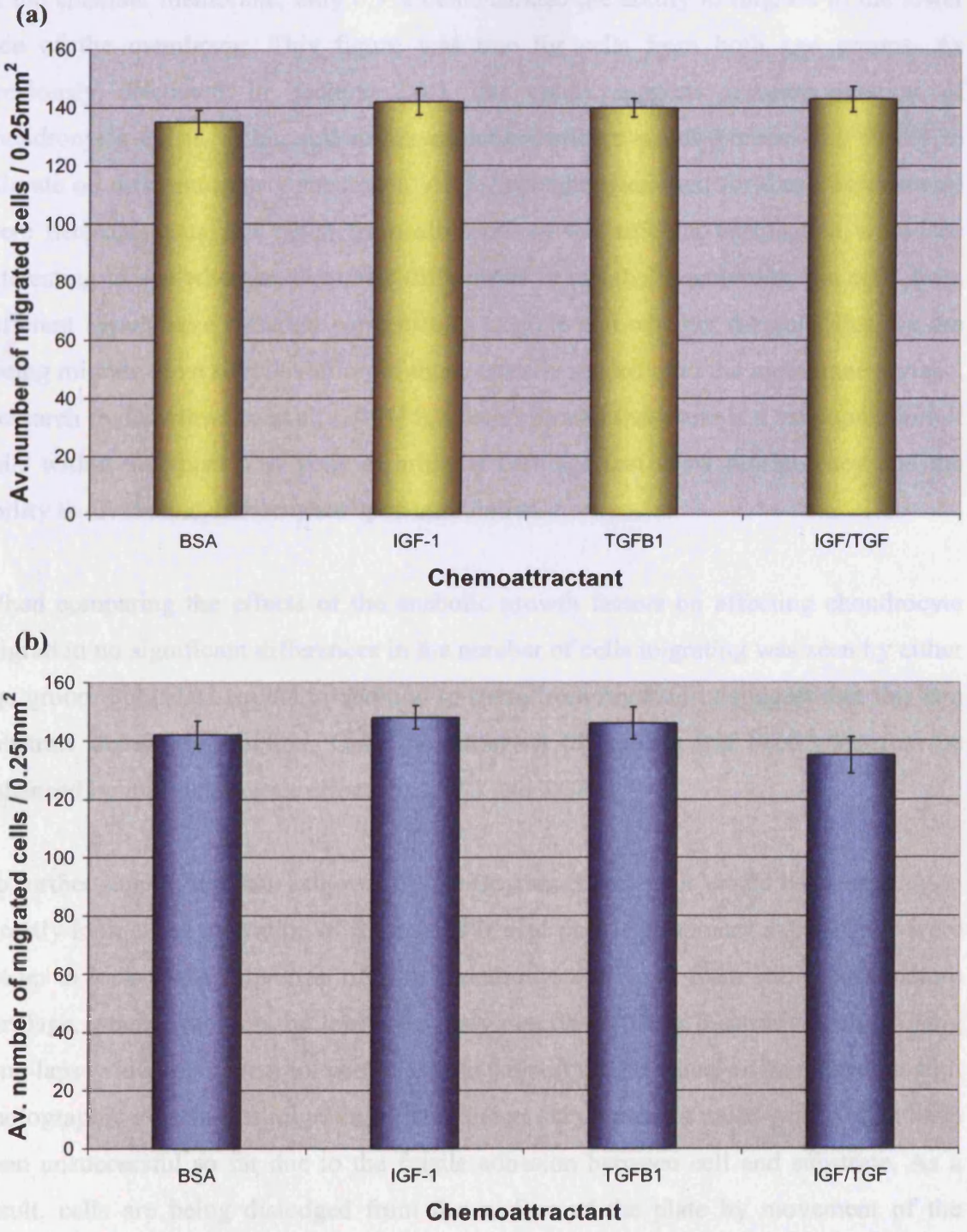


Fig. 2.15 Measuring the chemotactic effects of IGF-1 and TGFβ1 on the migration of chondrocytes isolated from (a) young and (b) mature cartilage, seeded onto chondroitinase ABC treated aggrecan, using a Boyden chamber system. Results are expressed as the average number of migrated cells/0.25mm^2 visual field with SEM bars.

substrate. A significant difference was seen at 24 hours with the chondrocytes isolated from young articular cartilage. Of those chondrocytes seeded onto the upper-most side of the chamber membrane, only 0.9% demonstrated the ability to migrate to the lower side of the membrane. This figure was true for cells from both age groups. As previously discussed in section 2.4.3 this result suggests a subpopulation of chondrocytes exists within full depth articular cartilage which possess the ability to migrate on different matrix substrates. As the chondrocytes used for these experiments were heterogeneous and taken from all zones of the articular cartilage it would be interesting to see whether, like their differences in metabolic activities, the cells from different layers have different capacities to migrate and whether the cells that we are seeing migrate are a subpopulation of those actually seeded onto the membrane surface. Research by Dowthwaite et al. (2004) has demonstrated that there is a subpopulation of cells within the superficial zone of articular cartilage that show pluripotency and the ability to divide and differentiate upon stimulation.

When comparing the effects of the anabolic growth factors on affecting chondrocyte migration no significant differences in the number of cells migrating was seen by either age group. Published results in addition to those from my studies suggest that this is a substrate dependent reaction. Only migration on fibronectin has been shown to be enhanced by the chemotactic effects of IGF-1 and TGFβ1.

To further support the data achieved by the Boyden chambers it would be interesting to directly look at the migration of these cells in real time. Preliminary experiments were set up to look at the migration of both chondrocytes isolated from young and mature cartilage, when coated on the four previously described matrix macromolecules. Using time-lapse video-microscopy I was hoping to support the previously discussed data with photographic evidence of migration. Experiments set up using a multi-well system have been unsuccessful so far due to the fragile adhesion between cell and substrate. As a result, cells are being dislodged from the surface of the plate by movement of the microscope stage. Whilst it is important to allow an appropriate amount of time for the chondrocytes to adhere to the plate surface, it is also important to remember that these cells will dedifferentiate if left coated on a surface for prolonged periods. It has, to date, not proved possible to establish the necessary conditions in order to obtain data for this experiment; however the data would be extremely beneficial to confirming the findings using the Boyden chamber system for measuring cell migration.

In summary:

- Chondrocytes isolated from young and skeletally mature bovine articular cartilage have the ability to migrate.
- CS chains play an inhibitory role in the migration of chondrocytes.
- Only 0.9% of chondrocytes, isolated from both age groups of cartilage, demonstrated the ability to migrate, suggesting a subpopulation of chondrocytes which have the ability to migrate and that are maintained through development.
- IGF-1 and TGFβ1 demonstrated a substrate dependent chemotactic effect on chondrocyte migration, with enhanced migration only seen on cells seeded onto fibronectin.
- IGF-1 and TGFβ1 did not exert a chemotactic response on chondrocytes migrating on chondroitinase ABC treated aggrecan.

Chapter 3

Enhancing the Integration of Transplanted Chondrocytes in an *in vitro* Model of Cartilage Injury

3.1 Background

Many studies using animal models of cartilage injury and repair have demonstrated the ability to stimulate filling of the defect with repair tissue, hyaline or fibrocartilaginous. This repair tissue invariably degenerates with time due to poor integration with the endogenous cartilage (Cook et al., 2003; Han et al., 2003; Shao et al., 2006). The successful repair of articular cartilage is not solely dependent on the ability of chondrocytes to migrate to a site of injury, or in the case of cell-based surgical repair strategies, to adhere to the injured cartilage and fill the defect with new tissue. The answer to effective articular cartilage repair may not lie in the composition of the repair tissue but whether it is stably integrated with the pre-existing cartilage. Integration is crucial for long-term biomechanical stability of the cartilage during loading and the associated shearing forces within the synovial joint.

The Boyden chamber system described in chapter 2 demonstrated that both chondrocytes isolated from young and skeletally mature articular cartilage do have the ability to migrate, but that this process is dependent on the matrix molecule onto which they are seeded (see section 2.4.3). As discussed in section 1.13.2 the ability of native aggrecan, as well as the SLRPs decorin and biglycan, to inhibit cell adhesion (Merle et al., 1997; Winnemoller et al., 1991; Winnemoller et al., 1992) and migration (Merle et al., 1999) is well characterised and is hypothesised to be through their GAG chains.

Anti-adhesive properties of aggrecan have also been attributed to its G1 domain. Within ageing, injured or diseased cartilage there is an accumulation of G1 aggrecan as turnover of whole aggrecan increases, leaving just the HA-bound domain within the cartilage (Lohmander et al., 1993; Poole et al., 1994; Sandy et al., 1991; Saxne and Heinegard, 1992). G1 aggrecan has the ability to accumulate around the chondrocytes, within the matrix and be released into the synovial fluid (Lark et al., 1997). Experiments using chicken chondrocytes transfected with a G1 domain construct demonstrated that not only did this domain inhibit cell adhesion, but also induced apoptosis as a result of this lack of attachment (Cao and Yang, 1999).

Taking these factors into account, and knowing that injured cartilage demonstrates an initial phase of increased proteoglycan synthesis close to the wound surface (Jeffrey et al., 1997;

Walker et al., 2000), in theory it would be advantageous to remove the wound surface proteoglycans to enhance the migration of transplanted chondrocytes into the injured tissue. Removal of surface proteoglycans would not only increase adhesion, and therefore retention of the transplanted cells within the defect, but also expose underlying pro-adhesive matrix proteins such as collagen fibrils and fibronectin for the chondrocytes to adhere and migrate on. Numerous groups have investigated the effects of enzymatically removing wound surface proteoglycans in enhancing coverage and adhesion of transplanted chondrocytes (Hunziker and Kapfinger, 1998; Hunziker and Rosenberg, 1996; Lee et al., 2000), but none have investigated whether these adhered chondrocytes are migrating into the digested tissue. Matrix macromolecules have been digested using different enzymes such as hyaluronidase as well as more aggressive methods such as guanidine hydrochloride and trypsinisation, which appear to be less specific (Bos et al., 2002; Quinn and Hunziker, 2002; van de Breevaart Bravenboer et al., 2004) Interestingly some of the best results have been seen using the enzyme chondroitinase ABC, which directly targets the GAG chains of the proteoglycan molecules (Lee et al., 2000).

Chondroitinase ABC is an endolytic GAG lyase derived from the bacterium *Proteus vulgaris*. The enzyme demonstrates broad specificity, with the ability to endolytically degrade CS, DS and HA (although at a much slower rate) by the elimination of the 1,4 hexosaminidic bond to unsaturated disaccharides and tetrasaccharides (Huang et al., 2003). The optimal temperature for enzyme activity is 37°C (Yamagata et al., 1968) and the pH will depend on the substrate to be digested. The optimum pH for the digestion of CS is pH 8 whereas for HA it is 6.8. The crystal structure of chondroitinase ABC has demonstrated 3 domains arranged in a linear fashion (see fig.3.1). The N-terminal domain has a similar organisation to the carbohydrate binding domains of xylanases, and therefore has been hypothesised to help in binding and presenting the GAG chain to the horseshoe-shaped, central catalytic domain. Both the middle and C-terminal domains show similarity to the domains of chondroitinase AC and bacterial hyaluronidases. Loops extending from the C-terminal into the catalytic domain are likely to be involved in substrate binding (Huang et al., 2003).

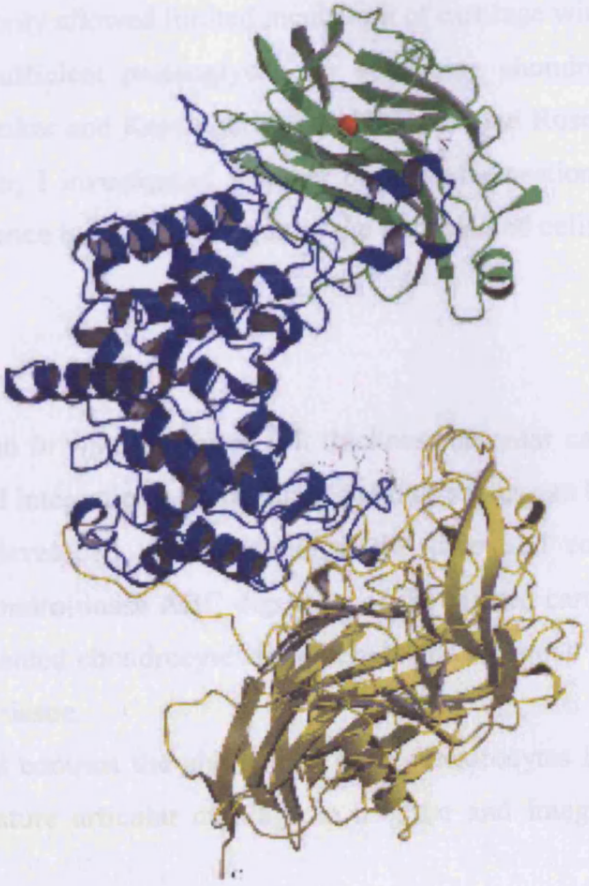


Fig.3.1 Ribbon drawing illustrating the 3 structural domains of chondroitinase ABC. N-terminal domain (green), middle, catalytic domain (blue), C-terminal domain (yellow) (reproduced from Huang et al., 2003)

Enzymatic digestion using a specific enzyme such as chondroitinase ABC provides directed and specific GAG degradation without damage to the actual architecture of the cartilage. Studies to date have only allowed limited incubation of cartilage with chondroitinase ABC in order to digest sufficient proteoglycan for enhancing chondrocyte adherence to the wound surface (Hunziker and Kapfinger, 1998; Hunziker and Rosenberg, 1996; Lee et al., 2000). In this chapter, I investigated whether prolonged digestion with this enzyme will allow not only adherence but also migration of the transplanted cells.

Aims:

1. To develop an *in vitro* model of full thickness articular cartilage injury where the migration and integration of transplanted chondrocytes can be monitored with time.
2. Using this developed model, optimise the time and concentration needed for sufficient chondroitinase ABC digestion of the injured cartilage surface in order to allow transplanted chondrocytes to adhere to the wounded surface and migrate into the damaged tissue.
3. Compare and contrast the abilities of both chondrocytes isolated from young and skeletally mature articular cartilage to migrate and integrate within the digested tissue.

3.2 Materials

All materials were obtained from Sigma Chemical Company, UK unless otherwise stated within the protocol.

3.3 Methods

3.3.1 Optimisation of Chondroitinase ABC Treatment for Proteoglycan Digestion of Young Bovine Cartilage Explants

Aims:

1. Digestion of GAG chains deep enough into all zones of the cartilage to promote sufficient migration of transplanted chondrocytes for the integration of repair and endogenous tissue.
2. Optimise the degree of digestion for the enhancement of integration but maintain the degree of digestion at a level that the proteoglycans can be replaced by the endogenous and transplanted chondrocytes.

Full thickness articular cartilage explants were excised from the medial and lateral condyles of the metacarpophalangeal or metatarsophalangeal joints of 7 day old calves. The explants were injured using a sharp number 11 scalpel blade and embedded in 2% agarose (Flowgen, UK) dissolved in PBS (pH 7.4) and previously equilibrated with media. The explant was orientated such that only the wounded surface was left exposed with superficial through to deep zones of the cartilage present (see fig.3.2). The agarose was allowed to set before the addition of the chondroitinase ABC solution.

Lyophilised chondroitinase ABC was reconstituted in PBS (pH 7.4) at a concentration of either 1U/ml or 10U/ml. The solution (250µl) was added to the exposed wound surface and incubated for a period of either 1, 4, 8 or 24 hours at 37°C. (Previous experiments using chondroitinase ABC at 1U/ml for 5, 15 and 30 minutes showed very limited digestion of CS chains). Following incubation, the solution was removed and the explant washed 3 times in PBS (pH 7.4) for periods of 5 minutes.

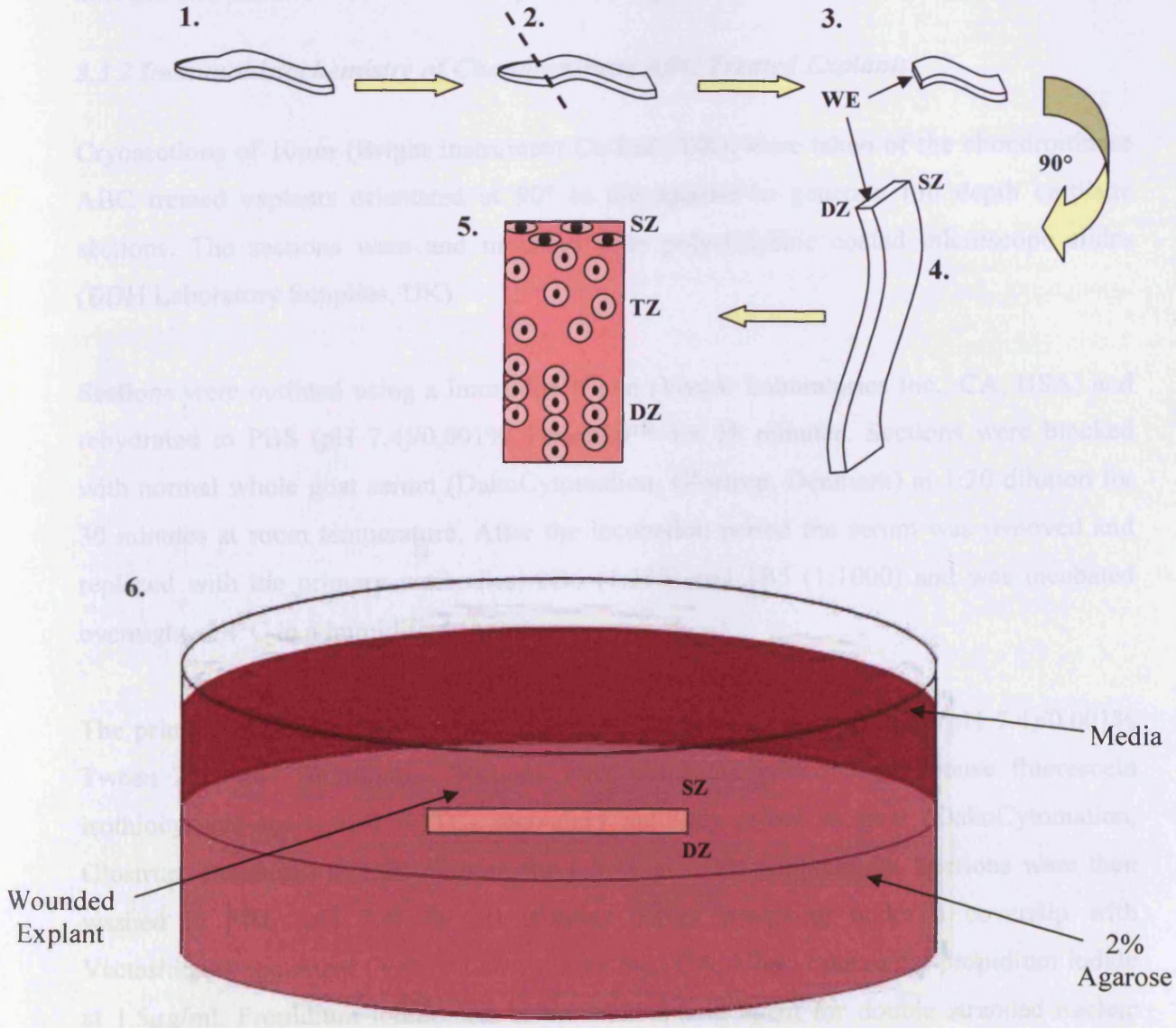


Fig. 3.1 Schematic representation of the cartilage explant model for measuring chondrocyte migration and integration. **1.** The explant after excision from the bovine metacarpalphalangeal/metatarsophalangeal joint **2.** The explant was wounded using a sharp scalpel **3.** The wounded 5mm x 4mm full thickness explant **4.** The wounded cartilage was rotated 90° so that the all zones were left exposed to the chondroitinase ABC treatment **5.** The wound edge (WE), demonstrating wounding through all zones of the cartilage **6.** The explant embedded in agarose with only the wound surface exposed.

All explants were washed three times in PBS (pH 7.4), immediately snap frozen in liquid nitrogen and stored at –80°C before cryosectioning.

3.3.2 Immunohistochemistry of Chondroitinase ABC Treated Explants

Cryosections of 10μm (Bright Instrument Co Ltd., UK), were taken of the chondroitinase ABC treated explants orientated at 90° to the agarose to generate full depth cartilage sections. The sections were and mounted onto poly-L-lysine coated microscope slides (BDH Laboratory Supplies, UK).

Sections were outlined using a ImmEdge™ pen (Vector Laboratories Inc., CA, USA) and rehydrated in PBS (pH 7.4)/0.001% Tween20™ for 10 minutes. Sections were blocked with normal whole goat serum (DakoCytomation, Glostrup, Denmark) at 1:20 dilution for 30 minutes at room temperature. After the incubation period the serum was removed and replaced with the primary antibodies, 2B6 (1:100) and 1B5 (1:1000) and was incubated overnight at 4°C in a humidified chamber.

The primary antibodies were removed and the sections washed in PBS (pH 7.4)/0.001% Tween 20™ for 10 minutes. Sections were incubated with an anti-mouse fluorescein isothiocyanate-conjugated (FITC) secondary antibody raised in goat (DakoCytomation, Glostrup, Denmark) at 1:50 dilution for 1 hour at room temperature. Sections were then washed in PBS (pH 7.4) for 10 minutes before mounting under a coverslip with Vectashield® mountant (Vector Laboratories Inc., CA, USA) containing propidium iodide at 1.5μg/ml. Propidium iodide acts as an intercalating agent for double stranded nucleic acids and in this case is being used as a counterstain for cell nuclei (Jones and Kniss, 1987). The slides were sealed with clear nail varnish and stored at 4 °C in the dark.

Control slides were incubated in PBS (pH 7.4)/0.001% Tween 20™ instead of primary antibody were performed to confirm specific binding. Sections were viewed using an upright fluorescent microscope (Laborlux 12, Leitz DRMB, Leica, Germany) at x 40 magnification.

3.3.3 Visualising Cell Death at the Wound Edge as a Measure of the Cytotoxicity of Chondroitinase ABC

Ethidium homodimer was used as a measure of cell death at the wound edge of treated explants to investigate whether chondroitinase ABC at the chosen concentration and incubation time period was toxic to the chondrocytes within the cartilage explant. Ethidium homodimer binds to the DNA of cells whose cell membrane has been compromised and are therefore dead. This compound will primarily detect necrotic cell death, but will label those cells which are in the final stages of apoptosis. Ethidium homodimer has an excitation wavelength of 528nm and an emission of 617nm which can be visualised by fluorescence microscopy.

Cell death after incubation with chondroitinase ABC at 1U/ml for 8 hrs was compared to controls of PBS treatment (8 hrs) and also explants which had been wounded and immediately snap-frozen in liquid nitrogen. Replicate samples (n=3) were used in all experimental groups. Explants were treated with chondroitinase ABC or PBS in the manner previously described in section 3.3.1 and subsequently washed in PBS (pH 7.4) three times over a period of 15 minutes before incubation with ethidium homodimer. Ethidium homodimer was reconstituted at a concentration of 2μM in PBS (pH 7.4). The treated and washed explants were incubated in 1ml of 2μM ethidium homodimer for a period of 1.5 hrs at 37°C. Explants were then washed in PBS (pH 7.4) three times over 15 minutes before being snap frozen in liquid nitrogen. Cryosections were cut from the explants (10μm) and mounted under a coverslip using Vectashield® mountant. The sections were visualised by fluorescence microscopy at x20 magnification.

3.3.4 Visualising Chondrocyte Migration by Fluorescence Labelling

A vial of 50μg lyophilised 5'chloromethylfluorescein diacetate (CMFDA) (Molecular Probes Europe BV, The Netherlands) was warmed to room temperature and dissolved in 90μl of anhydrous dimethylsulfoxide (DMSO) and mixed well by vortexing. The stock concentration of dye (1.94mM) was diluted 1:1000, to a working concentration of 1.94μM

in sterile PBS, (pH 7.4). The diluted dye (20ml) was added to tubes containing 1 x 10^7 isolated chondrocytes (optimal concentration decided by personal communication with Dr. Tony Hayes). The chondrocytes were resuspended in the dye and incubated at 37°C for 1 hour. Subsequent to incubation, the cells were centrifuged at 500 x g (gravitational force) for 10 minutes and the supernatant removed. The labelled cells were resuspended at 2 x 10^6 cells/ml in basal media and 1ml was seeded directly onto the chondroitinase ABC treated and control (PBS treated) explants. The labelled cells were cultured with the explants for periods of up to 28 days in basal media /50μg/ml ascorbic acid /2% FBS. Media was changed every 72 hours.

3.3.5 Visualisation of Chondroitinase ABC Generated CS-Stubs Using Immunohistochemistry

Immunohistochemistry with antibodies 2B6 and 1B5 was performed using the same protocol as previously described in section 3.3.2; except that a Texas Red® conjugated secondary antibody raised in donkey (1:50 dilution) (GE Healthcare UK Ltd., UK) was used to counterstain the CMFDA labelled cells. Sections were also blocked with whole donkey serum (1:20 dilution) prior to incubating with the primary antibody. Sections were mounted using a Vectashield® mountant containing no propidium iodide.

3.4 Results and Discussion

3.4.1 The Effect of Concentration and Incubation Time of Chondroitinase ABC on the Digestion of Articular Cartilage Explants

It was important to ascertain an optimal concentration and incubation time for digestion of the injured cartilage explants with chondroitinase ABC in order to obtain a balance between sufficient digestion for the migration and integration of transplanted cells without causing permanent damage to the cartilage. Full thickness articular cartilage explants from young bovine metacarpophalangeal/metatarsophalangeal joints were embedded in agarose leaving only the wounded surface exposed. The injured surface was treated with either 1U/ml or 10U/ml chondroitinase ABC for varying time periods of up to 24 hours. The explants were sectioned and labelled for CS-stubs generated by the chondroitinase digestion. In order to obtain a biomechanically stable repair tissue that will not degenerate with time, it is important that the endogenous and repair tissue are integrated through all the different zones of the cartilage. In choosing an optimal concentration for chondroitinase ABC digestion it was therefore important to see sufficient generation of CS-stubs through all the zones of the cartilage.

Initial time course experiments using published concentrations and incubation times for chondroitinase ABC digestion (Hunziker and Kapfinger, 1998; Hunziker and Rosenberg, 1996; Lee et al., 2000), 1U/ml chondroitinase ABC for periods between 0-1hr, demonstrated no detectable digestion of the GAG content of the cartilage beyond the wound surface (data not shown). The described results therefore used a 1 hour incubation time and 1U/ml concentration of enzyme as the minimum conditions. Figures 3.3-3.6 (a)–(f) demonstrate the labelling of C-0-S and C-4-S stubs using the monoclonal antibodies 2B6 and 1B5 together with a FITC conjugated secondary antibody. Nuclei of the chondrocytes within the cartilage have been counterstained with propidium iodide.

The rate and amount of digestion between zones varied due to the differing concentrations of proteoglycans within these zones. The fixed charge density, (FCD) of articular cartilage

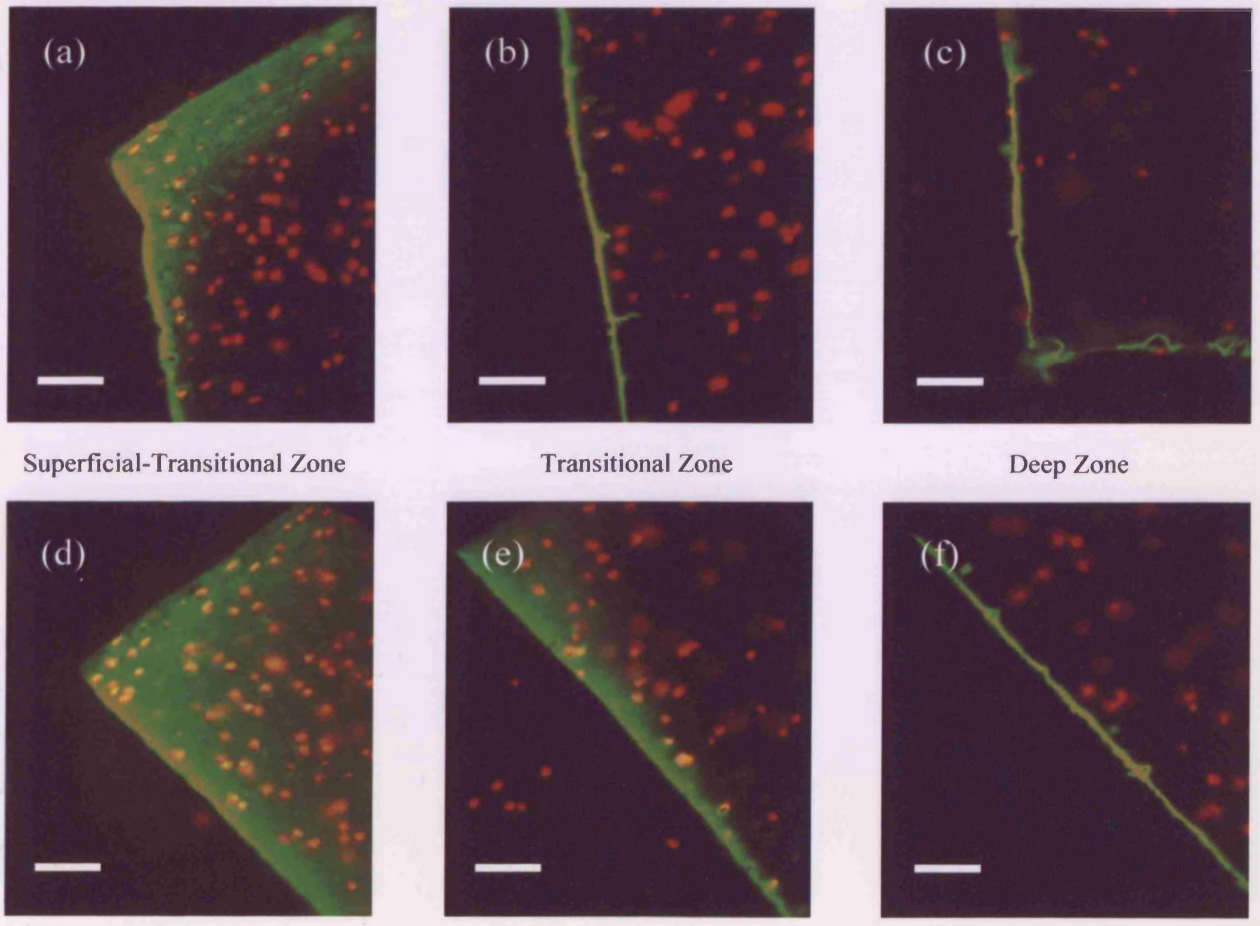


Fig.3.3 Immunofluorescence photomicrographs illustrating chondroitinase ABC generated CS-stubs, after 1 hour, using the antibodies 2B6 and 1B5 together and a FITC conjugated secondary antibody (cells counterstained with propidium iodide). (a)-(c) superficial-transitional, transitional and deep zones respectively treated with 1U/ml chondroitinase ABC, (d)-(f) superficial-transitional, transitional and deep zones respectively treated with 10U/ml chondroitinase ABC
Bar = 50μm

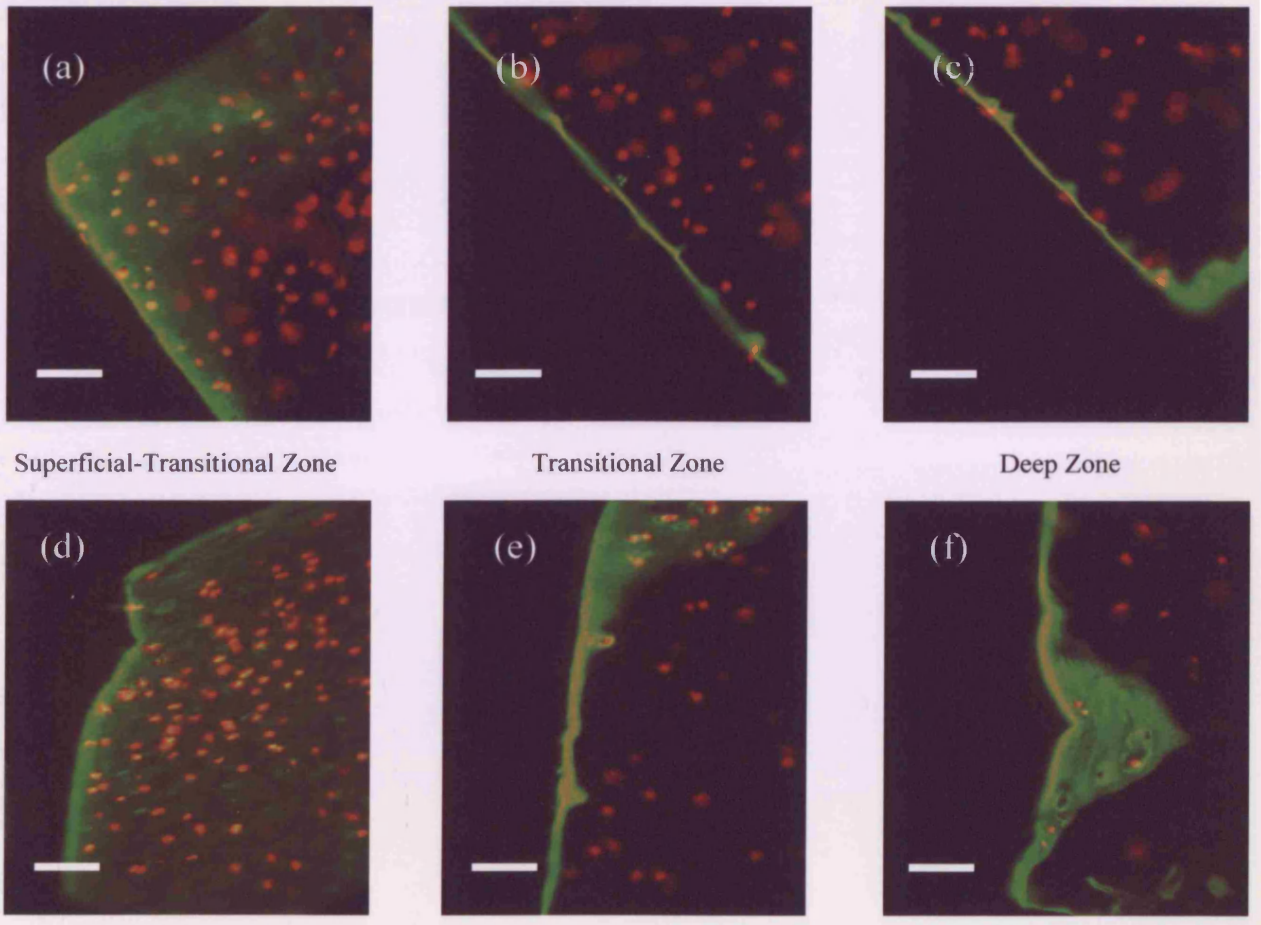


Fig.3.4 Immunofluorescence photomicrographs illustrating chondroitinase ABC generated CS-stubs, after 4 hours, using the antibodies 2B6 and 1B5 together and a FITC conjugated secondary antibody (cells counterstained with propidium iodide). (a)-(c) superficial-transitional, transitional and deep zones respectively treated with 1U/ml chondroitinase ABC, (d)-(f) surface-transitional, transitional and deep zones respectively treated with 10U/ml chondroitinase ABC
Bar = 50μm

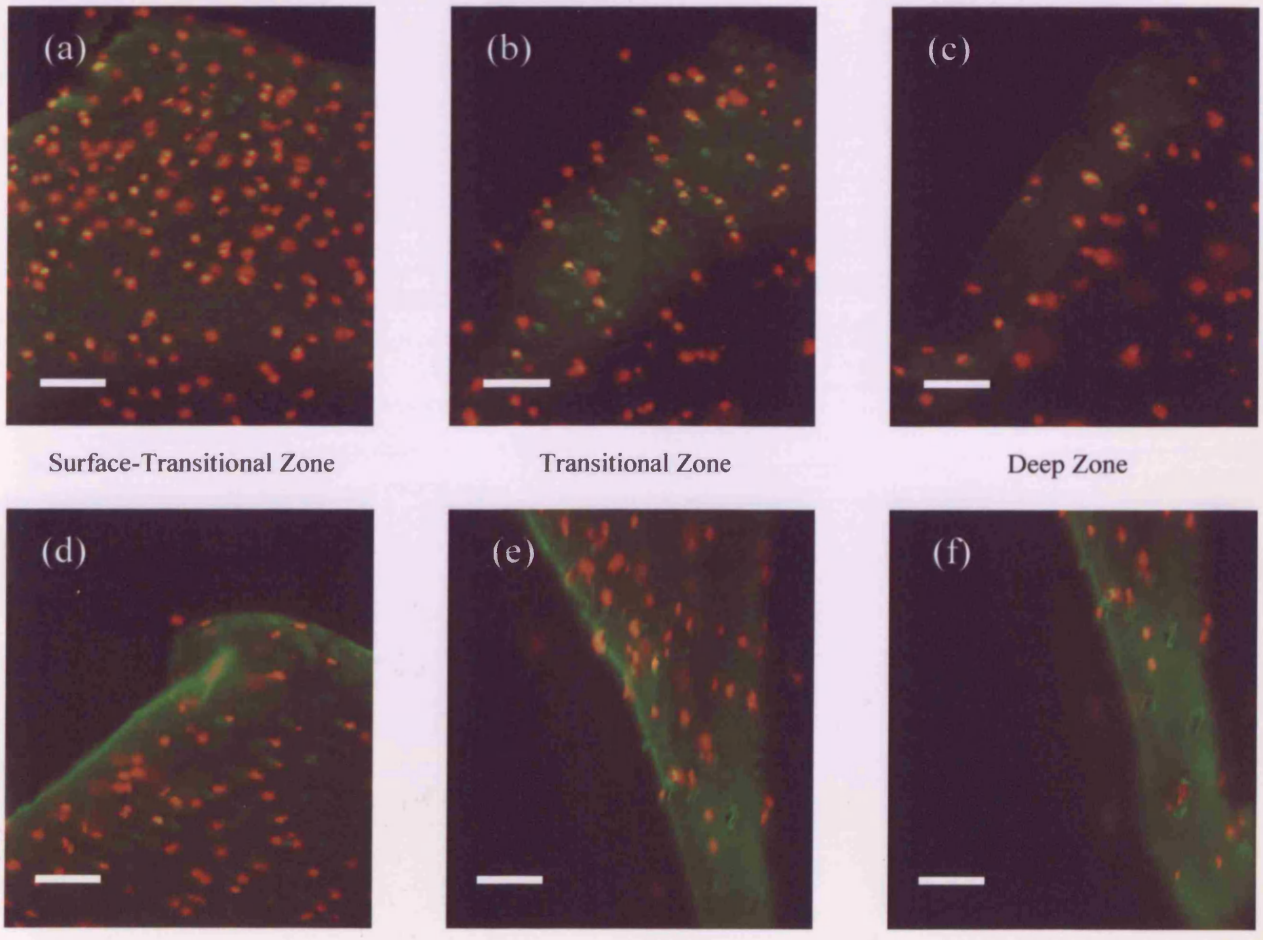


Fig.3.5 Immunofluorescence photomicrographs illustrating chondroitinase ABC generated CS-stubs, after 8 hours, using the antibodies 2B6 and 1B5 together and a FITC conjugated secondary antibody (cells counterstained with propidium iodide). (a)-(c) surface-transitional, transitional and deep zones respectively treated with 1U/ml chondroitinase ABC, (d)-(f) surface-transitional, transitional and deep zones respectively treated with 10U/ml chondroitinase ABC
Bar = 50μm

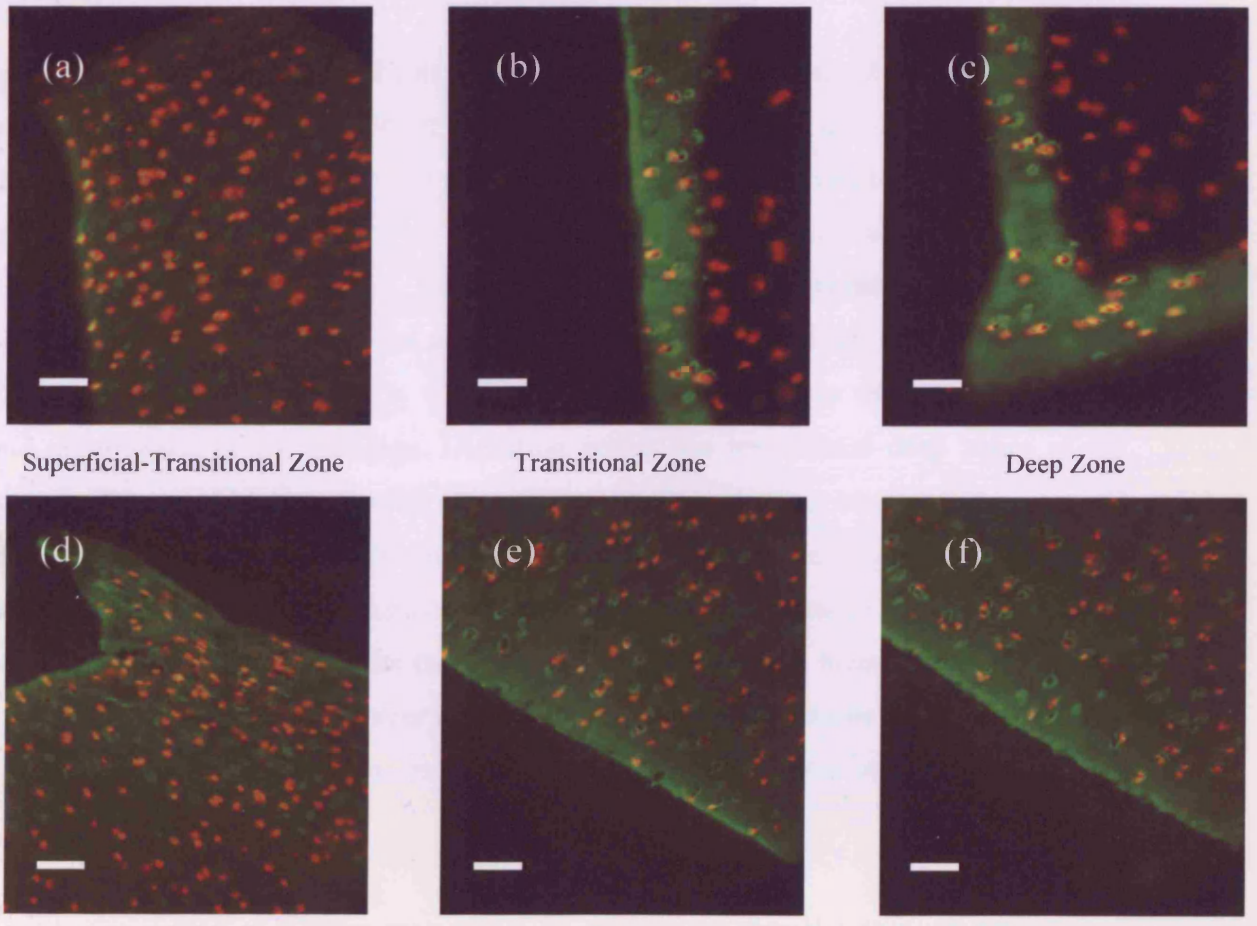


Fig.3.6 Immunofluorescence photomicrographs illustrating chondroitinase ABC generated CS-stubs, after 24 hours, using the antibodies 2B6 and 1B5 together and a FITC conjugated secondary antibody (cells counterstained with propidium iodide). (a)-(c) superficial-transitional, transitional and deep zones respectively treated with 1U/ml chondroitinase ABC, (d)-(f) surface-transitional, transitional and deep zones respectively treated with 10U/ml chondroitinase ABC
Bar = 50μm

is low in the superficial zone and can be seen to increase with depth of the tissue from the articular surface (Maroudas, 1979). It would therefore be expected that the relative amount of digestion would be highest in the superficial zone with progressively less towards the deeper zones of the tissue. However, it is also important to take into account the fact that the SLRPs decorin and biglycan, with their GAG chains, are found in relatively high concentrations within the superficial and transitional zones (Poole et al., 1996; Rodrigues et al., 2005). As expected, digestion within the superficial zone was more extensive than within other zones of the cartilage. Digestion within the transitional–deep zones of the tissue after 1 hour of incubation with either 1U/ml or 10U/ml of the enzyme did not extend beyond the wound surface. While this is probably enough to encourage the adherence of chondrocytes to the injured surface it is insufficient to enhance the migration of transplanted chondrocytes into the cartilage (see fig.3.3). At four hours, further digestion was seen within the superficial layer as expected, but within the deeper layers digestion of the cartilage was still seen to be minimal and insufficient for the enhancement of cell migration (see fig.3.4).

An incubation period of 8 hours appeared to be optimal for digestion through all zones of the tissue (see fig.3.5a-c). The depth of digestion from the exposed surface had increased within all zones, although digestion was far more limited in the transitional and deep zones in comparison to that of the upper region of the tissue. A similar result was visualised in the tissue digested with 10U/ml of chondroitinase ABC (see fig.3.5d-f). Allowing a digestion period of 24 hours with 1U/ml chondroitinase ABC did not see a substantial difference in digestion within the transitional-deep zones than that already achieved at 8 hrs (see fig.3.6b-c). Digestion within the superficial zone extended throughout the complete explant. However, allowing an incubation period of 24 hours with 10U/ml of enzyme saw complete digestion of the CS chains with a blanket of chondroitinase ABC generated CS-stubs being seen throughout the tissue (see fig.3.6d-f). Complete digestion in such a manner would ultimately result in permanent damage to the cartilage and the ability of the cartilage to withstand compressive forces would be diminished. The natural FCD of the tissue would alter as its permeability would increase and the water trapped by the large aggregates of proteoglycans would be lost. An optimal concentration of 1U/ml chondroitinase ABC for an incubation period of 8 hours was therefore chosen for subsequent experiments using this *in vitro* model for cartilage injury and repair.

3.4.2 Does Chondroitinase ABC Treatment of Articular Cartilage Induce Cell Death?

On establishing an optimal concentration and incubation time for treatment of the wounded articular cartilage explants with chondroitinase ABC it was important to confirm that these conditions did not induce cell death of the chondrocytes within the digested explant. Cell death at the wound edge is an inevitable consequence of articular cartilage surgery (Bennett et al., 1932). Reparative methods, whether cell-based such as ACT or implant methods such as mosaicplasty, invariably involve debridement and cleaning of the lesion site. Cell death at the wound edge may in itself be a limiting factor for cartilage-cartilage interface integration (Tew et al., 2000). Any integration that does occur therefore is occurring within a zone of dead tissue which will degenerate with time and therefore limit the integration between pre-existing and newly generated cartilage (Wakitani et al., 1994). It is therefore important to consider any factors that would induce more cell death than that already seen as a result of initial wounding. Chondrocyte cell death was measured using ethidium homodimer, a DNA intercalating compound which can only travel through the compromised cell membranes of dead cells (Gaugain et al., 1978a; Gaugain et al., 1978b). Ethidium homodimer is a red, fluorescent compound which can be detected by fluorescence microscopy.

Explants treated with chondroitinase ABC for the optimised time and concentration (1U/ml for 8 hours) were incubated with 2μm ethidium homodimer for 1.5 hours. Wounded and PBS treated explants served as controls for this experiment. Previous experiments have demonstrated that the process of wounding articular cartilage does induce cell death at the wound edge, however, this can be dramatically reduced by wounding with a sharp scalpel blade as opposed to a blunt instrument such as a trephine (Redman, et al., 2004). Cell death at wound edges has previously been shown to be due to necrosis, with a zone of apoptotic cells directly behind this (Tew et al., 2000), therefore some cell death at the wound edge was expected with this experiment.

Explants that were wounded with a scalpel blade and immediately incubated with ethidium homodimer demonstrated some cell death at the wound edge through all zones of the

cartilage (fig. 3.7a). As expected the level of cell death within the deep zone was highest and this can be attributed to cell death induced during removal of the explant from the bone for experimentation. Cell death however was limited to the wound edge and extended back on average 15μm into the superficial zone of the tissue (defined as 100μm from the top of the explant) to 89μm in the deep zone (defined as 100μm from the base of the explant). Explants treated with PBS as a control instead of chondroitinase ABC for 8 hours demonstrated a similar distribution of cell death at the wound edge within the superficial and transitional zones (see fig. 3.7b). Within this treatment group an average zone of cell death 27μm from the wound edge was seen in the surface of the explant and this remained unchanged in the deep zone, with cell death extending back into the tissue for an average of 31μm. Differences in the amount of cell death seen within the deep zone can be attributed to the effects of explant excision from the bone as previously discussed.

The explants treated with chondroitinase ABC showed a very similar pattern of cell death to the controls, confirming that any cell death at the wound edge was as a direct result of wounding to the tissue and not due to incubation of the explant with chondroitinase ABC (fig. 3.7c). Although not calculated here, the zone of GAG digestion seen in the explants treated with chondroitinase ABC for 8 hours within section 3.3.1 (see fig. 3.5) was in excess of the zone of cell death demonstrated here. For example, the zone of GAG digestion within the superficial zone extended for the complete depth of the explant. Within this treatment group cell death within the defined superficial zone extended back from the wound edge into the treated tissue for an average of 17μm. Within the deep zone this increased only to an average of 31μm. These results confirm that this chondroitinase ABC treatment, under the conditions described above, is not toxic to the chondrocytes within the treated explant and therefore it can be used at this concentration and incubation time to investigate the migration of isolated chondrocytes at the wound edge into the digested tissue.

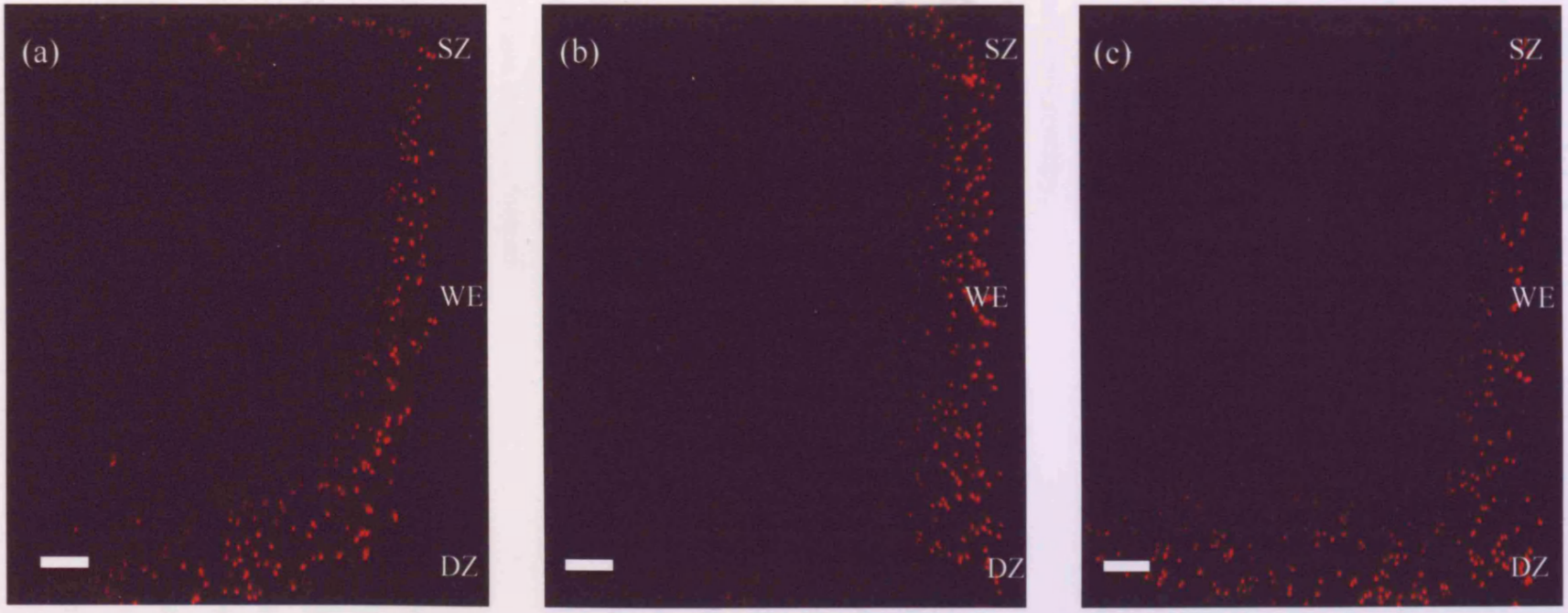


Fig.3.7 Fluorescent photomicrographs illustrating ethidium homodimer (dead) labelled chondrocytes within full depth articular cartilage explants (a) scalpel wounded explant, (b) PBS treated explant, (c) chondroitinase ABC treated explant.
SZ = superficial zone, DZ = deep zone and WE = wound edge
Bar = 50μm

3.4.3 The Effect of Chondroitinase ABC Digestion on Enhancing the Migration of Chondrocytes Isolated from Young and Mature Articular Cartilage into Cartilage Explants

As previously investigated, an optimal concentration of the enzyme chondroitinase ABC of 1U/ml and an incubation period of 8 hours was chosen. Isolated chondrocytes from the both the young and skeletally mature articular cartilage were labelled with the green fluorescent, cytoplasmic dye CMFDA and seeded onto the surface of the pre-digested explants. Labelled cells were seeded onto the surface of PBS treated explants to serve as controls. The chondrocytes were allowed to adhere to the wound surface and migrate into the digested tissue for a period of up to 28 days before being sectioned and viewed using fluorescence microscopy.

Using grids of $0.1mm^2$ it has been possible to quantify the migration of labelled cells into the explants over the 28 day culture period. The number of labelled cells present within the superficial, transitional and deep zones of the explants were counted and averaged (n=3). As stated in section 3.4.2, the superficial zone has been defined as the region extending from the articular surface to a depth of 100μm into the tissue. The deep zone has been defined for this purpose as the region extending 100μm above the start of the calcified zone. As expected variation between explants and batches of cells means that the standard error figures are high and for this reason graphical representation presented in fig.3.8 is purely to indicate the trends and differences between control and treatment groups. No statistics have been performed.

The data obtained support the hypothesis that GAG chains inhibit the migration of chondrocytes, and complement the data described in chapter 2. A negligible amount of migration was seen with chondrocytes from both the young and skeletally mature cartilage into the PBS treated control explants over the initial 7 day culture period (see fig.3.9 and fig.3.13). The data provided by the Boyden chambers suggesting the anti-adhesive properties of GAG chains and the ability of proteoglycans to mask other pro-adhesive matrix macromolecules present within the cartilage prevents chondrocyte migration. These data are also in line with that previously published demonstrating the ability of anti-

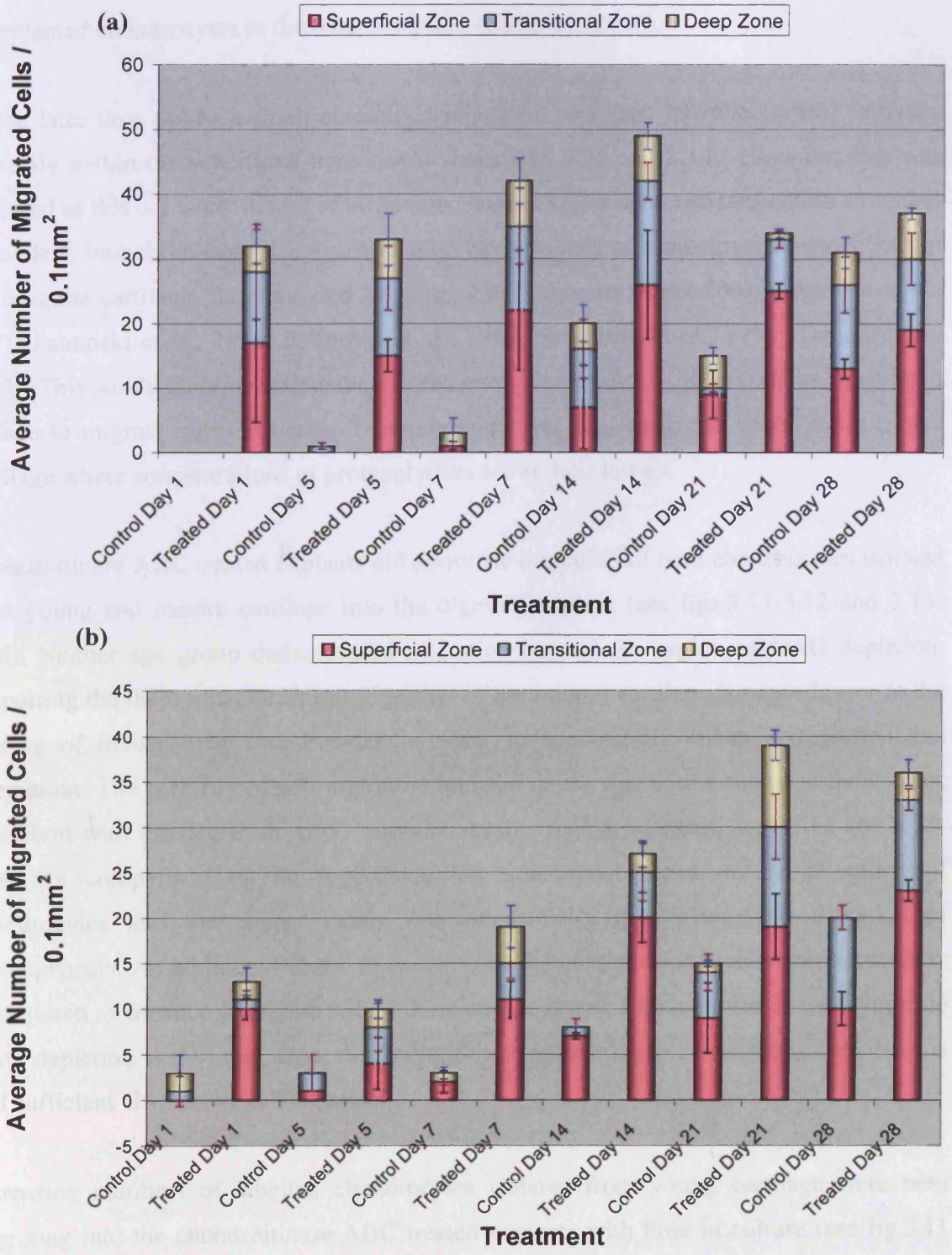


Fig. 3.8 Graphical representation of the number of CMFDA labelled chondrocytes isolated from (a) young and (b) mature cartilage migrating into PBS (control) and chondroitinase ABC treated explants over periods of 1-28 days after seeding onto the wound surface. The superficial zone is defined here as extending from the articular surface to 100μm into the tissue, the deep zone being 100μm from the point of excision (excluding any calcified cartilage).

adhesive proteoglycans present at articular cartilage wound edges to reduce the adhesion of transplanted chondrocytes to the wound surface (Lee et al., 2000).

At the later time points a small amount of migration was seen into the control explants, primarily within the superficial-transitional zones (fig. 3.10 and 3.14). However, this was expected as this is a static model of migration. As previously demonstrated within a number of models, immobilisation of a synovial joint leads to loss of proteoglycans and CS from the articular cartilage (Caterson and Lowther, 1978; Haapala et al., 2001; Kiviranta et al., 1987; Palmoski et al., 1979; Palmoski et al., 1980; Saamanen et al., 1990; Tammi et al., 1983). This would therefore allow the migration of chondrocytes still present on the wound surface to migrate into the tissue. This migration was limited to the upper zones of the cartilage where concentrations of proteoglycans are at their lowest.

Chondroitinase ABC treated explants did allow the migration of both chondrocytes isolated from young and mature cartilage into the digested explant (see figs.3.11-3.12 and 3.15-3.16). Neither age group demonstrated migration beyond the region of GAG depletion; supporting the theory that sufficient digestion of the injured cartilage is needed prior to the seeding of transplanted chondrocytes in order to successfully enhance migration and integration. The majority of cell migration into the tissue was within the superficial zone, consistent with the degree of GAG digestion in this region (see figs. 3.11-3.12 and 3.15-3.16). In comparison to the superficial and transitional zones, active migration of chondrocytes into the deeper zones was substantially less, even with digestion of proteoglycans. An additional factor to encourage migration such as a chemoattractant may be required to enhance migration within these deeper zones. Although these results indicate GAG depletion within this zone, this method is non-quantitative. It is likely that there is still sufficient GAG to inhibit migration.

Increasing numbers of labelled chondrocytes isolated from young cartilage were seen migrating into the chondroitinase ABC treated explants with time in culture (see fig.3.11 and 3.12). This trend appeared to plateau at 14 days with no further increase in the number of labelled chondrocytes seen migrating into the explants (see fig.3.12). By days 21 and 28 fluorescently labelled migrated chondrocytes were demonstrating the correct morphology

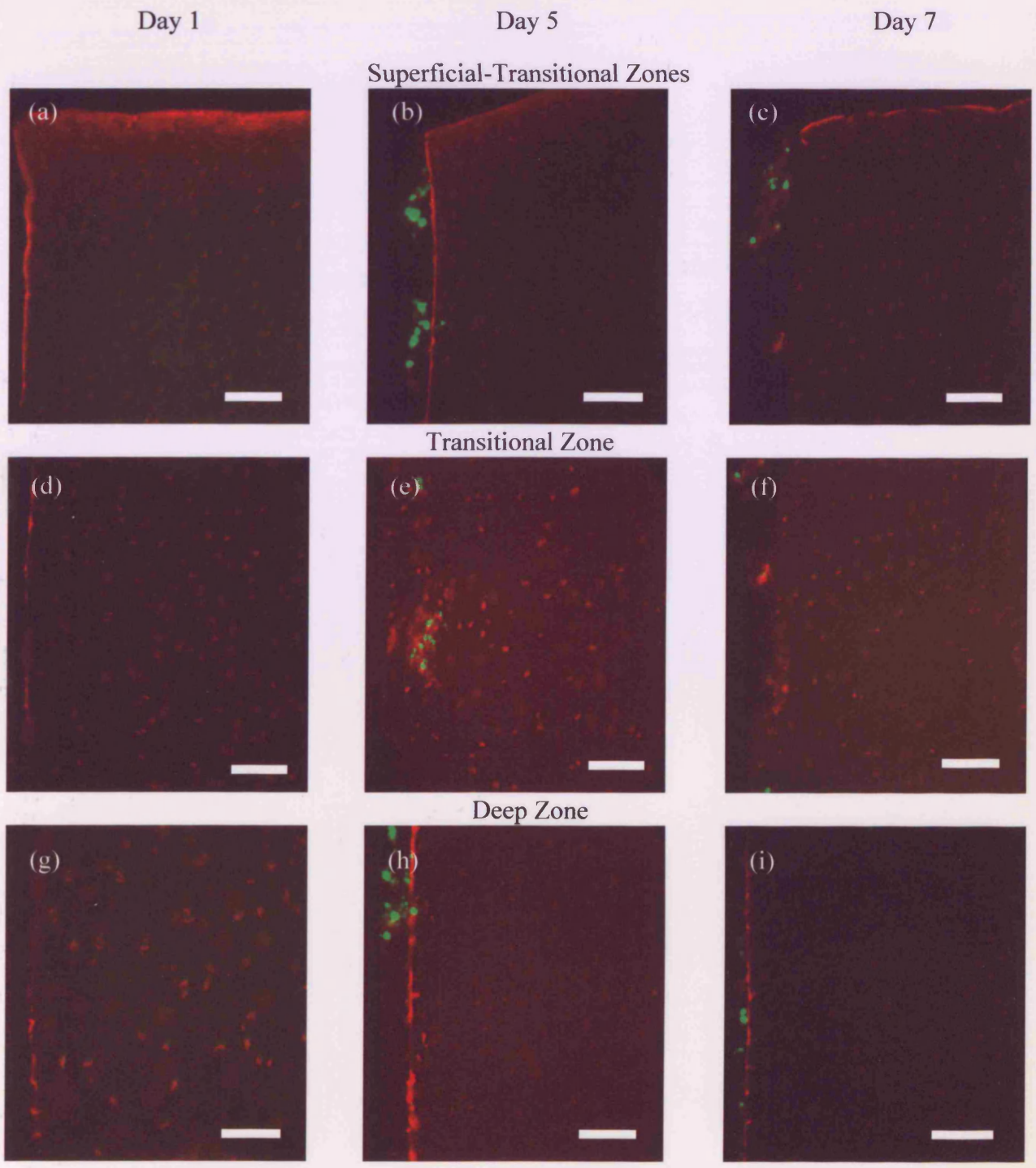


Fig.3.9 Immunofluorescence photomicrographs illustrating the migration of CMFDA labelled chondrocytes (green) isolated from young articular cartilage into the (a-c) superficial-transitional, (d-f) transitional and (g-i) deep zones of PBS treated cartilage explants after (a, d, g) 1, (b, e, h) 5, and (c, f, i) 7 days of culturing. The antibodies 2B6 and 1B5 and a Texas Red® conjugated secondary antibody have been used to highlight the chondroitinase ABC generated CS-stubs.

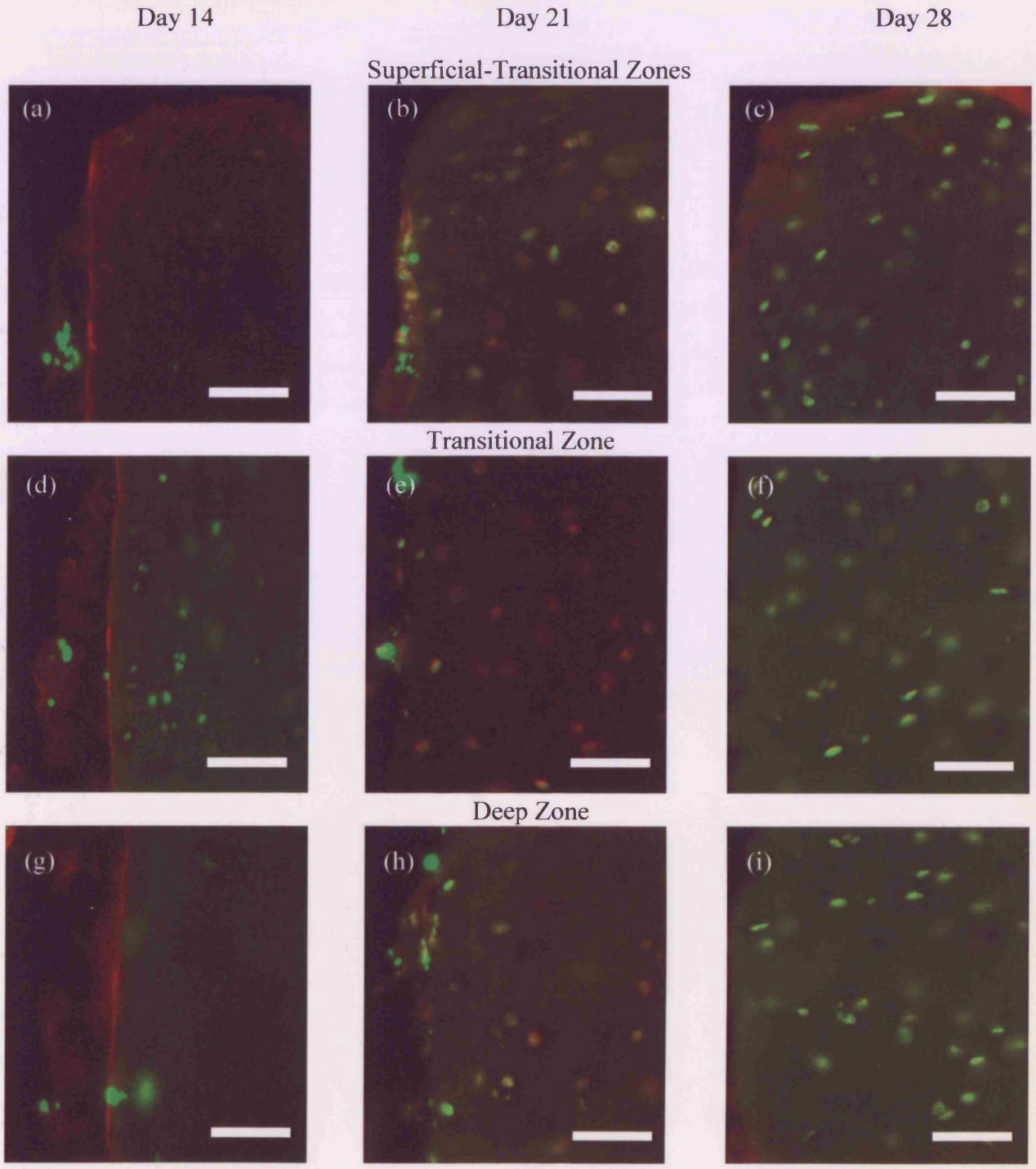


Fig.3.10 Immunofluorescence photomicrographs illustrating the migration of CMFDA labelled chondrocytes (green) isolated from young articular cartilage into the (a-c) superficial-transitional, (d-f) transitional and (g-i) deep zones of PBS treated cartilage explants after (a, d, g) 14, (b, e, h) 21, and (c, f, i) 28 days of culturing. The antibodies 2B6 and 1B5 and a Texas Red® conjugated secondary antibody have been used to highlight the chondroitinase generated CS-stubs.
Bar = 50μm

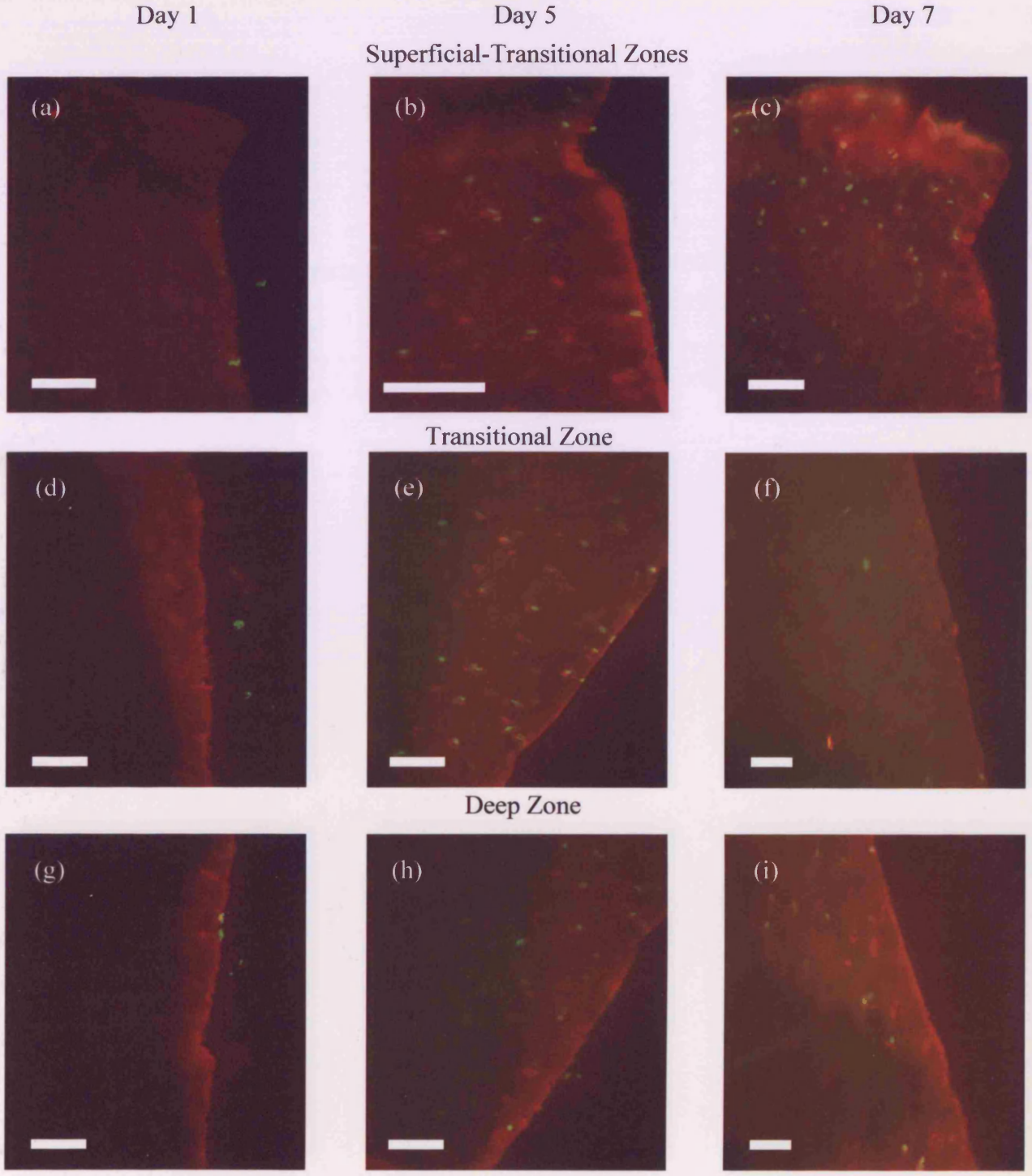


Fig.3.11 Immunofluorescence photomicrographs illustrating the migration of CMFDA labelled chondrocytes isolated from young articular cartilage into the (a-c) superficial-transitional, (d-f) transitional and (g-i) deep zones of chondroitinase ABC treated cartilage explants after (a, d, g) 1, (b, e, h) 5, and (c, f, i) 7 days of culturing. The antibodies 2B6 and 1B5 and a Texas Red® conjugated secondary antibody have been used to highlight the chondroitinase generated CS-stubs.
Bar = 50μm

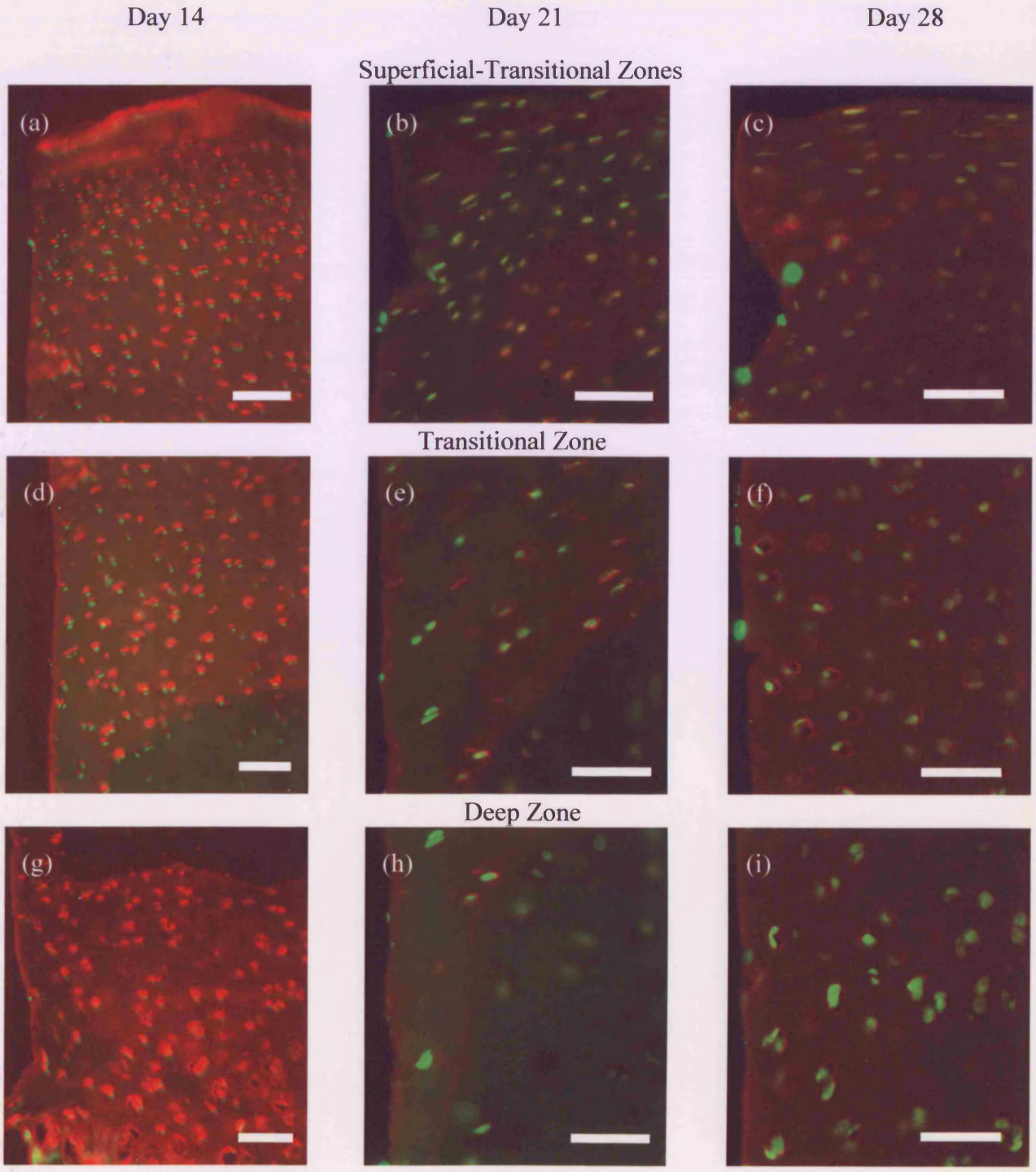


Fig.3.12 Immunofluorescence photomicrographs illustrating the migration of CMFDA labelled chondrocytes (green) isolated from young articular cartilage into the (a-c) superficial-transitional, (d-f) transitional and (g-i) deep zones of chondroitinase ABC treated cartilage explants after (a, d, g) 14, (b, e, h) 21, and (c, f, i) 28 days of culturing. The antibodies 2B6 and 1B5 and a Texas Red® conjugated secondary antibody have been used to highlight the chondroitinase generated CS-stubs.
Bar = 50μm

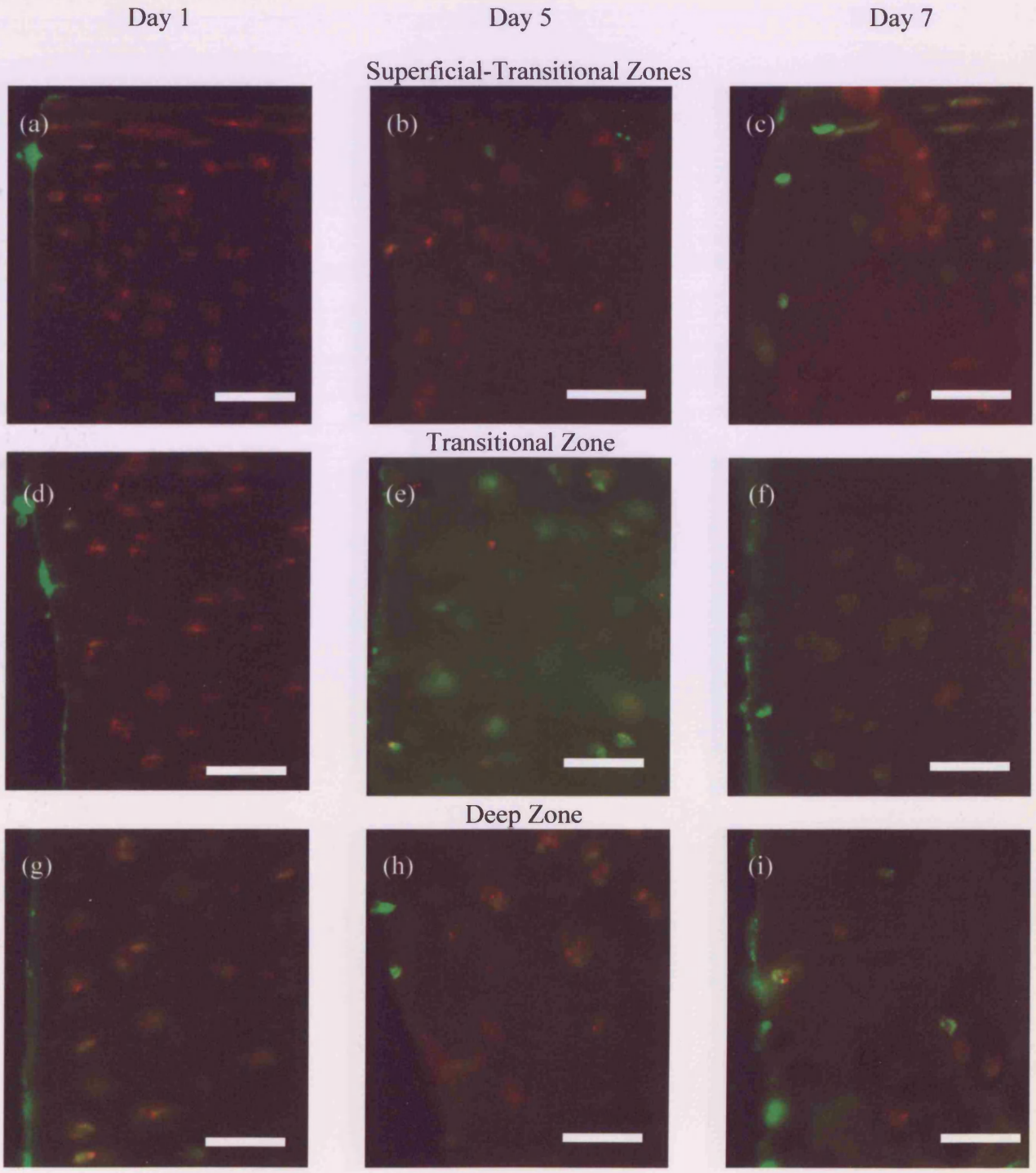


Fig.3.13 Immunofluorescence photomicrographs illustrating the migration of CMFDA labelled chondrocytes (green) isolated from mature articular cartilage into the (a-c) superficial-transitional, (d-f) transitional and (g-i) deep zones of PBS treated cartilage explants after (a, d, g) 1, (b, e, h) 5, and (c, f, i) 7 days of culturing. The antibodies 2B6 and 1B5 and a Texas Red® conjugated secondary antibody have been used to highlight the chondroitinase generated CS-stubs.
Bar = 50μm

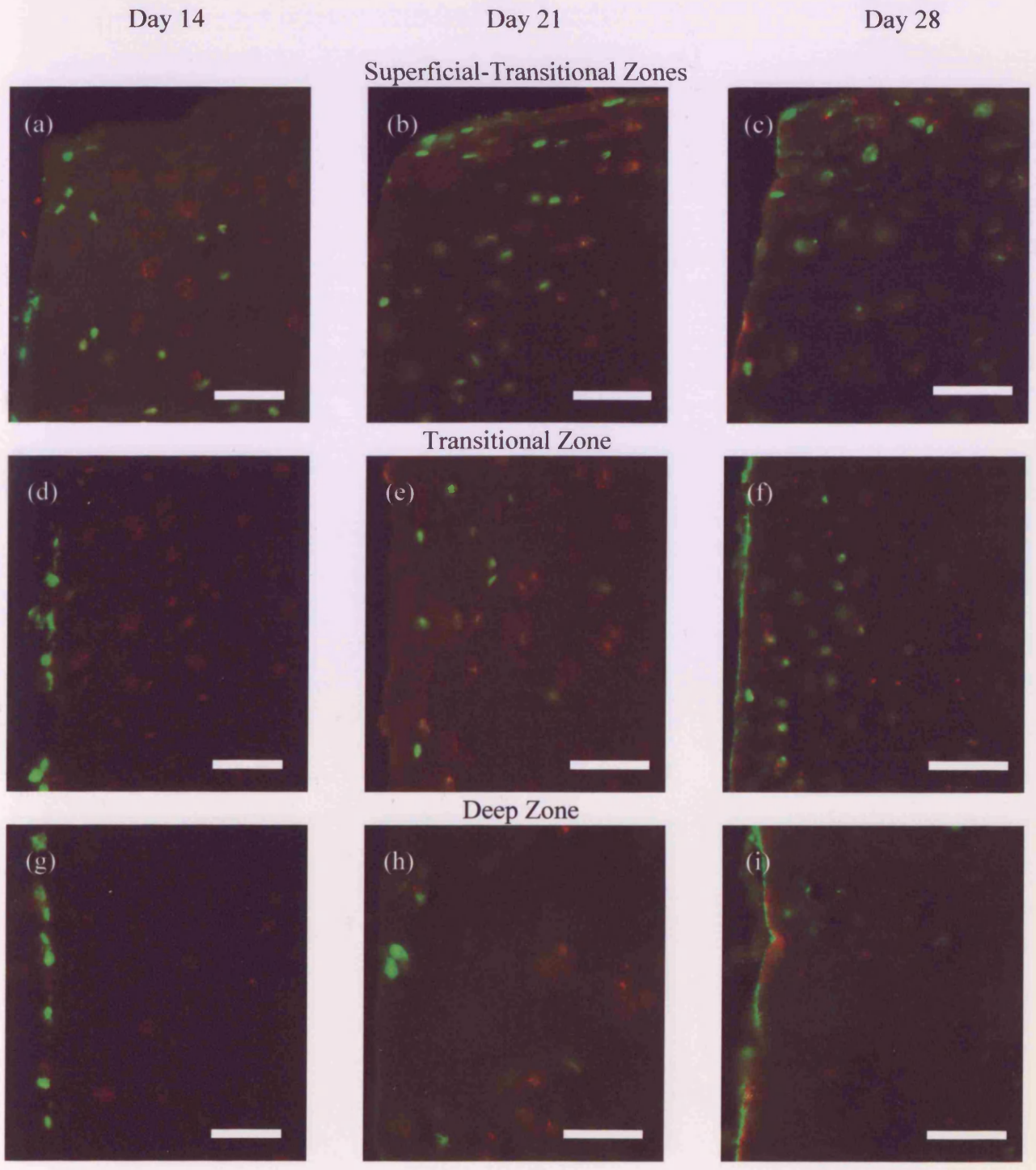


Fig.3.14 Immunofluorescence photomicrographs illustrating the migration of CMFDA labelled chondrocytes (green) isolated from mature articular cartilage into the (a-c) superficial-transitional, (d-f) transitional and (g-i) deep zones of PBS treated cartilage explants after (a, d, g) 14, (b, e, h) 21, and (c, f, i) 28 days of culturing. The antibodies 2B6 and 1B5 and a Texas Red® conjugated secondary antibody have been used to highlight the chondroitinase generated CS-stubs.
Bar = 50μm

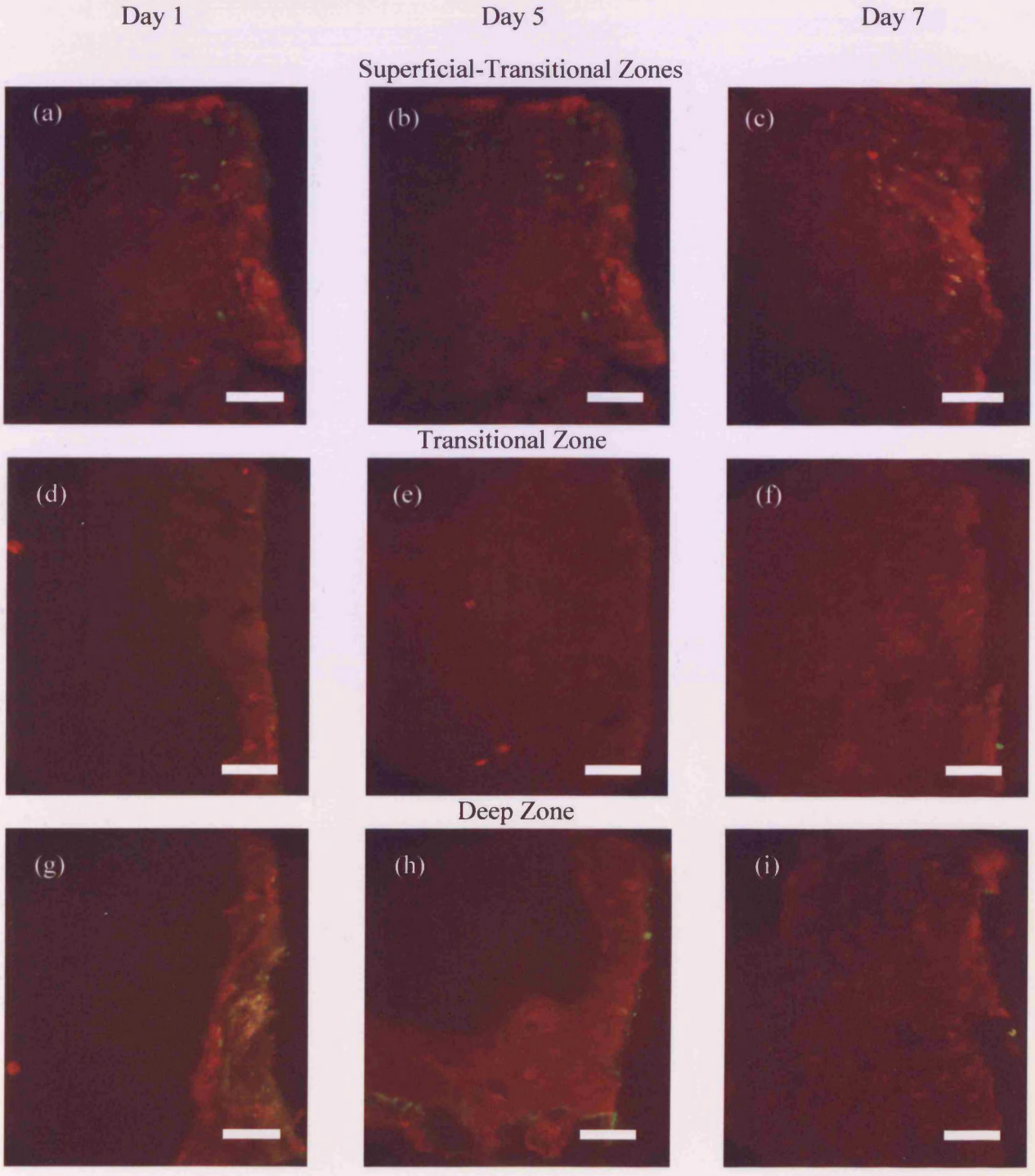


Fig.3.15 Immunofluorescence photomicrographs illustrating the migration of CMFDA labelled chondrocytes (green) isolated from mature articular cartilage into the (a-c) superficial-transitional, (d-f) transitional and (g-i) deep zones of chondroitinase ABC treated cartilage explants after (a, d, g) 1, (b, e, h) 5, and (c, f, i) 7 days of culturing. The antibodies 2B6 and 1B5 and a Texas Red® conjugated secondary antibody have been used to highlight the chondroitinase generated CS-stubs.
Bar = 50μm

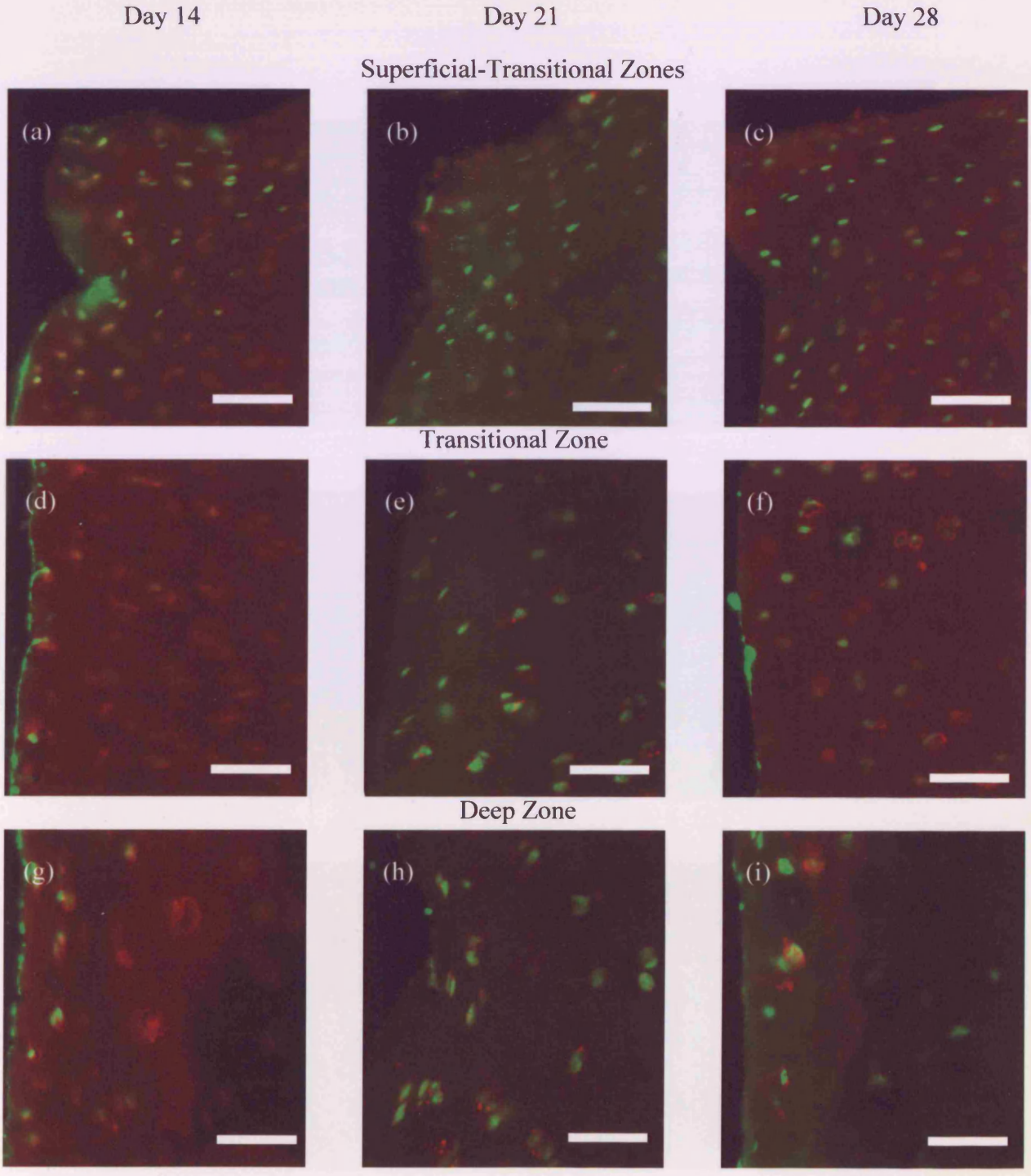


Fig.3.16 Immunofluorescence photomicrographs illustrating the migration of CMFDA labelled chondrocytes (green) isolated from mature articular cartilage into the (a-c) superficial-transitional, (d-f) transitional and (g-i) deep zones of chondroitinase ABC treated cartilage explants after (a, d, g) 14, (b, e, h) 21, and (c, f, i) 28 days of culturing. The antibodies 2B6 and 1B5 and a Texas Red® conjugated secondary antibody have been used to highlight the chondroitinase generated CS-stubs.
Bar = 50μm

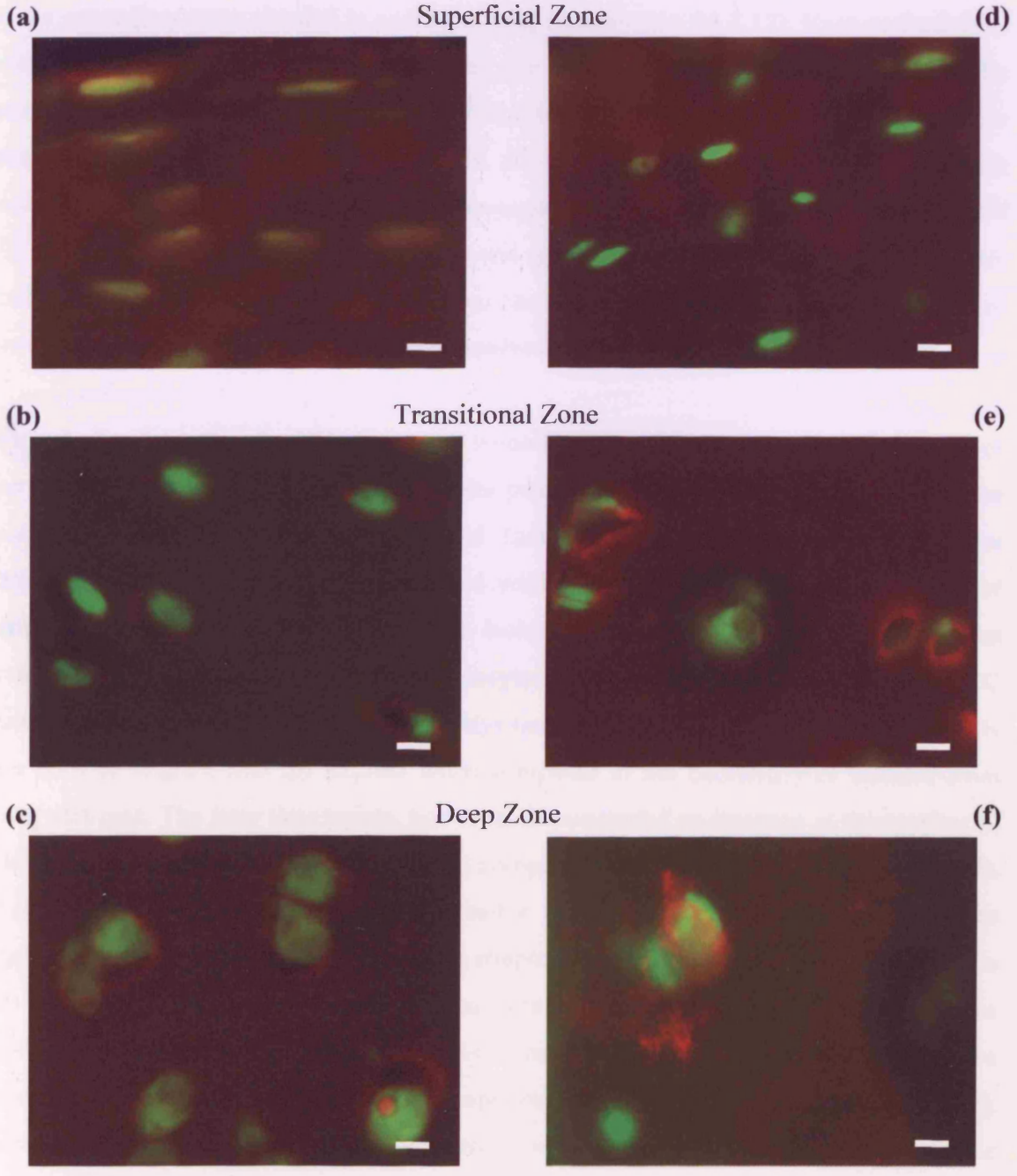


Fig. 3.17 Magnified images of migrated CMFDA labelled chondrocytes (green) isolated from (a-c) young and (d-f) mature articular cartilage into the (a and d) superficial, (b and e) transitional, (c and f) deep zones of chondroitinase ABC treated cartilage explants after 28 days of culturing. The photomicrographs illustrate the ability of the migrated chondrocytes to adapt to the cellular morphology of the zone that they have migrated into.
Bar = 10μm

for the zones they were situated in and occupying lacunae (see fig.3.17). Even at these later time points, no migration was seen beyond the zone of digestion. These data raise the question of whether the migrating chondrocytes are occupying existing lacunae, or generating new lacunae around themselves after migrating into the digested matrix. It would be interesting to investigate this phenomenon further with chondrocytes transfected with a type VI collagen expressing vector and green fluorescent protein (GFP) tag. This would give a clear indication as to whether the transfected, migrating chondrocytes are generating a pericellular matrix around themselves, once within the digested tissue.

Labelled chondrocytes were present at the wound edge replacing those lost as a result of injury to the articular cartilage. These results present a basis for live chondrocytes at the wound edge able to synthesis matrix and form a biomechanically stable region for integration with any neo-cartilage produced within the lesion site. A similar pattern of migration was seen with the chondrocytes isolated from mature cartilage. A consistent increase in the number of labelled chondrocytes found within the chondroitinase ABC treated explants was seen over the first 21 days (see fig.3.15 and 3.16). Initially fewer cells were seen to migrate into the explant when compared to the chondrocytes isolated from young cartilage. The later time points, however, demonstrated an increase in the number of cells migrating into the tissue, with numbers comparable to those seen with the young cells by day 28 (see fig.3.16). It is however possible that this increase in cells is not due to migration, but cell division. As discussed in chapter 2 (see section 2.4.3), only 0.9% of the cells seeded onto the Boyden chambers demonstrated the ability to migrate, leading to the hypothesis that there may be a subpopulation of cells within articular cartilage which have the ability to migrate. A hypothesis which supports the work by Dowthwaite et al., (2004), which describes the presence of a chondroprogenitor population within the superficial zone of articular cartilage. These chondroprogenitor cells have a phenotypic plasticity and like other undifferentiated cells are able to form colonies. This ability to divide may explain why increases in the number of labelled cells were found residing in the explant with time. It would be interesting to separate the chondrocytes from the different zones of articular cartilage, both young and skeletally mature, and investigate the migratory abilities of cells from the different zones to see if this is the case. These undifferentiated cells are also characterised by their adhesiveness to fibronectin. It is well documented that the GAG chains do inhibit cell adhesion to fibronectin found within the matrix by blocking the cell

binding domain (Lewandowska et al., 1987). Removal of these inhibitory GAG chains from the wound surface would therefore in theory expose other matrix macromolecules, such as fibronectin, for these cells to bind and adhere to.

The data achieved from this work pose the questions of whether digestion of the proteoglycans within the cartilage matrix increases the migration of chondrocytes because of the reduced FCD and therefore increased the permeability of the tissue or whether it is due to the unmasking of pro-adhesive matrix proteins allowing chondrocytes to adhere to the cartilaginous matrix and migrate into the injured tissue.

3.5 Conclusions

In support of the data obtained in chapter 2, these studies have demonstrated the anti-adhesive properties of proteoglycans and the role of the GAG chains in preventing the adhesion and migration of chondrocytes into injured cartilage explants. An *in vitro* model of articular cartilage injury has been developed and the optimal conditions for chondroitinase ABC digestion of the wound surface ascertained. The data demonstrates the ability of both chondrocytes isolated from young and skeletally mature cartilage to migrate into pre-digested tissue, but only to the depth to which digestion occurred, reinforcing this initial point.

Maximal migration of both age groups was seen in the superficial zones, with minimal migration into the deeper zones of the wounded cartilage, coinciding with the relative concentration of proteoglycans within these zones. Combining digestion of the tissue with other factors such as the use of a chemoattractant or growth factor to enhance migration may further encourage the motility of these cells and their integration into the articular cartilage matrix.

Increasing number of cells isolated from both young and skeletally mature cartilage were seen within the upper zones of the cartilage explants with time. Whether this was as a result of increased migration with time, or due to division of the migrated cells remains unknown. As discussed in chapter 2, a subpopulation of chondrocytes appear to have the

ability to migrate and isolating chondrocytes from their respective zones and repeating this experiment may be fruitful in ascertaining whether these migratory cells are those previously described as chondroprogenitors.

Supplementary to this work it would be interesting to investigate the biosynthetic activities of the migrated cells once they are within the cartilage matrix. Using $^{35}SO_4$ and ^{3}H-proline labelling of chondrocytes prior to transplantation within this model it would be possible to look at their respective proteoglycan and collagen production after migration into the injured tissue. In addition to this it would also be possible to look at the ability of growth factors such as those used in chapter 2, IGF-1 and TGFβ1, in enhancing the migratory process and the biosynthetic activity of the cells once they are within the cartilage.

In summary:

- The inhibitory role of CS in chondrocyte migration and integration using an *in vitro* model of cartilage injury corroborated with the data described in chapter 2.
- Chondrocytes isolated from young and skeletally mature cartilage migrated into chondroitinase ABC treated cartilage explants.
- Migrated chondrocytes at day 21 and 28 were seen to have adopted the morphology of the endogenous cells within the zone into which they have migrated.

Chapter 4

Investigating the Ability of IGF-1 and TGFβ1 to Enhance Matrix Biosynthesis in Articular Chondrocytes

4.1 Background

The previous experimental chapters have discussed the importance of combining both cellular migration and integration in the formation of a biomechanically stable tissue for cartilage repair. The composition and attributes of the newly formed cartilage are crucial for conveying its stability. As discussed in section 1.4.4, it is the balance of the tensile strength providing collagens and compressive force resisting proteoglycans that gives articular cartilage its ability to function. This chapter describes work aimed at enhancing matrix biosynthesis for the production of a functionally integrated repair tissue. The effects of the anabolic growth factors, IGF-1 and TGFβ1 have been investigated with reference to their ability to alter the synthesis of proteoglycans and collagens whilst maintaining the chondrocytic phenotype. We have previously demonstrated the ability of IGF-1 and TGFβ1 to enhance the migration of chondrocytes and also their co-operation and synergising effects when used in combination (see chapter 2).

The nature and attributes of these growth factors have been discussed in sections 1.15.1 and 1.15.2. The positive role of IGF-1 in aiding cartilage homeostasis and the maintenance of the mechanical properties of the tissue, including the equilibration modulus and electrokinetic coefficient is well documented (Sah et al., 1996). IGF-1 was chosen as a target growth factor in this study due to its well characterised anabolic effects within chondrocytes as well as other cell types. Specifically within cartilage, IGF-1 has been implicated in matrix homeostasis and in maintenance of the chondrocytic phenotype (Guerne et al., 1994; Tyler, 1989). IGF-1 is known to increase both proteoglycan and collagen biosynthesis (Bonassar et al., 1997) and can also enhance cellular proliferation and differentiation with an increase in DNA synthesis (Trippel, 1997). These effects are accompanied by decreased expression of degradative factors such as MMPs and up-regulation of TIMP expression (Hui et al., 2001a; Hui et al., 2001b). In addition to this, IGF-1 is able to counteract the catabolic effects of inflammatory cytokines such as IL-1 and TNFα (Tyler, 1989).

The metabolic effects of TGFβ1 on cartilage are less well characterised and conflicting results have been published. These conflicting reports appear to be dependent on the target cell's cell cycle stage. TGFβ1 will enhance proliferation of those cells within S phase,

whereas those dividing slowly within G_1 will be inhibited (Trippel, 1995; Vivien et al., 1992; Vivien et al., 1990). Some studies have demonstrated TGFβ1 to be stimulatory on proteoglycan synthesis (Glansbeek et al., 1998) and cell proliferation (Darling and Athanasiou, 2005a). Other publications indicate no such effects on chondrocytes or even the reverse with inhibition of proteoglycan synthesis seen (van der Kraan et al., 1992). As with IGF-1, TGFβ1 has the ability to reduce pro-inflammatory cytokine-induced proteoglycan degradation. Studies with IL-1 have shown that TGFβ1 is able to counteract the degradative effects this cytokine can induce (van Beuningen et al., 1993; van Beuningen et al., 1994). Despite conflicting reports this growth factor was chosen for investigation as, unlike other growth factors older cells maintain the ability to respond to TGFβ1. In a study by Guerne et al. (1994), all three isoforms of TGFβ proved to be the most effective growth factor in old chondrocytes in comparison to IGF-1, FGF2 and PDGF. However, a recent study by Hickery et al. (2003) suggests that TGFβ1 may not enhance proteoglycan synthesis in mature cells. This report suggests that differences in sensitivity to growth factors such as TGFβ1 might be due to differences in ECM composition. Since we know that substantial differences in the ECM structure develop with age this theory may be plausible.

It is well accepted that cellular ageing is accompanied by a decreased ability to divide and respond to growth factors. The reasons and mechanisms for this are still to be determined. Most hypotheses centre on an alteration in the expression of the specific growth factor receptors and/or the affinity of the ligand for the receptor. Ribault et al. (1998) demonstrated in the case of EGF that the number of these receptors decreased on the surface of rat articular chondrocytes with increasing age. Martin et al. (1997) also showed that there are fewer binding sites for IGF-1 in ageing chondrocytes but the affinity of the ligand for its receptor is not altered. In the case of IGF-1 this decrease in responsiveness by the ageing chondrocytes may be due to the increasing numbers of IGFBPs, especially 3 and 4 (Martin et al., 1997). These binding proteins would, in theory, compete at the cell surface for IGF-1 binding to its receptor, resulting in reduced IGF-1 activity. This work is supported by *in vivo* studies where a diminished sensitivity to growth factors is seen in osteoarthritic chondrocytes directly due to increased expression of IGFBPs (Dore et al., 1994; Tardif et al., 1996). Osteoarthritic chondrocytes are less responsive to the anabolic effects of IGF-1 even if there is increased availability of the growth factor within the matrix

and synovial fluid (Chevalier and Tyler, 1996; Dore et al., 1994; Tavera et al., 1996). Messai et al. (2000) demonstrated that old rat chondrocytes maintain the ability to bind IGF-1 the ligand to its specific receptors and suggested that despite this binding a decreased responsiveness is due to an altered intracellular signalling pathway. The activation of adenylcyclase and subsequently the production of cAMP result in the activation of PKC. However, levels of cAMP and therefore downstream effectors were much lower in the older cells suggesting less efficient signalling machinery in the older chondrocytes than the young cells.

Aims:

1. Determine the effects of IGF-1 and TGFβ1 on the biosynthesis of proteoglycans and collagen.
2. Confirm that the biosynthetic effects of IGF-1 and TGFβ1 are accompanied by maintenance of the chondrocytic phenotype.
3. Investigate changes in target gene expression, including those encoding for IGF-1, TGFβ1 and their respective receptors induced by these growth factors at the mRNA level.

4.2 Materials

All materials were obtained from Sigma Chemical Company, UK unless otherwise stated within the protocol.

4.3 Methods

4.3.1 Isolation and Culturing of Chondrocytes from Bovine Cartilage

Chondrocytes from young and mature cartilage were isolated and resuspended in basal media as previously described in section 2.3.2. Cells were seeded into 48 well culture plates at a concentration of 5 x 10^5 cells/well and left overnight to stabilise.

4.3.2 Labelling of Chondrocytes with ^{35}Sulphate and ^{3}H-proline

Subsequent to the stabilisation period, media was aspirated off the chondrocytes and replaced with 500µl of basal media containing 1 of the following:

1. 10ng/ml IGF-1
2. 10ng/ml TGFβ1
3. 10ng/ml IGF-1 and 10ng/ml TGFβ1

Media alone served as a control. All treatment groups were further supplemented with 10µCi/ml (microcuries) $^{35}SO_4$ and 20µCi/ml ^{3}H-proline (GE Healthcare Ltd., UK) and incubated for periods of 1, 3 or 7 days.

4.3.3 Calculating Proteoglycan and Collagen Biosynthesis Using ^{35}Sulphate and ^{3}H-Proline

A measure of the biosynthesis of sGAG (representative of proteoglycan biosynthesis) and collagen was calculated within the media (radiolabel secreted by the chondrocytes) and also within the cell lysate (radiolabelled intracellular and extracellular associated material).

4.3.3.1 Equilibriation of Ultrafree®-MC Centrifugal Filter Units

Unincorporated radiolabel was removed from media and lysate samples using Ultrafree®-MC centrifugal filter units (Millipore (UK) Ltd., UK). These filter units contain a regenerated cellulose membrane with a cut-off value of 10KDa. Prior to addition of the sample, the units required equilibriation using a buffer consisting of 1% BSA and 1% sucrose. Units were centrifuged with 400µl of the buffer for 20 minutes at 2000 x g at room temperature. The buffer was aspirated from both the upper and lower chambers before addition of the sample.

4.3.3.2 Removal of Unincorporated Radiolabel using Ultrafree®-MC Centrifugal Filter Units

4.3.3.2.1 Processing of Media Samples

Protease inhibitor cocktail (500µM AEBSF hydrochloride, 150nM aprotinin, 1µM E-64 protease inhibitor, 0.5mM EDTA disodium, 1µM leupeptin hemisulphate), 4µl of a 1 x stock (Calbiochem®, UK), was added to the culture media before aspiration from the 48 well plate. This prevented any breakdown of newly synthesised matrix molecules within the sample. The aspirated media was transferred to a pre-equilibriated Ultrafree®-MC centrifugal filter unit and centrifuged for 20 minutes at 2000 x g at room temperature. A further 5 x 20 minute spins were performed with the addition of 100µl of PBS (pH 7.4) to the upper chamber between each centrifugation step (work by Dr E. Blain and Dr S. Gilbert within our laboratory had previously demonstrated that 5 wash spins was sufficient to remove all unincorporated label from media samples). After the removal of unincorporated radiolabel, a 1µl aliquot of sample remaining in the upper chamber was removed for scintillation counting.

4.3.3.2.2 Processing of Cell Lysates and Associated Material

Cultured chondrocytes were lysed in 0.9% Triton® X 100 containing protease inhibitors at the above concentrations. The cells and their associated matrix were removed from the 48 well plate and transferred to pre-equilibrated Ultrafree®-MC centrifugal filter units. The

culture plates were scraped with a pipette tip to ensure any matrix remaining bound to the plastic was removed and included within the subsequent experiments. The aspirated lysates were spun for 20 minutes at 2000 x g at room temperature. A further 4 x 20 minute spins were performed with the addition of 100µl of PBS (pH 7.4) between centrifugation steps. After complete removal of the unincorporated radiolabel, a 1µl aliquot was removed from the upper cup for scintillation counting.

4.3.3.2.3 Calculating Collagen Biosynthesis

Tritiated proline served as a measure of total protein biosynthesis within the media and cell lysate samples. As discussed within section 1.4.4.3, all collagens are characterised by the presence of continuous or interrupted triple helices containing the amino acid repeat sequence Gly-X-Y, where X and Y are typically proline or hydroxyproline. The use of tritiated proline in calculating protein biosynthesis within chondrocytes therefore biases the results towards collagen biosynthesis. To accurately calculate what percentage of the total protein biosynthesis was in fact collagen, samples previously processed through the filter units to remove unincorporated radiolabel were collagenase digested and run through the filter units again to remove the digested collagen. A measurement of non-collagenous protein could then be calculated and subtracted from the total protein value in order to ascertain the amount of collagen synthesised. Data are expressed in counts per minute (CPM).

A 50µl aliquot of the filtered media or cell lysate sample was removed and digested with 8U of type III bacterial collagenase, isolated from *Clostridium histolyticum*, (Worthington Biochemical Corporation, NJ, USA) overnight at 37°C with agitation. Post-digestion, 10µl was removed and counted using the scintillation counter. The remaining 40µl was added to the top of a pre-equilibrated filter unit and processed using the methods outlined in section 4.3.3.2.1 for media and section 4.3.3.2.2 for cell lysates. A 10µl aliquot of the processed sample was then removed for scintillation counting.

The mean $\pm$ the SEM were calculated for both the sGAG and collagen biosynthesis samples. All data were assessed for normal distribution using the Anderson-Darling test

(P>0.05). Statistical significance was calculated using a one-way ANOVA and Bonferroni post-test where P<0.05.

4.3.4 Calculating Cytotoxicity and Total Cell Number Using the Cytotox 96® Assay

As previously described in section 2.3.6, the Cytotox 96® assay can be used to calculate both total cell number and cytotoxicity by measuring LDH levels within cell lysates and culture media respectively.

Samples generated from a 'cold' experiment were run alongside the radiolabelled samples. As in the original setup described in section 4.3.1 and 4.3.2, chondrocytes were cultured for 1, 3 or 7 days in the presence of 10ng/ml of IGF-1, TGFβ1 or both growth factors. Media alone served as a control. No radiolabel was added to this experiment. The media was aspirated off the plates and the cells lysed in 0.9% Triton® X 100. LDH levels were measured within both the culture media and cell lysates to determine whether IGF-1 and/or TGFβ1 treatment was cytotoxic or effected total cell number at these time points. The mean ± SEM was calculated for each treatment group. The data were assessed for normality and statistical analysis performed using the methods outlined in section 4.3.3.2.3.

4.3.5 Confirming the Chondrocytic Phenotype by Western Blotting for Type II Collagen

4.3.5.1 Separation of Proteins Using SDS-PAGE

SDS-PAGE allows the separation of proteins within a sample according to relative molecular weight. Prior denaturation using sample buffer containing the anionic detergent SDS allows dissociation of proteins into their individual polypeptide subunits and minimises aggregation (Laemmli, 1970).

For the separation of collagen within the samples a 7.5% resolving gel was used with a 4% stacking gel (see table 4.1). Gel mixtures were polymerised with the final addition of tetramethylethylenediamine (TEMED).

Table 4.1 SDS-PAGE gel mixes for 7.5% resolving and 4% stacking gels

Gel Component	Resolving Gel (7.5%)	Stack Gel (4%)
Distilled Water	8.16ml	4.075ml
10% SDS	100µl	50µl
10% APS †	75µl	37µl
Tris-HCl pH 8.8	3.63ml	-
Tris-HCl pH 6.8	-	1.3ml
40% Bisacrylamide	2.72ml	575µl
TEMED	15µl	7.5µl

† Abbreviation of ammonium persulphate

Samples were generated from a 'cold' experiment similar to that described in section 4.3.4. Chondrocytes were cultured for 1, 3 or 7 days in the presence of 10ng/ml of IGF-1, TGFβ1 or both growth factors. Media alone served as a control. No radiolabel was added to this experiment. At the end of the culture period, the treatment containing media was replaced and the chondrocytes cultured for a further 3 days. The media was aspirated off the plates and the cells lysed in 0.9% Triton® X 100. Previous work within the laboratory demonstrated that matrix macromolecule biosynthesis is increased by this treatment and therefore generating enough protein for successful detection using Western blotting.

The cell lysates and their associated ECM material were denatured and reduced in 2 x sample buffer (0.06M Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (w/v) glycerol, 2mg/ml Bromophenol Blue) containing 2.5% β-mercaptoethanol for 30 minutes at 60°C. This ensured that any pro-collagen present would be reduced to its constitutive α chains. Samples (15µl) and a bovine type II collagen standard (5µl) were run on a 7.5% resolving SDS-PAGE gel at 150V in 1 x Laemmli buffer [see appendix 5.1.3] until the dye approached the bottom of the gel. A molecular weight marker (Biorad Laboratories Ltd., UK) was loaded for the identification of proteins and their relative molecular sizes. Each gel was run in duplicate, acting as a positive and negative control for binding of the secondary antibody.

4.3.5.2 Western Blotting for Type II Collagen

Samples were transferred from the SDS gel onto Immobilon-P PVDF transfer membrane in transfer buffer at a constant 20V overnight. As detailed in section 2.3.5.2, on completion of transfer, membranes were washed 3 times over 30 minutes in blocking buffer with 3% skimmed milk powder to block any remaining binding sites before incubation with the primary antibody.

4.3.5.2.1 Primary Antibody – AVT-6E3

The primary monoclonal antibody, AVT-6E3 (courtesy of Dr. A Vaughan-Thomas, The Faculty of Veterinary Science, University of Liverpool, UK) was raised in mouse and specifically recognises an epitope within the triple helical domain of type II collagen as well as the ¾ fragment produced by collagenase degradation. Positive control membranes were exposed to AVT-6E3 at a 1:5 dilution in blocking buffer overnight on a roller at 4°C. Corresponding primary antibody negative controls were exposed to blocking buffer in the same conditions. On removal of the primary antibody, all membranes were soaked in blocking buffer for 1 hour with 3 changes and subsequently exposed to the secondary antibody (sheep anti-mouse) conjugated to HRP (1:10,000 diluted in blocking buffer) for detection, for 2 hours at room temperature on a roller. The membranes were again washed in blocking buffer for 2 hours with 6 changes before detection using the ECL™ method previously described in section 2.3.5.2.2. Membranes were exposed to Hyperfilm™ ECL™ overnight in an X-ray cassette before developing in standard solutions using an automatic developer.

4.3.6 Investigating Changes in mRNA Levels of Target Genes by Exogenous IGF-1 and TGFβ1

As described in section 4.3.2 isolated chondrocytes were incubated with 10ng/ml of IGF-1, TGFβ1 or both growth factors for periods of up to 7 days. As with those samples generated for the purpose of Western blotting, the samples required for RNA analysis had no radiolabel added to the culture media. Samples were otherwise treated the same and after the required incubation period the media was aspirated and the cells lysed in Trizol®

(Invitrogen Life Technologies Ltd., UK) for the extraction of RNA. The purpose of these experiments was to investigate the effects of these growth factors on a number of target genes within both chondrocytes isolated from young and mature cartilage.

4.3.6.1 Extraction of Total RNA

Isolated chondrocytes were resuspended at a concentration of 1 x 10^6 cells/ml. One ml of suspension was aliquoted into a 1.5ml microcentrifuge tube and centrifuged at 100 x g for 5 minutes to pellet the cells. The supernatant was replaced by 1ml of Trizol® and the suspension was left at room temperature for 5 minutes to lyse the cells. One hundred and seventy five µl of chloroform was added. The chloroform was mixed by inverting the tube and allowed to stand at room temperature for a further 3 minutes. The chloroform/Trizol® mixture was transferred to a phase-lock gel™ (PLG) tube (Eppendorf AG, Hamburg, Germany) and the RNA containing aqueous phase was separated by centrifugation at 13,000 x g for 2 minutes. The aqueous phase was then transferred to a fresh 1.5ml tube. All plastic-ware used was RNase free.

4.3.6.2 Precipitation of RNA

RNA was precipitated from the aqueous phase by the addition of an equal volume of isopropanol. The tubes were mixed by inversion and left at -20°C overnight to precipitate. The RNA pellet was isolated by centrifugation at 12,000 x g for 10 minutes at 4°C. The supernatant was removed and the pellet washed in 75% ethanol. The pellet was spun again at 7500 x g for 5 minutes at 4°C and the ethanol supernatant removed. The RNA pellet was air-dried for 5 minutes before resuspending in 43µl of RNase (ribonuclease) free water.

4.3.6.3 DNase Treatment of Extracted RNA

Isolated RNA (43µl) was treated with 1U DNase [deoxyribonuclease] (Promega, UK), 40U RNasin® recombinant ribonuclease inhibitor (Promega, UK) and buffered with 1 x DNase buffer (Promega, UK) to remove any genomic DNA contamination. The final volume of

50µl was heated to 65°C for 5 minutes to aid resuspension and then transferred to 37°C for 15 minutes before then cooling on ice and storing at -80°C.

4.3.6.4 Purification of DNase Treated RNA

The RNA was purified from any contaminating reagents such as DNases using the RNeasy® MinElute™ protocol for RNA cleanup (Qiagen, UK) (see fig.4.1). Briefly, an equal volume of RNase free water was added to the RNA suspension. Three hundred and fifty µl of buffer RLT (1% β-mercaptoethanol) was added and mixed thoroughly by vortexing. Two hundred and fifty µl of ice-cold 100% ethanol was added and mixed well by pipetting. The sample was transferred to an RNeasy® spin column and centrifuged for 1 minute at 8000 x g.

The eluate was disposed of and 500µl of buffer RPE was added to the column and spun again at 8000 x g for 1 minute. This step was repeated but the column was spun at 14,000 x g for 2 minutes to dry the membrane. The purified RNA was eluted by addition of 50µl of RNase free water to the spin column and centrifuging at 8000 x g for 1 minute. Purified RNA was stored at -80°C.

4.3.6.5 Confirming the Presence of RNA by Agarose Gel Electrophoresis (Sambrook et al., 1989)

The presence of ribosomal RNA was confirmed by running purified RNA samples, combined with a loading dye (10mM Tris-Cl, pH 7.5, 50mM EDTA, 10% Ficoll® 400, 0.255% Bromophenol blue, 0.25% Xylene Cyanol FF, 0.4% Orange G) (Promega, UK) at a 1:5 ratio, on a 1% agarose gel containing 0.5% (v/v) ethidium bromide. The gel was buffered in 1 x Tris-Borate ethylenediaminetetraacetic acid (TBE) (89mM Tris-Borate, 2mM EDTA, pH 8.3) and run at 50V for 2 hours. Molecular weights were estimated by running a 100bp DNA ladder (Promega, UK) on the same gel. The products were visualised using a transilluminator.

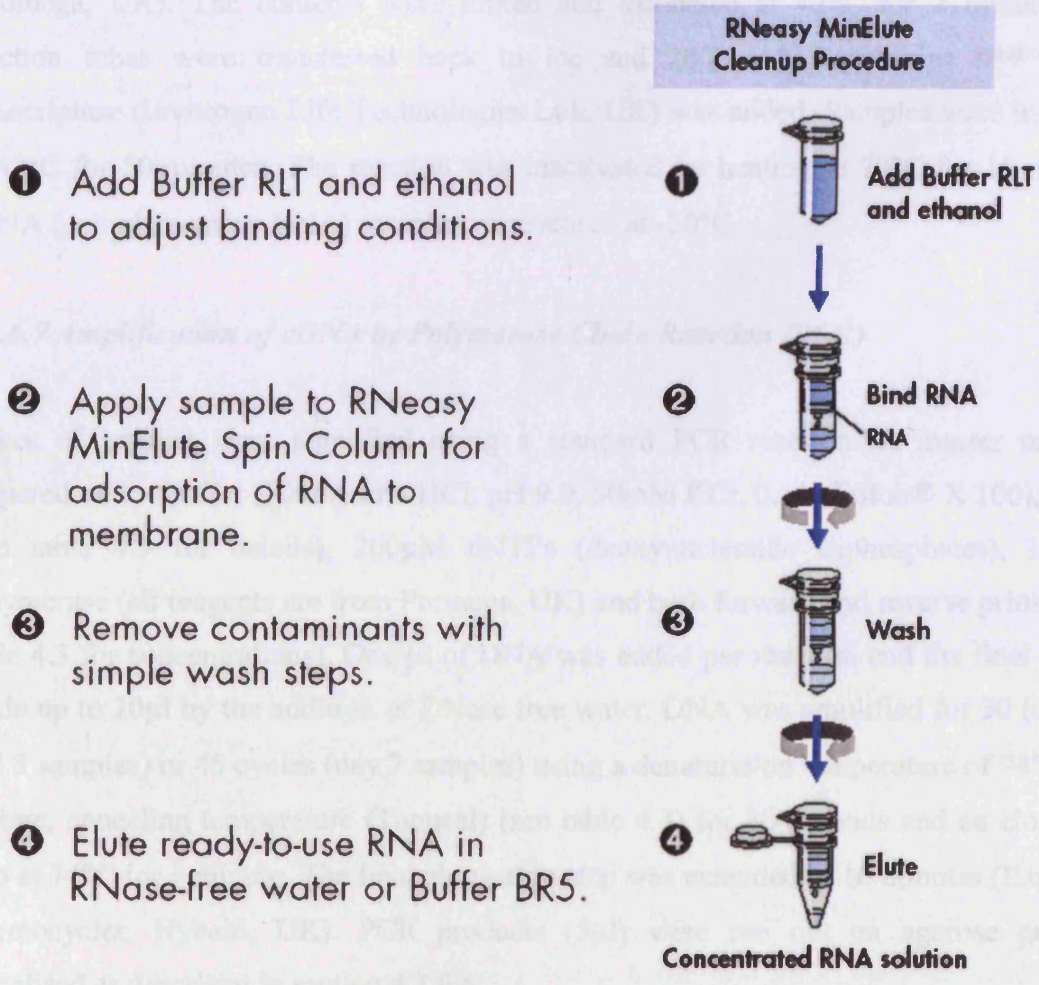


Fig.4.1 Summary of the RNeasy® MinElute™ cleanup procedure (reproduced from Qiagen, 2003)

4.3.6.6 Generation of cDNA by Reverse Transcription

Ten µl of RNA was primed with 0.5µg/µl oligo $(dT)^{15}$ (Promega, UK) at 65°C for 5 minutes. The reaction mix was immediately chilled on ice and a master mix of 4µl of 5 x first strand buffer, 2µl 0.1M DTT (dithiothreitol), and 1µl of RNAsin® was added (Promega, UK). The contents were mixed and incubated at 42°C for 2 minutes. The reaction tubes were transferred back to ice and 200U of Superscript II™ reverse transcriptase (Invitrogen Life Technologies Ltd., UK) was added. Samples were incubated at 42°C for 50 minutes. The reaction was inactivated by heating to 70°C for 15 minutes. cDNA (complementary DNA) samples were stored at -20°C.

4.3.6.7 Amplification of cDNA by Polymerase Chain Reaction (PCR)

Genes of interest were amplified using a standard PCR reaction. A master mix was prepared of 1 x buffer (10mM Tris-HCl, pH 9.0, 50mM KCl, 0.1% Triton® X 100), $MgCl_2$ (see table 4.3 for details), 200µM dNTPs (deoxynucleotide triphosphates), 1U Taq Polymerase (all reagents are from Promega, UK) and both forward and reverse primers (see table 4.3 for concentrations). One µl of DNA was added per reaction and the final volume made up to 20µl by the addition of DNase free water. DNA was amplified for 30 (day 0, 1 and 3 samples) or 45 cycles (day 7 samples) using a denaturation temperature of 94°C for 1 minute, annealing temperature (Tanneal) (see table 4.3) for 30 seconds and an elongation step at 74°C for 1 minute. The final elongation step was extended to 10 minutes (Express™ thermocycler, Hybaid, UK). PCR products (5µl) were run out on agarose gels and visualised as described in section 4.3.6.5.

Table 4.2 Forward and reverse primer sequences for the target genes, Sox 9, Col 2a/2b, IGF-1, TGFβ1, IGF-1 receptor and TGFβ1 receptor and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

[1] Primer sequence courtesy of Drs E.Blain and S. Gilbert (School of Biosciences, Cardiff University, UK)
[2] Primer sequence courtesy of Dr. I Khan (School of Biosciences, Cardiff University, UK)
[3] Primer sequence taken from Valcourt et al., 1999. Primers designed against the murine sequence for type II collagen and cross-react with the bovine sequence

Gene	Accession Number	Primer Sequence	Position (bp)
GAPDH[1]	U85042	F 5' TGG TCA CCA GGG CTG CTT TTA 3' R 5' CTC CTG CTT CAC CAC CTT CT 3'	14-34 759-739
Sox 9[2]	AF27803	F 5' CTC AAG GGC TAC GAC TGG AC 3' R 5' CGT TCT TCA CCG ACT TCC TC 3'	241-260 553-534
Col2a/2b[3]	-	F 5' GCC TCG CGG TGA GCC ATG ATC 3' R 5' CTC CAT CTC TGC CAC GGG GT 3'	-
IGF-1	X15726	F 5' GGA CCC GAG ACC CTC TGC GGG 3' R 5' GGC CGA CTT GGC GGG CTT GA 3'	145-165 354-335
TGFβ1	M36271	F 5' GCC CTG GAC ACC AAC TAC TGC TTC AGC TCC 3' R 5' GCT GCA CTT GCA GGA GCG CAC GAT CAT GTT 3'	612-641 947-918
IGF-1R	X54980	F 5' TCA AGG ACG GAG TCT TCA CC 3' R 5' GCT CAA ACA GCA TGT CAG GA 3'	1370-1389 1549-1530
TGFβ1-R	NM_174621	F 5' GGA CTT GCC CAT CTT CAC AT 3' R 5' CAC TCT GTG GTT TGG AGC AA 3'	967-986 1152-1133

Table 4.3 PCR reaction conditions and expected product sizes for the amplification of Sox 9, Col2a/2b, IGF-1, TGFβ1, IGF-1R, TGFβ1R and the housekeeping gene, GAPDH.

Gene	$MgCl_2$ Concentration (mM)	Primer Concentration (μM)	Tanneal (°C)	Product Size (bp)
GAPDH	2.5	0.25	60	746
Sox 9	1.5	1	53	313
Col2a/2b	2	0.2	60	2A-472 2B-268
IGF-1	2	0.25	65	210
TGFβ1	2	1.1	55	336
IGF-1R	2	1.7	52	180
TGFβ1R	2	1.7	52	186

4.4 Results and Discussion

4.4.1 The Effects of IGF-1 and TGFβ1 Treatment on Sulphated GAG Biosynthesis in Articular Chondrocytes

Radiolabelled sulphate ($^{35}SO_4$) was used to measure the effects of the anabolic growth factors, IGF-1 and TGFβ1 on sGAG biosynthesis. In this experiment it was possible to quantify levels of biosynthesis as well as the incorporation of sGAGs into a cell associated matrix by measuring levels of the radiolabelled compound in both the conditioned media and cell lysates with their associated material. Experiments using chondrocytes isolated from young articular cartilage demonstrated a significant increase in sGAG biosynthesis with TGFβ1 treatment at 24 hours when comparing levels of radiolabel within the control media [$P<0.01$] (see fig.4.2a). This trend was similar to the combined treatments of IGF-1 and TGFβ1 [$P<0.05$] (see fig.4.2a). No differences were seen at later time points between any of the treatment groups and their respective time point controls. Data obtained from the cell lysates and their cell associated material indicate a significant increase in radiolabel in the TGFβ1 treatment group compared to controls at the 3 day time point [$P<0.05$] (see fig.4.2b). Such a result would indicate that at this later time point no significant difference in sGAG is being seen with the conditioned media as this macromolecule is being laid down as ECM. Further results at the day 7 time point indicate no differences of sGAG concentrations between controls and treatment groups (see fig.4.2b). This may suggest that matrix being synthesised and laid down by the cells is being degraded at later time points.

Interestingly, the effects of these growth factors on articular chondrocytes isolated from skeletally mature cartilage did not reflect those demonstrated by the younger cells. In contrast to the diminished response expected by these more mature cells, a strong response was seen to both growth factors. Within the conditioned media samples a significant increase in sGAG biosynthesis was seen at 24 hours in response to all three treatments when compared to controls [$P<0.001$ in all treatment groups] (see fig.4.3a). This response was also maintained at the 3 day time point, although the statistical significance was lower [$P<0.05$ with IGF-1 treatment and $P<0.01$ in the TGFβ1 and combined treatment groups].

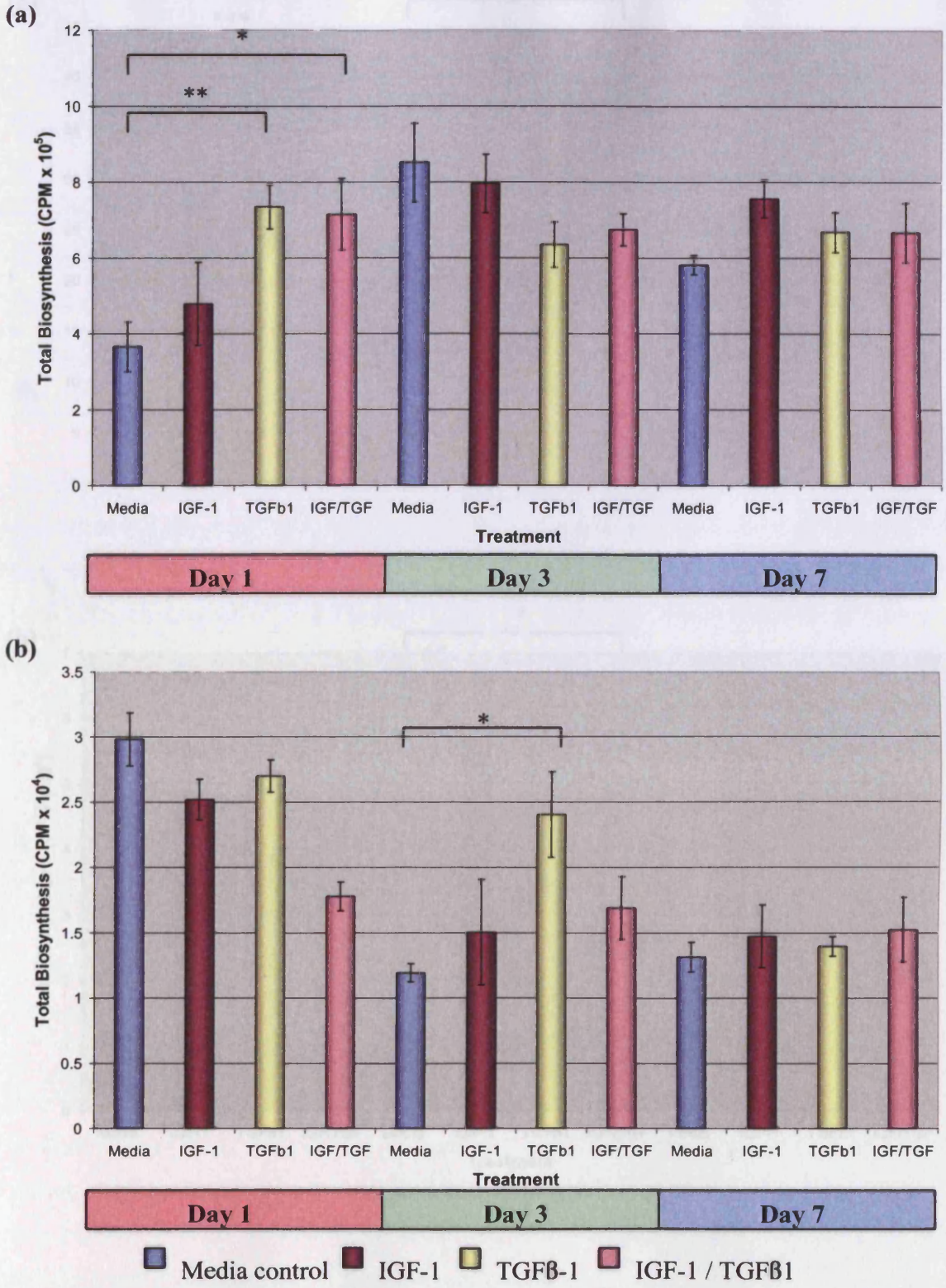


Fig.4.2 sGAG biosynthesis as assessed by incorporated $^{35}S0_4$ within (a) conditioned media and (b) cell lysates and associated material of treated chondrocytes isolated from young articular cartilage. Cells have been treated with IGF-1 and/or TGFβ1 and cultured for periods of up to 7 days. Media alone serves as a control. Values are expressed ± SEM. N=4
* P<0.05 ** P<0.01

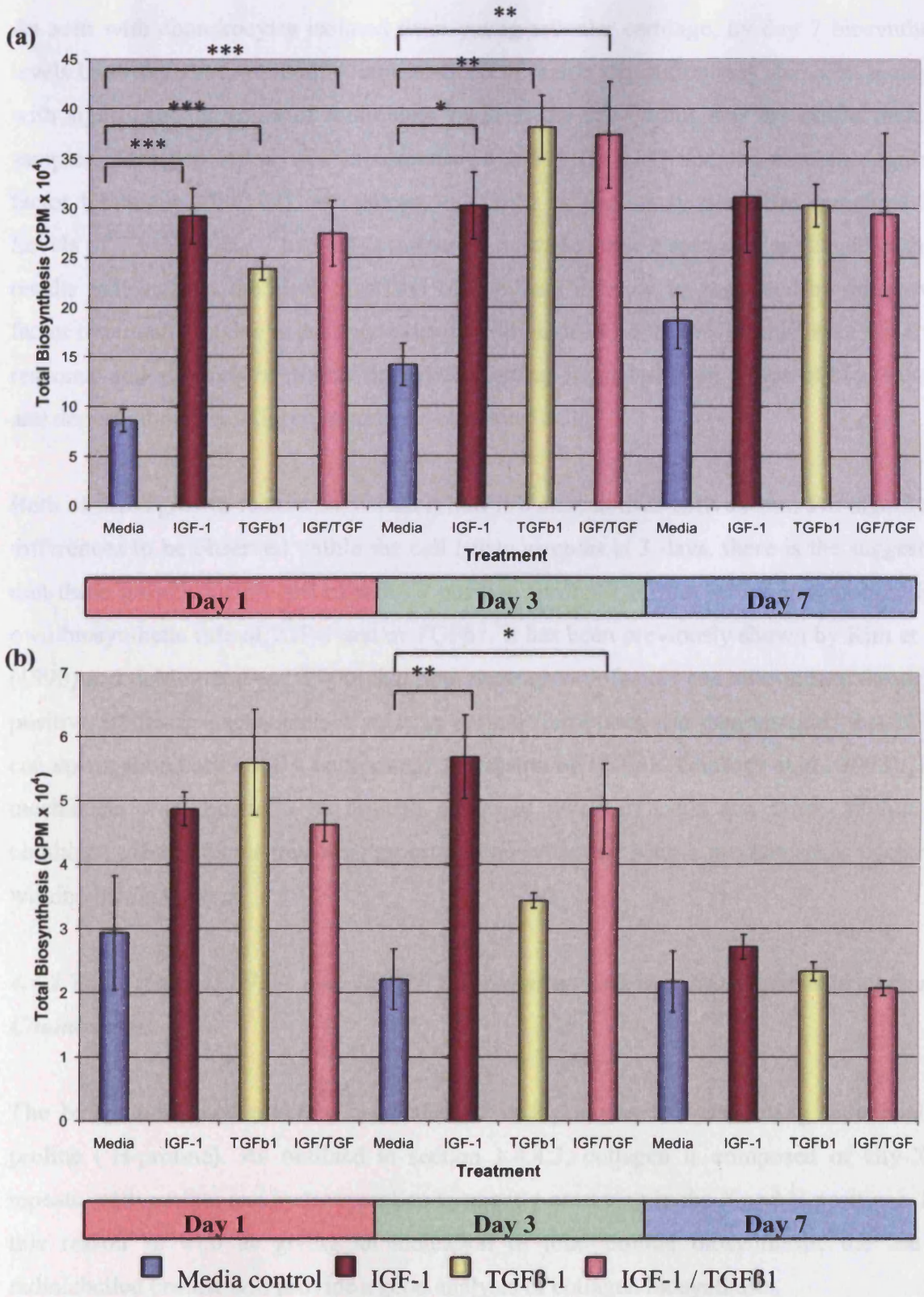


Fig.4.3 sGAG biosynthesis as assessed by incorporated $^{35}S0_4$ within (a) conditioned media and (b) cell lysates and associated material of treated chondrocytes isolated from mature articular cartilage. Cells have been treated with IGF-1 and/or TGFβ1 and cultured for periods of up to 7 days. Media alone serves as a control. Values are expressed ± SEM. N=4
* P< 0.05 ** P<0.01 ***P<0.001

As seen with chondrocytes isolated from young articular cartilage, by day 7 biosynthesis levels had returned to normal. A similar trend of matrix deposition was also seen at day 3, with significant increases of radiolabel found in the cell lysates and associated material samples. This deposition was in response to IGF-1 [$P<0.01$] and the combined growth factor treatments [$P<0.05$], in contrast to TGFβ1 as previously described (see fig.4.3b). Levels of sGAG at day 7 had also returned to normal within these samples. Together these results indicate that the level of sGAG biosynthesis is being up-regulated by the growth factor treatment and this is leading to the deposition of ECM. However, this is a short-lived response and as levels of matrix degradation are up-regulated these levels of biosynthesis and deposited matrix will return to those of control cells.

Both of these growth factors have a short half life once in their active form. For significant differences to be observed within the cell lysate samples at 3 days, there is the suggestion that these growth factors are exerting a positive feedback on the cells up-regulating their own biosynthetic rate of IGF-1 and/or TGFβ1. It has been previously shown by Kim et al., (1990) and deMoura at el., (2000) that both these growth factors can auto-induce through a positive feedback mechanism. A study in dermal fibroblasts also demonstrated that IGF-1 can up-regulate both mRNA and protein expression of TGFβ1 (Ghahary et al., 1998). This mechanism was shown to be through increased levels of c-fos and c-jun. Within the combined growth factor treatment group it is possible that such a mechanism is occurring within chondrocytes.

4.4.2 The Effects of IGF-1 and TGFβ1 Treatment on Collagen Biosynthesis in Articular Chondrocytes

The biosynthesis and possible deposition of collagen was analysed using radiolabelled proline (^{3}H-proline). As outlined in section 1.4.4.3, collagen is composed of Gly-X-Y repeats, with proline and hydroxyproline frequently occurring in the X and Y positions. For this reason as well as giving an indication of total protein biosynthesis, the use of radiolabelled proline will provide a good analysis of collagen biosynthesis.

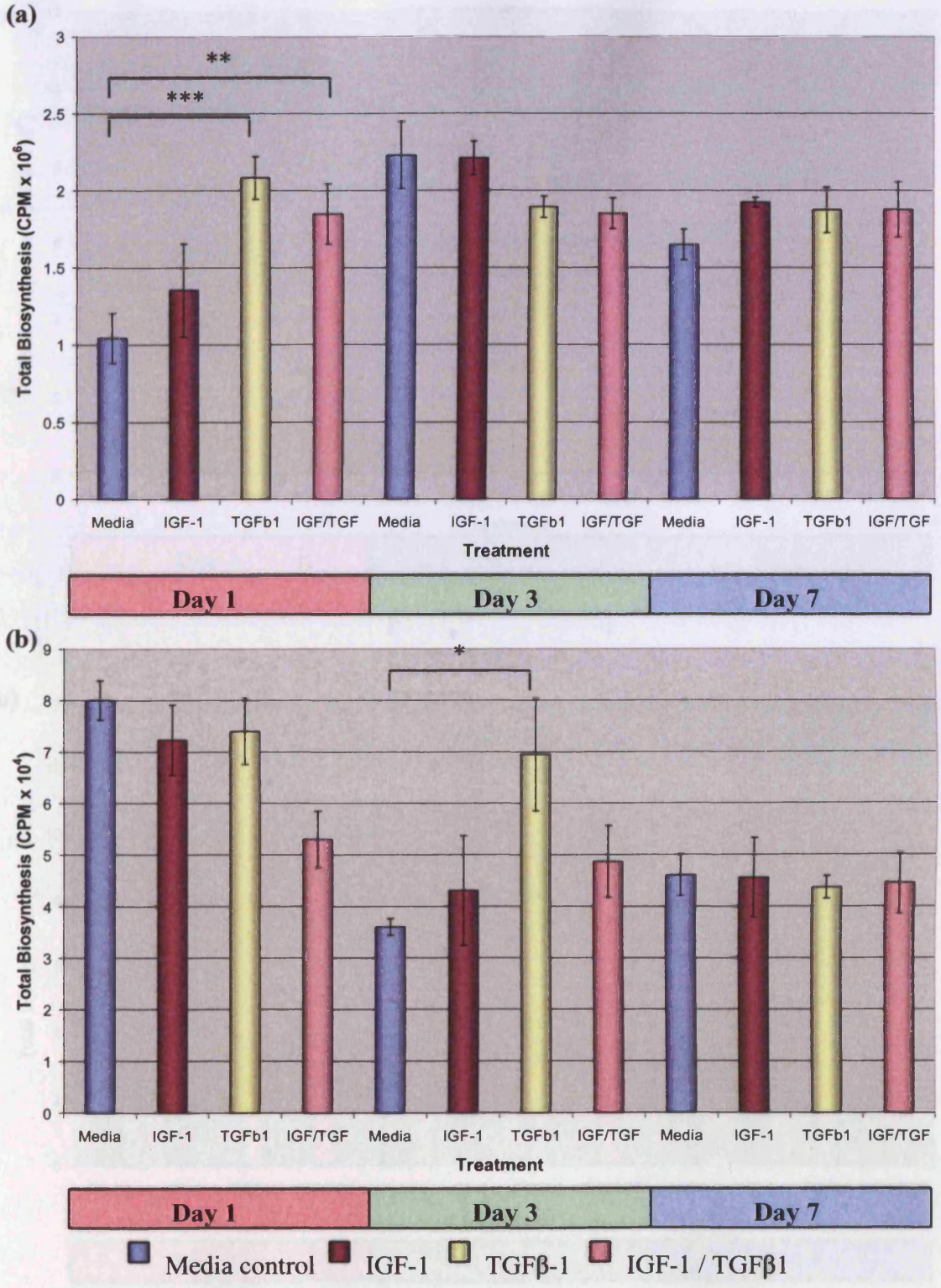


Fig.4.4 Collagen biosynthesis as assessed by incorporated ^{3}H-proline within (a) conditioned media and (b) cell lysates and associated material of treated chondrocytes isolated from young articular cartilage. Cells have been treated with IGF-1 and/or TGFβ1 and cultured for periods of up to 7 days. Media alone serves as a control. Values are expressed ± SEM. N=4
* P<0.05 ** P<0.01 ***P<0.001

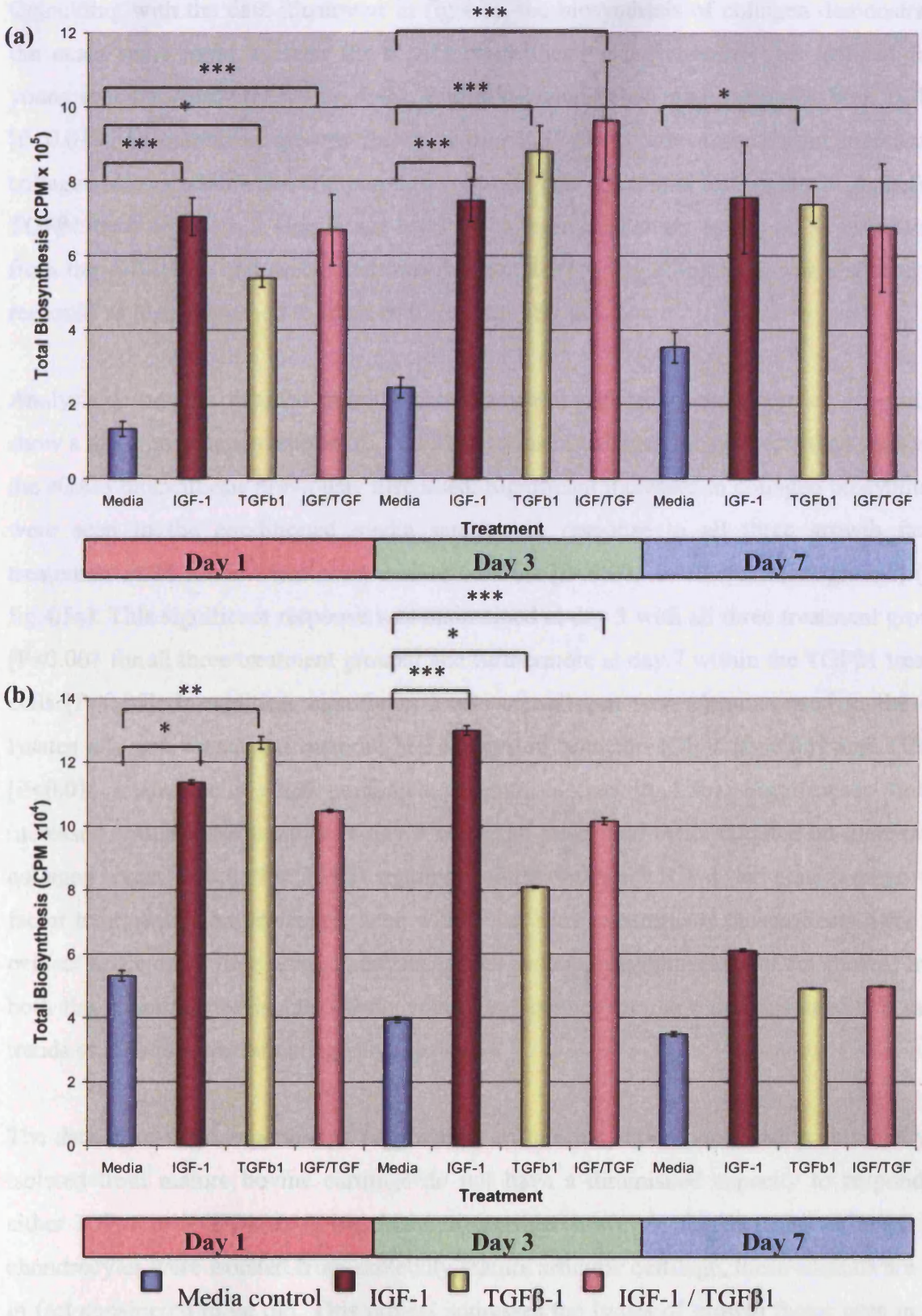


Fig.4.5 Collagen biosynthesis as assessed by incorporated ^{3}H-proline within (a) conditioned media and (b) cell lysates and associated material of treated chondrocytes isolated from mature articular cartilage. Cells have been treated with IGF-1 and/or TGFβ1 and cultured for periods of up to 7 days. Media alone serves as a control. Values are expressed ± SEM. N=4 * P<0.05 ** P<0.01 ***P<0.001

Coinciding with the data illustrated in fig.4.2a, the biosynthesis of collagen demonstrated the exact same trend as seen for sGAG biosynthesis using chondrocytes isolated from young articular cartilage (see fig.4.4a). Within the conditioned media samples, both TGFβ1 [$P<0.01$] and combined growth factor treatment [$P<0.05$] saw a significant increase in collagen biosynthesis when compared to controls. This effect was lost by day 3. As before, TGFβ1 treatment saw a significant level of collagen deposition at day 3, as ascertained from the cell lysate and associated material samples (see fig.4.4b). This was a short-lived response as levels returned to those of the controls by day 7.

Analysis of the data obtained from the chondrocytes isolated from mature articular cartilage show a more prolonged response to TGFβ1 in terms of collagen biosynthesis than seen with the sGAG biosynthesis previously discussed. Significant increases in collagen biosynthesis were seen in the conditioned media samples in response to all three growth factor treatments at 24 hours when compared to controls [$P<0.001$ in all treatment groups] (see fig.4.5a). This significant response was maintained at day 3 with all three treatment groups [$P<0.001$ for all three treatment groups] and furthermore at day 7 within the TGFβ1 treated cells [$P<0.05$]. In addition, significant levels of collagen were demonstrated in the cell lysates and cell associated material at 24 hours in both the IGF-1 [$P<0.05$] and TGFβ1 [$P<0.01$] treated cells when compared to controls (see fig.4.5b). Significance further increased within these samples at day 3 where all three treatments initiated an increase in collagen levels [$P<0.05$ for TGFβ1 treatment and $P<0.001$ for IGF-1 and combined growth factor treatments]. As previously seen within the other experiments these effects were not evident at the day 7 time point. Analysis of total protein biosynthesis (data not shown) from both the chondrocytes isolated from young and mature cartilage demonstrated the same trends as those shown for collagen biosynthesis.

The data from these experiments suggest that within this experimental setup chondrocytes isolated from mature bovine cartilage do not have a diminished capacity to respond to either IGF-1 or TGFβ1. It is important to consider however, the fact that although the chondrocytes were isolated from skeletally mature articular cartilage, these animals are not in fact considered to be old. This project addresses the issues of growth factor uses in the repair of articular cartilage defects within skeletally mature, but relatively young individuals that have obtained the injury through trauma or sport.

4.4.3 The Effects of IGF-1 and TGFβ1 Treatment on Chondrocyte Cell Number

In order to ascertain whether the effects seen by IGF-1 and TGFβ1 treatment (10ng/ml for both growth factors) were a true indication of the up-regulation of matrix biosynthesis, changes in cell number were calculated over the 7 day culture period. As previously discussed in section 4.1, these growth factors have been previously shown to up-regulate DNA synthesis and cellular proliferation levels (Darling and Athanasiou, 2005a; Trippel, 1995; Trippel, 1997; Vivien et al., 1992; Vivien et al., 1990). Chondrocytes cultured over periods of up to 7 days and treated with IGF-1 and/or TGFβ1 were lysed and their LDH levels measured using the Cytotox 96® assay.

No significant change in cell number was seen on treatment of either chondrocytes isolated from young (see fig.4.6a) or mature articular cartilage (see fig.4.6b) when compared to media controls. LDH levels in samples from chondrocytes isolated from young articular cartilage initially increased between 1 and 3 days in culture, although a statistical increase was only demonstrated in the TGFβ1 and combined growth factor treatment groups [$P<0.05$ for both treatments]. Significant decreases in LDH in all treatment groups were seen however between the 3 day and 7 day time periods [$P<0.001$ for all treatments]. In contrast to this, no increase in LDH was seen in the chondrocytes isolated from mature articular cartilage, with decreases in LDH being seen at each time point. All the treatment groups saw significant decreases in LDH between 1 and 3 days [$P<0.001$ for all treatments], and both the IGF-1 and TGFβ1 treatment groups saw further significant decreases in LDH between 3 and 7 days [$P<0.001$ for IGF-1 and $P<0.05$ for TGFβ1]. Decreasing levels of LDH within the cell lysates with time in culture may be indicative of a decrease in cell number. Data obtained from this experiment must be interpreted with caution though. Recent research suggests that within monolayer culture chondrocytes increase their oxygen consumption, decreasing their levels of glycolysis (Heywood and Lee, 2006). This may in turn affect levels of LDH found within the cells, with a down-regulation of the enzyme with time in culture. Such an effect may suggest that the data obtained from this experiment is due to the down-regulation of LDH expression, and not a decrease in cell number. In order to confirm that none of the growth factor treatments were exerting a toxic effect on the chondrocytes, the LDH levels within the conditioned media from these cells was also measured.

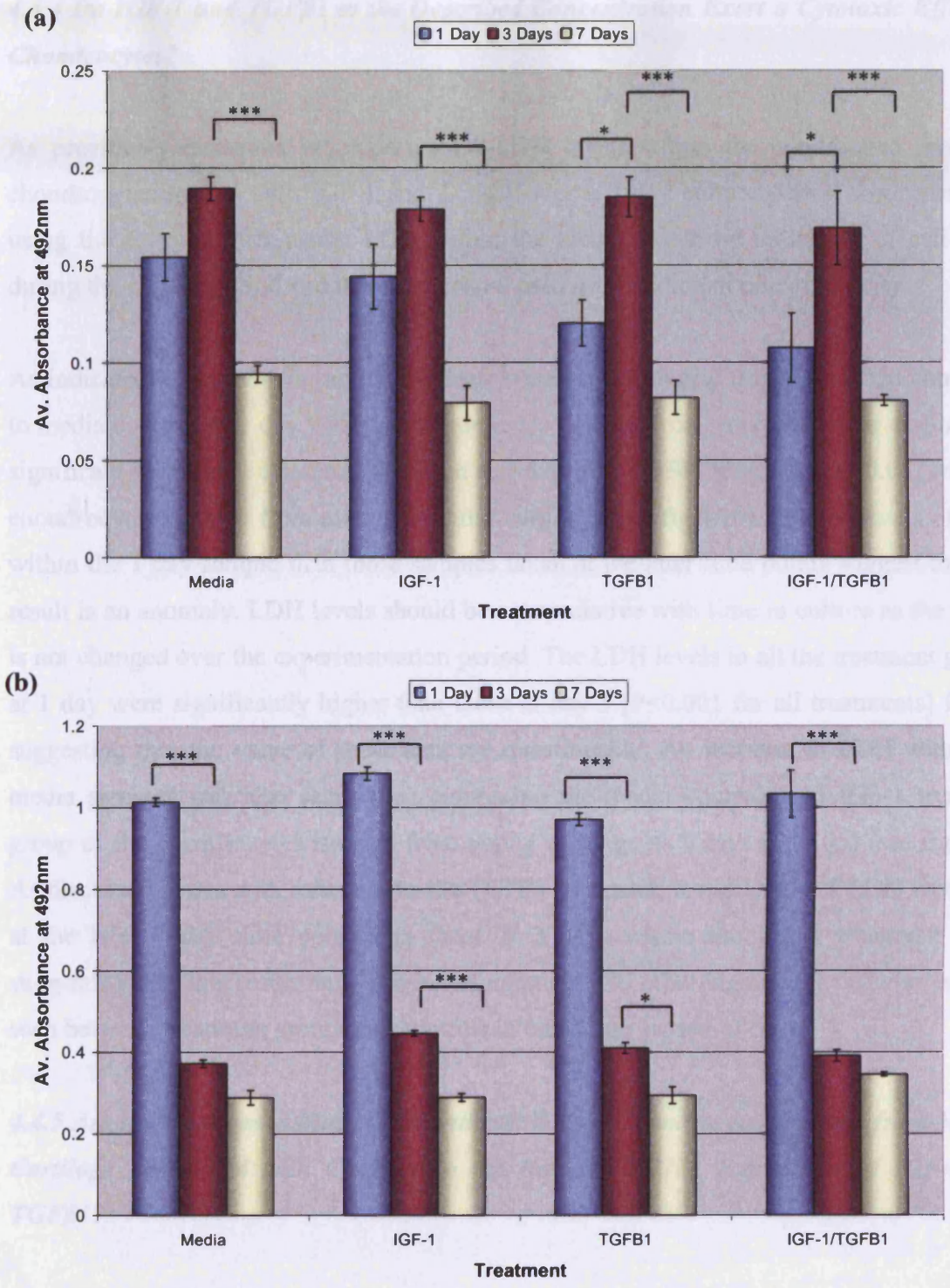


Fig. 4.6 The proliferation of chondrocytes isolated from (a) young and (b) mature articular cartilage in response to IGF-1 and/or TGFβ1 treatment. Average absorbencies are shown with standard error bars. N=4

* $P<0.05$

*** $P<0.001$

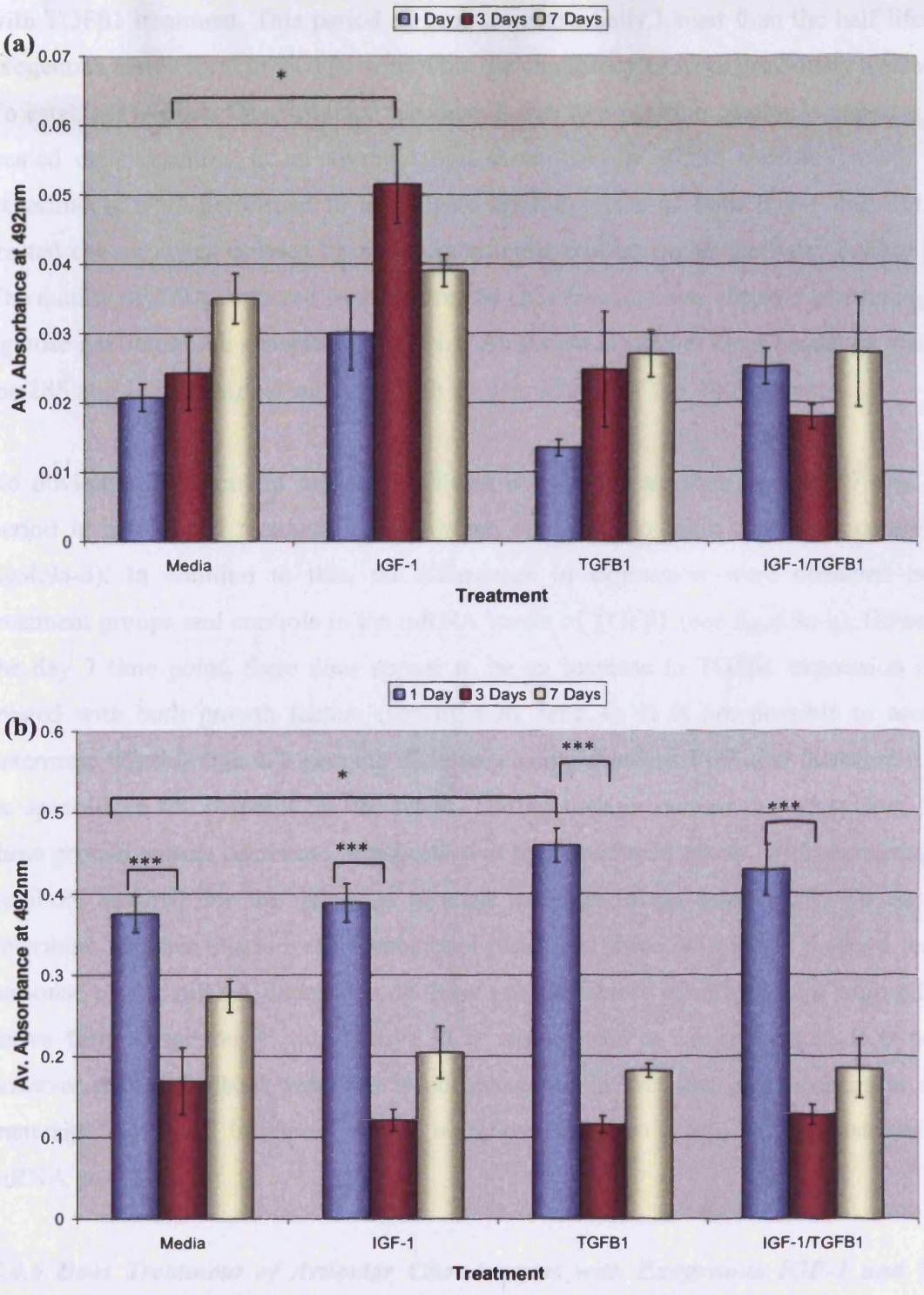


Fig.4.7 The toxic effects of IGF-1 and/or TGFβ1 treatment on chondrocytes isolated from (a) young and (b) mature articular cartilage. Average absorbencies are shown with standard error bars. N=4
* P<0.05
*** P<0.001

This response was sustained for periods of up to 7 days in the case of collagen biosynthesis with TGFβ1 treatment. This period of time is substantially longer than the half life of the exogenous active form of TGFβ1 with what the chondrocytes were previously treated with. To establish whether this sustained response is due to a positive feedback response on the treated cells resulting in an up-regulation in expression of the specific growth factor, experiments were performed to investigate mRNA levels of both IGF-1 and TGFβ1 in treated chondrocytes isolated from mature articular cartilage over the 7 day culture period. The quality of RNA extracted from the treated chondrocytes was checked by running on an agarose gel before the generation of cDNA. As shown in fig.4.8, clear bands are visible for the 28S and 18S ribosomal subunits, with no degradation of the RNA apparent.

No obvious differences in the mRNA levels of IGF-1 were seen over the 7 day culture period in any of the treatment groups when compared to their respective controls (see fig.4.9a-d). In addition to this, no differences in expression were observed between treatment groups and controls in the mRNA levels of TGFβ1 (see fig.4.9e-h). However, at the day 7 time point, there does appear to be an increase in TGFβ1 expression in cells treated with both growth factors (see fig.4.9h, lane 4). It is not possible to accurately determine whether this is a genuine difference using standard PCR and therefore it would be speculative to comment on this result. During time in culture the expression of both these growth factors decreased, irrespective of their treatment group, with increasing cycle numbers required for the detection of their message levels using PCR. To accurately determine whether this is a representational result and there is in fact a positive feedback response on the mRNA expression of these growth factors when cells are exposed to the active form exogenously, quantitative PCR would need to be performed. It is possible however that a feedback response is occurring but in fact this is happening at a post-transcriptional level, in which case no significant changes would be demonstrated at the mRNA level.

4.4.6 Does Treatment of Articular Chondrocytes with Exogenous IGF-1 and TGFβ1 Lead to an Increase in the mRNA Expression of Their Respective Receptors?

It has been previously demonstrated that the presence of TGFβ1 can, in chondrocytes, increase the level of expression of the IGF-1R (Tsukazaki et al., 1994). These experiments

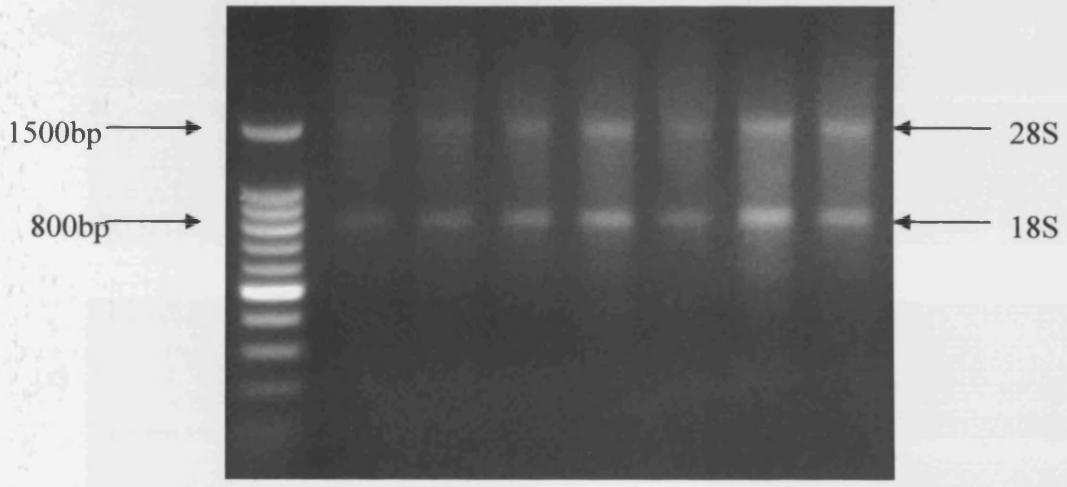


Fig.4.8 RNA extracted from chondrocyte monolayer cultures, separated on a 1% agarose gel. Bands representing the 28S and 18S ribosomal subunits have been marked. Molecular sizes were determined using a 100bp DNA ladder

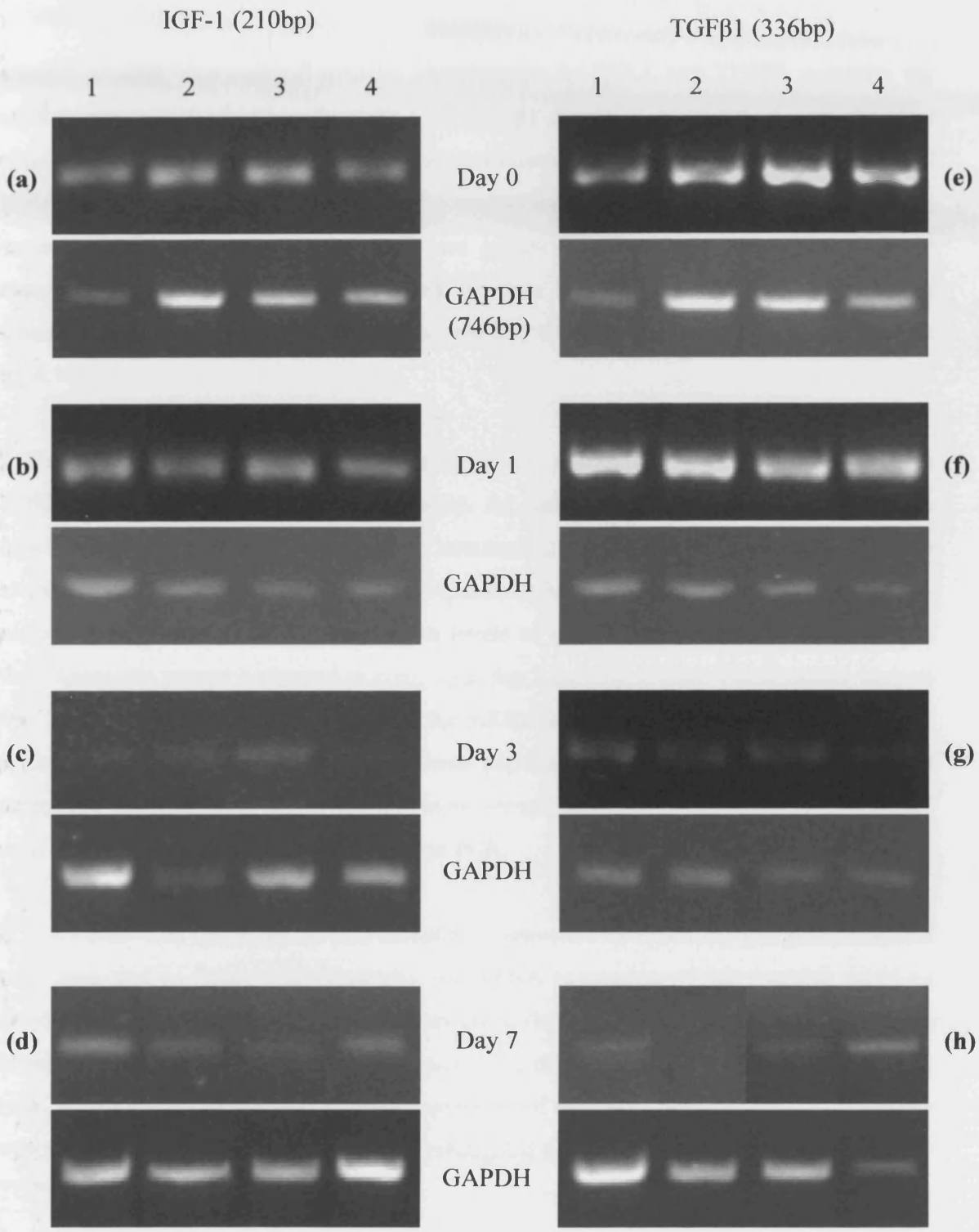


Fig.4.9 Comparing the relative mRNA expression of IGF-1 (a)-(d) and TGFβ1 (e)-(h) in chondrocytes isolated from mature articular cartilage over a 7 day culture period. Lane 1 indicates media controls, lane 2 IGF-1 treatment, lane 3 TGFβ1 treatment and lane 4 IGF-1 and TGFβ1 treatment. Relative levels of the house-keeping gene, GAPDH are shown below each panel.

address whether exposure of articular chondrocytes to IGF-1 and TGFβ1 regulates the mRNA expression of either the IGF-1 or TGFβ1 receptors. Coinciding with the data obtained for matrix biosynthesis discussed in sections 4.4.1 and 4.4.2, increased levels of IGF-1R mRNA was detected in the IGF-1 treated cells at day 3 when compared to both media controls and also the other treatment groups (see fig.4.10c). This effect was not apparent at day 7 however, corroborating the data from the radiolabelling experiments where any increases in biosynthesis were returned to basal levels by this time point (see fig.4.10d).

TGFβ1R expression was immediately affected by the treatment of chondrocytes with TGFβ1, either on its own or in combination. As figure 4.9e illustrates, TGFβ1R mRNA expression levels were lower within these treatment groups at day 0 (a 10 minute exposure to the growth factor). Repetition of this experiment has confirmed that these results were not due to experimental error. Expression levels of this receptor were also lower within these treatment groups compared to controls at day 3 (see fig.4.10g). These results suggest that exposure to TGFβ1 down-regulates the mRNA expression of its receptor on the cell surface of chondrocytes. At the day 7 time point mRNA levels of TGFβ1R were not detectable using PCR in any of the treatment groups (see fig.4.10h). These results would need to be further confirmed by quantitative PCR.

It is apparent that the levels of this receptor, irrespective of treatment group do diminish with time, and as figure 4.9h illustrates, no mRNA expression of this receptor could be detected at day 7. It is possible that this may occur due to the culturing of the chondrocytes in an artificial monolayer system, an unnatural 2-dimensional environment for this cell type. It is known that the cell surface expression of receptors does alter with monolayer culturing although no specific study has investigated this response in chondrocytes with the TGFβ1R.

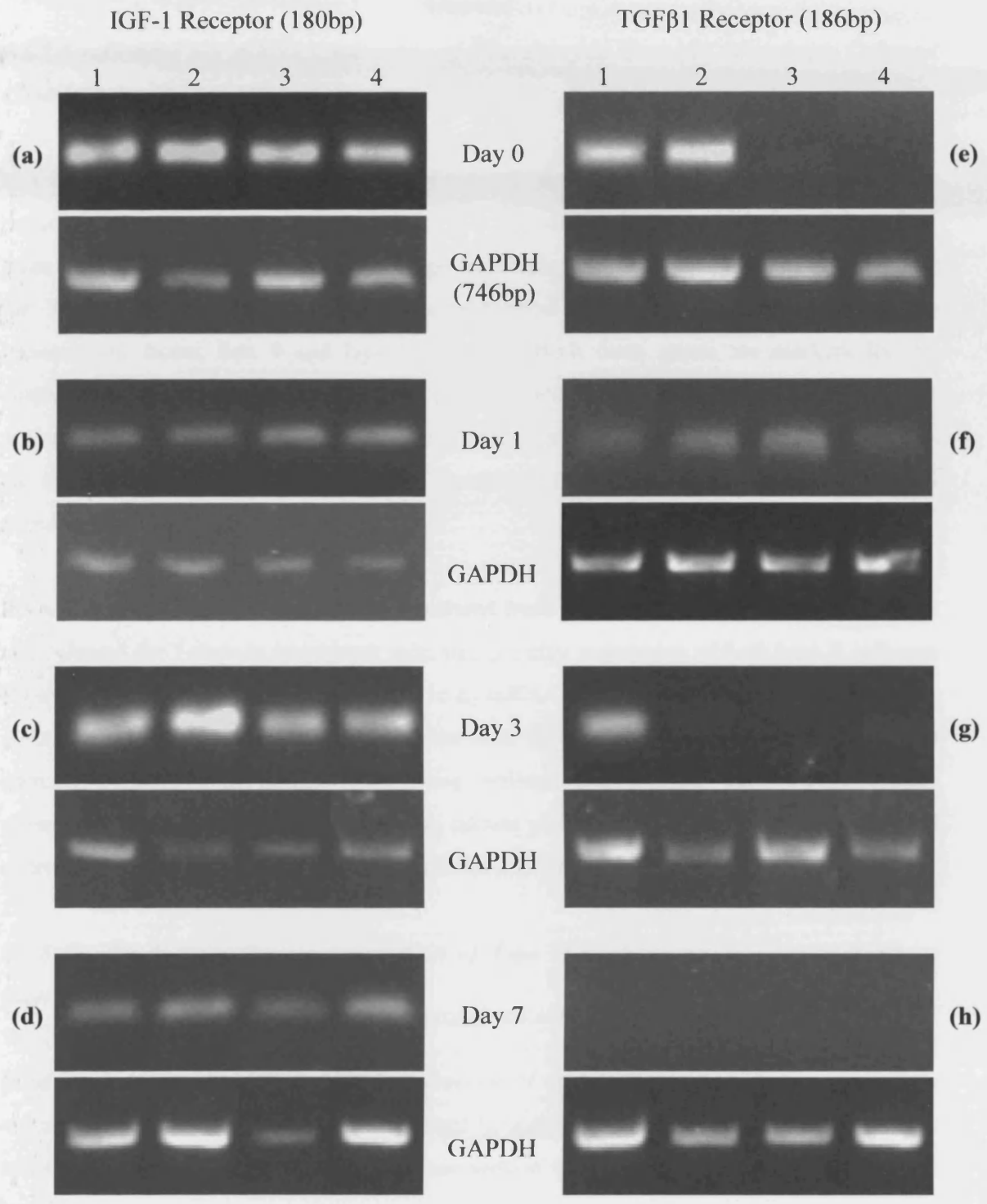


Fig. 4.10 Comparing the relative mRNA expression of IGF-1R (a)-(d) and TGFβ1R (e)-(h) in chondrocytes isolated from mature articular cartilage over a 7 day culture period. Lane 1 indicates media controls, lane 2 IGF-1 treatment, lane 3 TGFβ1 treatment and lane 4 IGF-1 and TGFβ1 treatment. Relative levels of the house-keeping gene, GAPDH are shown below each panel.

4.4.7 Confirming the mRNA Expression of Chondrocytic Genes in Monolayer Cultured Chondrocytes

It is widely accepted that chondrocytes are prone to dedifferentiation upon removal of their native ECM and culturing in monolayer (Darling and Athanasiou, 2005b). To confirm preservation of the chondrocytic phenotype in the chondrocytes which have been cultured for 7 days in monolayer, PCRs were performed using primers targeting both the transcription factor, Sox 9 and type II collagen. Both these genes are markers for the chondrocytic phenotype. In addition, the primer pair used for amplifying type II collagen will amplify both alternatively spliced forms of the gene, the embryonic type IIA as well as the mature form; type IIB. Expression of the former indicates dedifferentiation of the cell population.

RNA extracted from both chondrocytes isolated from young and mature articular cartilage and cultured for 7 days in monolayer indicated positive expression of both type II collagen B (see fig.4.11a-d) and Sox 9 (see fig.4.11e-h) mRNA. Only the results from chondrocytes isolated from mature articular cartilage have been shown, although an identical result was seen using chondrocytes isolated from young cartilage. This confirms that the chondrocytic phenotype was maintained throughout the culture period in both age groups of cells. No expression of the type II A form was detected at any time point.

4.4.8 Confirming the Protein Expression of Type II Collagen in Monolayer Cultured Chondrocytes

In addition to visualising the mRNA expression of chondrocytic genes within monolayer cultured chondrocytes, it was also important to confirm that the collagen synthesised, as measured previously by radiolabelling (see section 4.4.2) was the characteristic type II collagen.

Western blotting using a monoclonal antibody to type II collagen was performed using the cell lysates extracted from treated chondrocytes as described in section 4.3.5.2. As shown in figure 4.12a and b, type II collagen was being produced by both age groups of chondrocytes. When comparing to the type II bovine collagen standard, it is

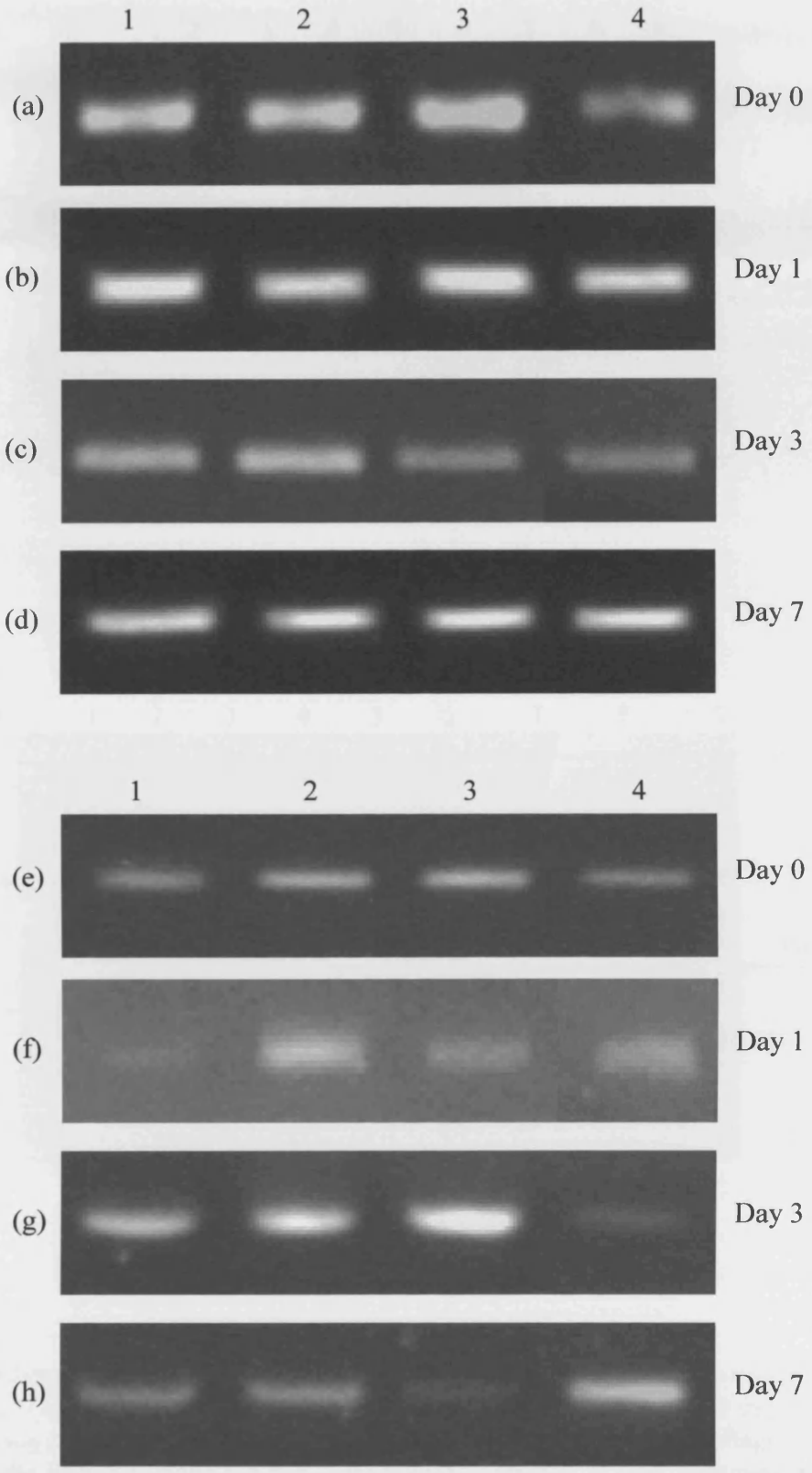


Fig.4.11 Confirming mRNA expression of type II collagen B [268bp] (a)-(d) and Sox 9 [313bp] (e)-(h) in chondrocytes isolated from mature articular cartilage over a 7 day culture period. Lane 1 indicates media controls, lane 2 IGF-1 treatment, lane 3 TGFβ1 treatment and lane 4 IGF-1 and TGFβ1 treatment.

apparent that the collagen being synthesised by both groups of cells is most likely to be pro-collagen. Here the pro-peptide of the collagen molecule has yet to be cleaved and therefore runs above the characteristic α1 chain on an SDS-PAGE gel. As the samples assessed for collagen content were cell lysates, the majority of the collagen was expected to be intracellular pro-collagen. It was interesting to note the low levels of type II collagen in control and IGF-1 treated chondrocytes from the young articular cartilage compared to the levels seen within both TGFβ1 treated groups. TGFβ1 obviously had a significant effect on type II collagen biosynthesis within these cells. In addition to this, extracellularly processed mature collagen was seen in these TGFβ1 treated young samples, which would have been from the ECM laid down by the cells and collected after lysis of the cells. No processing of pro-collagen into mature type II collagen was apparent with the chondrocytes isolated from mature articular cartilage, irrespective of their treatment. These results confirm that these monolayer cultured cells have maintained their chondrocytic phenotype, further supporting the data illustrated in section 4.4.7.

4.5 Conclusions

This chapter aimed to address the effects of the anabolic growth factors, IGF-1 and TGFβ1 as well as their combined treatment on the biosynthesis of sGAGs and collagen in chondrocyte monolayer culture systems. This work has demonstrated the ability of both factors to up-regulate the biosynthesis of these matrix molecules within chondrocytes isolated from young and mature articular cartilage, when used alone or in combination. It has also convincingly demonstrated that chondrocytes isolated from mature bovine articular cartilage do not have a diminished capacity to respond to these growth factors.

Data obtained from the radiolabelling experiments demonstrated an up-regulation of matrix biosynthesis in both age groups of cells with growth factor treatment (using conditioned media), and that significant levels were seen at the day 3 time point incorporated into the cell associated material extracted on lysis of the cells. With both age groups of cells however, matrix synthesis levels had returned to normal by the day 7 time point suggesting possible up-regulation of matrix degradation. The capacity of the chondrocytes isolated from mature articular cartilage to demonstrate increased biosynthetic levels of collagen and sGAG after the exogenous growth factor would have been depleted by natural half-life

turnover, suggested a possible positive feedback resulting in the cell up-regulating its own synthesis of the specific growth factor. PCR experiments using primers against both IGF-1 and TGFβ1 suggested that this may not be the case. However, this technique is non-quantitative and therefore experimentation using quantitative PCR would be required to confirm such an effect. It is also possible that a positive feedback effect is occurring but that this is post-transcriptional and therefore not detectable using the methods employed here.

The proliferative and toxic effects of these growth factors at the concentration used (10ng/ml for both growth factors) were investigated using the Cytotox 96® plate assay. No proliferative effects were seen with treatment confirming that the increased biosynthesis seen in the radiolabelling experiments was not as a result of increased cell numbers. Toxicity experiments suggested a significant increase in LDH levels in IGF-1 treated chondrocytes isolated from young articular cartilage at the 3 day time point and a similar effect with TGFβ1 at 1 day with the chondrocytes isolated from mature chondrocytes. Discrepancies between the data obtained at different time points may suggest that these results are anomalies.

Experiments looking at the relative mRNA expression of the growth factor receptors with treatment did suggest an up-regulation of the IGF-1R with IGF-1 treatment at the day 3 time point. Expression of the TGFβ1R was immediately affected by exogenous TGFβ1. A decrease in expression of the receptor was apparent 10 minutes after exposure to TGFβ1. Expression levels of the TGFβ1R were also lower at the 3 day time point within TGFβ1 treatment groups. These results suggest a down-regulation of the receptor in response to exposure to TGFβ1. Both PCR and Western blotting were used to confirm the phenotype of the treated chondrocytes after 10 days in culture. Sox 9 and type II collagen B mRNA expression was demonstrated at the 10 day time point in all treatment groups. Western blotting using a type II collagen antibody further confirmed production of type II pro-collagen in all of the samples.

In summary:

- The anabolic effects of IGF-1 and TGFβ1 on the biosynthesis of collagen and proteoglycans in both chondrocytes isolated from young and skeletally mature bovine articular cartilage was demonstrated.
- Synthesised matrix molecules were laid down into ECM by both age groups of chondrocytes.
- Western blotting and PCR for chondrocytic phenotype markers demonstrated maintenance of the chondrocyte characteristics within the cultured cells.
- Chondrocytes from skeletally mature articular cartilage were not seen to exhibit a diminished response to IGF-1 or TGFβ1.
- No proliferative effect was seen in either age group of cells by exposure to IGF-1 or TGFβ1.
- IGF-1R was suggested to be up-regulated on exposure to IGF-1, whereas TGFβ1 appeared to have a negative effect on the expression of its cell surface receptor (TGFβ1R).
- No changes in the expression of IGF-1 and TGFβ1 mRNA were seen in response to exposure to the growth factor exogenously.

Chapter 5

Over-expressing IGF-1 and TGFβ1 in Articular Chondrocytes

5.1 Background

This chapter addresses the possibility of over-expressing IGF-1 and TGFβ1 in chondrocytes isolated from young and mature articular cartilage. The data in chapter 2 demonstrated the chemotactic effect of both these growth factors on chondrocyte migration when the cells are seeded onto a fibronectin substrate. This chemotactic response appeared to be abolished in the presence of other ECM substrates such as type II collagen and aggrecan, suggesting a substrate dependent, chemotactic response. The effects seen in terms of both a chemotactic and biosynthetic response by the chondrocytes on exposure to IGF-1 and TGFβ1 would have been limited by the short half lives of these molecules. A limiting factor of this kind may well be overcome by over-expressing the molecule within chondrocytes.

As discussed in section 1.13.2 the key to biomechanically stable integration between pre-existing cartilage and any 'neo-cartilage' formed lies not only in the enhancement of cell migration but also in the stimulation and regulation of matrix biosynthesis to provide the appropriate composition and ratio of matrix molecules. In chapter 4, the data discussed demonstrated the ability of IGF-1 and TGFβ1 to enhance both proteoglycan and collagen biosynthesis while maintaining the chondrocytic phenotype. As seen with the effect on the chondrocytes isolated from mature articular cartilage, these growth factors have a short half life once in their active form, but may be able to initiate a positive feedback effect on the cells maintaining their stimulatory response for longer periods of time. In addition, it is well documented that maturation of chondrocytes leads to diminished sensitivity to growth factors with age (as discussed in section 1.15.8). Published data supporting this theory comes from the use of old chondrocytes, possibly explaining why a response to IGF-1 and TGFβ1 was still seen when using chondrocytes isolated from skeletally mature, but not old, articular cartilage. Combining the data achieved from the migration and biosynthesis experiments, the over-expression of IGF-1 and TGFβ1 may provide a solution to problems such as short half life and diminished sensitivity.

The successful transfection of chondrocytes has been demonstrated using both viral (Cucchiarini et al., 2005) and non-viral methods (Kaul et al., 2005; Madry et al., 2005). Non-viral methods, such as lipid-mediated gene transfer offer great advantages over viral methods in the context of *in vivo* gene delivery. The use of retroviruses, for example, carry

the risk of insertional mutagenesis, and adenoviruses the complication of immunogenicity (Madry and Trippel, 2000). Lipofectin® (Invitrogen Ltd., UK), a cationic liposome used within these experiments for transfection has the advantage of being easy and safe to use and removes the restriction of the size of DNA that can be delivered to the target cell (Gao and Huang, 1995). In the context of transfection for cell-mediated cartilage repair techniques, liposomal methods allow transient over-expression of the inserted gene for the relatively short time period during which integration will occur. The principle of this method is to use a liposome formulation that interacts with DNA, resulting in the formation of a lipid-DNA complex where the DNA is completely entrapped. These liposomes are able to fuse with the cell membrane and mediate uptake and subsequent expression of the DNA. The limiting factor of these lipid-mediated transfer methods is the low transfection efficiency associated with them. A study by Madry and Trippel (2000) compared the different lipid transfection systems on the market using both bovine and human articular chondrocytes. In this report they published that low transfection efficiencies within adult chondrocytes may be due to the low mitotic activity which they exhibit. They also emphasised the importance of optimising the ratio of lipid to DNA for transfection efficiency. A factor already raised by Caplen et al. (1995).

Within the context of application for clinical cases, the use of genes such as IGF-1 within localised areas of articular cartilage defects may offer the potential to enhance tissue healing and integration. The clinical application of soluble growth factors is limited though due to delivery problems. To deliver such a treatment in the concentrations and sustained time period required for any response would be to risk a reaction within non-target organs and therefore undesirable side effects (Evans et al., 2000). In such a context, the potential for delivery via genetic manipulation is huge. However, in order to provide sufficient concentrations of the gene for prolonged periods of time, a stable method of transfection would be required. The potential of IGF-1 and TGFβ1 has been demonstrated within numerous studies (Madry et al., 2005; Madry et al., 2001; Shuler et al., 2000; Smith et al., 2000) as well as the work presented within this thesis (chapters 2 and 4). TGFβ1 has been used in transfection studies with reference to both articular defects due to trauma and also OA. A recent study by Ulrich-Vinther et al. (2005) demonstrated the ability of adeno-associated viral transfer of TGFβ1 to normal chondrocytes to induce up-regulation of mRNA levels of type II collagen and aggrecan. In addition, MMP3 was seen to be down-

regulated, a response that they also demonstrated in human osteoarthritic chondrocytes. This study demonstrates the ability of TGFβ1 not only to increase anabolism within articular chondrocytes, but also to decrease the effect of degradative enzymes responsible for matrix degradation within degenerative diseases such as OA. These findings are not alone; studies by Lee et al. (2005) and Shuler et al. (2000) also demonstrated the up-regulation of matrix biosynthesis by transfection with TGFβ1 and the maintenance of the chondrocytic phenotype, as confirmed by the production of the characteristic cartilage matrix macromolecules, type II collagen and aggrecan.

The anabolic inducing characteristics of IGF-1 have also been widely demonstrated in articular chondrocytes. Recent reports have focussed on the ability of over-expressing IGF-1 and the ability of these transfected cells to enhance articular cartilage defects (Fortier et al., 2002; Madry et al., 2005; Madry et al., 2001). Madry et al. (2001) demonstrated that over-expression of human IGF-1 in chondrocytes induced both cell proliferation and matrix synthesis, culminating in new tissue formation when the transfected cells were transplanted onto the surface of articular cartilage explants. This study also suggested the possibility that the over-expression of IGF-1 within transplanted chondrocytes was able to induce activity within chondrocytes resident within the cartilage explants. Interestingly, the authors noted good integration between the newly formed tissue and the explant, which they have attributed to the IGF-1 over-expression and pre-treatment of the explant with chondroitinase ABC. A later study demonstrated IGF-1 to enhance the structural and functional integrity of tissue engineered cartilage as well (Madry et al., 2002). All these studies demonstrated the increased production of a type II collagen and aggrecan rich matrix confirming the maintenance of the chondrocytic phenotype. The chondroprotective effect of IGF-1 has already been noted as a response co-ordinated through the Sox 9 transcription factor (Kolettas et al., 2001).

This chapter focuses on the development of constructs for the over-expression of both IGF-1 and TGFβ1 in bovine articular chondrocytes. Whereas research already published has placed emphasis on the ability of over-expression of these growth factors to increase matrix biosynthesis and decrease matrix degradation, here the emphasis has been placed on whether over-expression of IGF-1 and TGFβ1 can induce an enhanced migratory response.

Aims:

1. To develop constructs for the over-expression of IGF-1 and TGFβ1 in chondrocytes isolated from young and mature articular cartilage.
2. Confirm protein and mRNA levels of over-expression and efficiency of transfection using a liposomal method of gene delivery.
3. Determine whether the over-expression of these growth factors enhances the migration of chondrocytes when seeded on chondroitinase ABC treated aggrecan.

5.2 Materials

All reagents were purchased from Promega, UK unless otherwise stated within the protocol.

5.3 Methods

5.3.1 Preparation of Plasmids Containing IGF-1 and TGFβ1

Clones encoding the mature peptides of bovine IGF-1 and TGFβ1 for over-expression in bovine chondrocytes were constructed from cDNA generated from bovine liver and cartilage respectively (cDNA was a generous gift from Dr. P Callender, School of Biosciences, Cardiff University, UK).

5.3.1.1 Polymerase Chain Reaction

Standard PCR reactions, as described in section 4.3.6.7, were employed to amplify the sequences encoding the mature peptides of bovine IGF-1 and TGFβ1 using the previously described primers (see table 4.3). Bovine liver and cartilage cDNAs were used as templates in reactions of 40 cycles.

5.3.1.2 Agarose Gel Electrophoresis

Amplification of the gene of interest was confirmed by running 5µl of the PCR reaction on a 2% agarose gel as previously described in section 4.3.6.5. Product sizes of 210bp and 336bp were expected for IGF-1 and TGFβ1 respectively.

5.3.1.3 PCR Product Purification

Amplified genes were purified using the QIAquick PCR purification kit (Qiagen, UK). This removes any primer dimers, buffer, unused nucleotides and polymerases from the reaction. This process has been summarised in fig. 5.1. As per the Qiagen handbook (Qiagen, 2002),

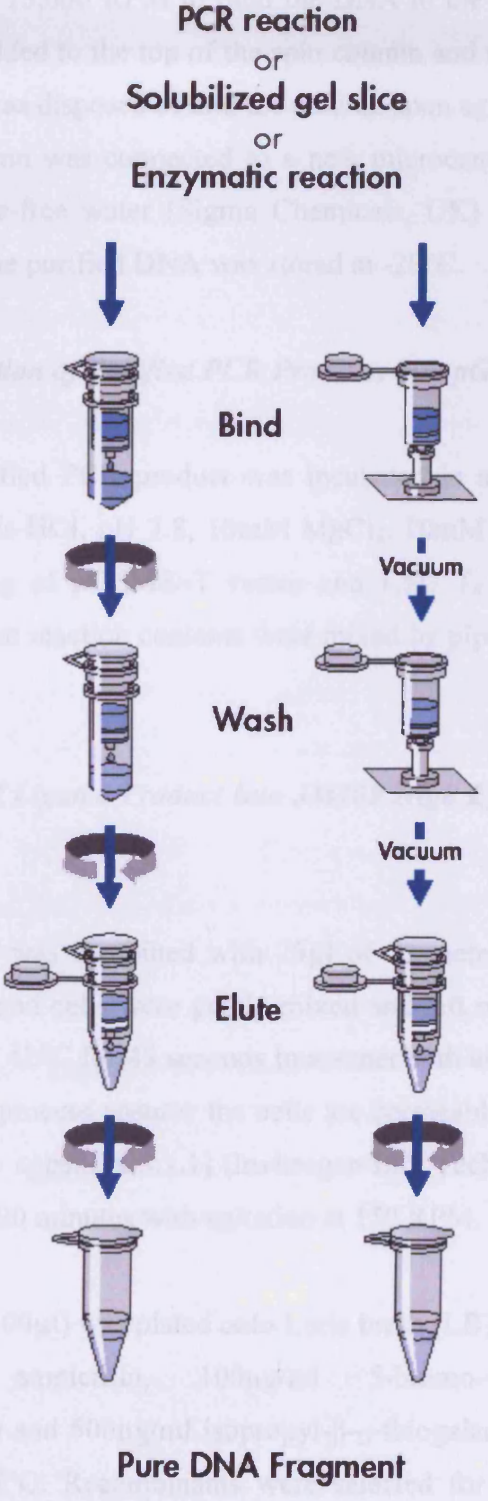


Fig.5.1 Summary of the QIAquick PCR purification procedure used for the removal of contaminants from PCR products (reproduced from Qiagen, 2002)

5 volumes of buffer PN was added to 1 volume of PCR product and pipetted directly onto the membrane of a QIAquick spin column connected to a 2ml collection tube. The column was spun for 1 minute at 13,000 RPM to bind the DNA to the membrane. Subsequently, 750µl of buffer PE was added to the top of the spin column and spun again at 13,000 RPM for 1 minute. The eluate was disposed of and the column spun again to remove any residual buffer PE. The spin column was connected to a new microcentrifuge tube and the DNA eluted in 200µl of DNase-free water (Sigma Chemicals, UK) by spinning for a further minute at 13,000 RPM. The purified DNA was stored at -20°C.

5.3.1.4 TA-Cloning: Ligation of Purified PCR Products into pGEM®-T Vector

A 1µl aliquot of the purified PCR product was incubated in a master mix of 1 x rapid ligation buffer (30mM Tris-HCl, pH 7.8, 10mM $MgCl_2$, 10mM DTT, 1mM ATP and 5% polyethylene glycol), 25ng of pGEM®-T vector and 1.5U T_4 DNA ligase with a final reaction volume of 5µl. The reaction contents were mixed by pipetting and left overnight at 4°C.

5.3.1.5 Transformation of Ligated Product into JM109 High Efficiency Competent Cells for Plasmid Purification

The ligated product (2µl) was combined with 25µl of competent JM109 *E.coli* (1 x 10^8 cfu/µg DNA). The DNA and cells were gently mixed and left on ice for 20 minutes. The cells were heat shocked at 42°C for 45 seconds in a water bath and returned back to ice for a further 2 minutes. This process ensures the cells are permeable to the vector and insert. SOC medium (475µl) [see appendix 3.1.1] (Invitrogen Life Technologies, UK) was added and incubated at 37°C for 90 minutes with agitation at 150 RPM.

The transformed culture (100µl) was plated onto Luria broth (LB) agar [see appendix 3.1.2] containing 100µg/ml ampicillin, 100µg/ml 5-bromo-4-chloro-3-indolyl β-$_D$-galactopyranoside (X-Gal) and 500µg/ml isopropyl-β-$_D$-thiogalactopyranoside (IPTG) and incubated overnight at 37°C. Recombinants were selected for using blue/white colony screening. Colonies containing the correct insert, white due to disruption of the lac Z gene

within the multiple cloning site (MCS) of pGEM®-T, were picked and grown up in 5ml of LB broth containing 100µg/ml ampicillin at 37°C overnight with agitation (225 RPM).

5.3.1.6 Plasmid DNA Purification

Amplified plasmid DNA was purified using the SV Wizard™ DNA purification mini-prep kit according to the manufacturers protocol. A 500µl aliquot of the bacterial culture was taken and combined with an equal volume of glycerol for storage at -80°C before the remaining DNA was purified. Bacterial cultures were pelleted by centrifugation at 3000 RPM for 10 minutes at room temperature. The pellet was resuspended in 250µl of cell resuspension solution (50mM Tris-HCl, pH 7.5, 10mM EDTA, 100µg/ml RNase A) and the mixture transferred to a sterile microcentrifuge tube. An equal volume of cell lysis solution (0.1M NaOH, 0.5% SDS) was added and the contents of the tube mixed by inversion. The solution becomes clear as the bacterial cell wall is destroyed and the plasmid is released. Alkaline phosphatase (10µl) was added, mixed by inversion and the mixture incubated for 5 minutes at room temperature. Alkaline phosphatase removes endonucleases and other proteins released during cell lysis which may interfere with the quality of the purified DNA. The reaction was terminated by the addition of 350µl of neutralisation solution (1.66M guanidine hydrochloride, 0.309M potassium acetate, 0.862M glacial acetic acid, final pH 4.2) and after thorough mixing the bacterial lysate was centrifuged at 14,000 RPM for 10 minutes at room temperature. The resulting supernatant was decanted into a mini-prep spin column and centrifuged at 14,000 RPM for 1 minute at room temperature to bind the DNA. The spin column membrane was washed twice with column wash solution (60mM potassium acetate, 8.3mM Tris-HCl, pH 7.5, 0.04mM EDTA, pH 8.0, 60% ethanol) by centrifugation at 14,000 RPM and the plasmid DNA eluted in 50µl of nuclease free water by centrifugation again at 14,000 RPM for 1 minute. Purified DNA was stored at -20°C.

5.3.1.7 Nco1 and Sal1 Restriction Digestion of Cloned Inserts

The restriction sites *Nco*1 and *Sal*1 within the MCS of the pGEM®-T vector were used to confirm insertion and cloning of the correct product. Purified plasmid DNA (0.5µg) was digested using 0.5U *Nco*1 and 0.5U *Sal*1 with 0.5µg/ml BSA, in 1 x digest buffer D (50mM

Tris-HCl, pH 7.9, 1.5mM NaCl, 60mM $MgCl_2$, 10mM DTT). The final reaction volume was made up to 20µl using DNase-free water. Restriction occurred at 37°C for 2 hours. Single digests using these restriction enzymes were also performed to confirm site specific cleavage. Restricted samples were run on 2% agarose gels alongside the undigested vector to confirm the presence of inserts. A 100bp DNA ladder was used to estimate the size of the insert.

5.3.1.8 Assessing the Concentration and Purity of Plasmid DNA

Spectrophotometry was used to determine the concentration and purity of the plasmid DNA obtained. Samples were diluted appropriately to keep absorbencies within the linear range, and transferred to a quartz cuvette. Absorbance was measured at 260nm (A_{260}) and 280nm (A_{280}). DNA concentration was determined using the extinction coefficients for DNA i.e. an absorbance of 1 is equal to 50µg/ml of DNA. Purity of the sample can be measured by calculating the ratio of A_{260} to A_{280}. A ratio of approximately 1.8 is expected for DNA, anything higher is indicative of ethanol contamination, and lower, protein contaminants.

5.3.1.9 DNA Sequencing

All sequencing was performed by an in house support facility (School of Biosciences, Cardiff University, UK) using the ABI Prism® Genetic Analyser (Applied Biosystems, UK). Sequencing reactions were prepared according to the ABI Prism® Big Dye Terminator Reaction Cycle Sequencing kit protocol. This involves the use of a ready reaction mix containing; dNTPs, AmpliTaq DNA Polymerase, $MgCl_2$ (at a concentration previously determined but not disclosed) and dye terminators. DNA samples were provided at a concentration of approximately 100µg/ml. The reaction mix consisted of 8µl of terminator ready reaction mix, 290ng of plasmid DNA, 2.4pmol of forward or reverse primer [for sequencing primers see appendix 4.1] and DNase-free water to make the reaction up to 20µl. Samples were briefly vortexed and spun before being subjected to 25 sequence cycles of 96°C for 20 seconds, 50°C for 10 seconds and 60°C for 4 minutes (Express thermocycler, Hybaid).

DNA extension products from the PCR were precipitated using an ethanol/sodium acetate solution (3µl 3M sodium acetate, pH 4.6, 62.5µl non-denatured 95% ethanol and made up to 80µl with deionised water) to remove any unincorporated Big Dye Terminators. The 20µl sample was added to 80µl of the ethanol/sodium acetate solution and briefly vortexed. The samples were incubated for 15 minutes at room temperature to allow precipitation to occur before spinning at 12,000 x g for 20 minutes. The pellet was washed in 250µl of 70% ethanol to remove any remaining residual salts before spinning again at 12,000 x g for 5 minutes. The pellet was air-dried for 10-15 minutes and resuspended in 20µl of HiDi® formamide (Applied Biosystems, UK) to denature the sample before running on the ABI Prism® 3100 Genetic Analyser (Applied Biosystems, UK). Resulting sequence chromatograms were viewed using the computer software program, Chromas, version 1.43 (Conor McCarthy, Griffith University, Brisbane, Australia). Text data were copied into Microsoft Word (and examined for homology on the NCBI database using the search engine, BLAST (www.ncbi.nlm.gov/BLAST).

5.3.2 Over-expression of IGF-1 and TGFβ1 in Primary Chondrocytes

In order to over-express the above growth factors in primary articular chondrocytes, the pGEM®-T cDNA clones needed to be subcloned into the mammalian expression vector, pSecTag2. Construction of this vector for transfection required multiple steps which have been summarised in fig. 5.2.

5.3.2.1 Amplification of Target DNA and Addition of Restriction Sites for Insertion into pSecTag2

To aid the insertion of the IGF-1 or TGFβ1 cDNA sequences into the expression vector, the DNA was amplified using primers designed to add restriction enzyme sites to both the 5' and 3' ends of the cDNA sequences. The use of different restriction sites at each end of the cDNA allowed directional cloning of the insert. The resulting PCR products were TA cloned into pGEM®-T to confirm sequence homology and in frame expression of the insert. DNA sequences of the inserts were also run through the Webcutter 2.0 (www.firstmarket.com/cutter/cut2.html) database to confirm that these restriction enzymes would not cut anywhere within the target sequence. The original primer sequences for both

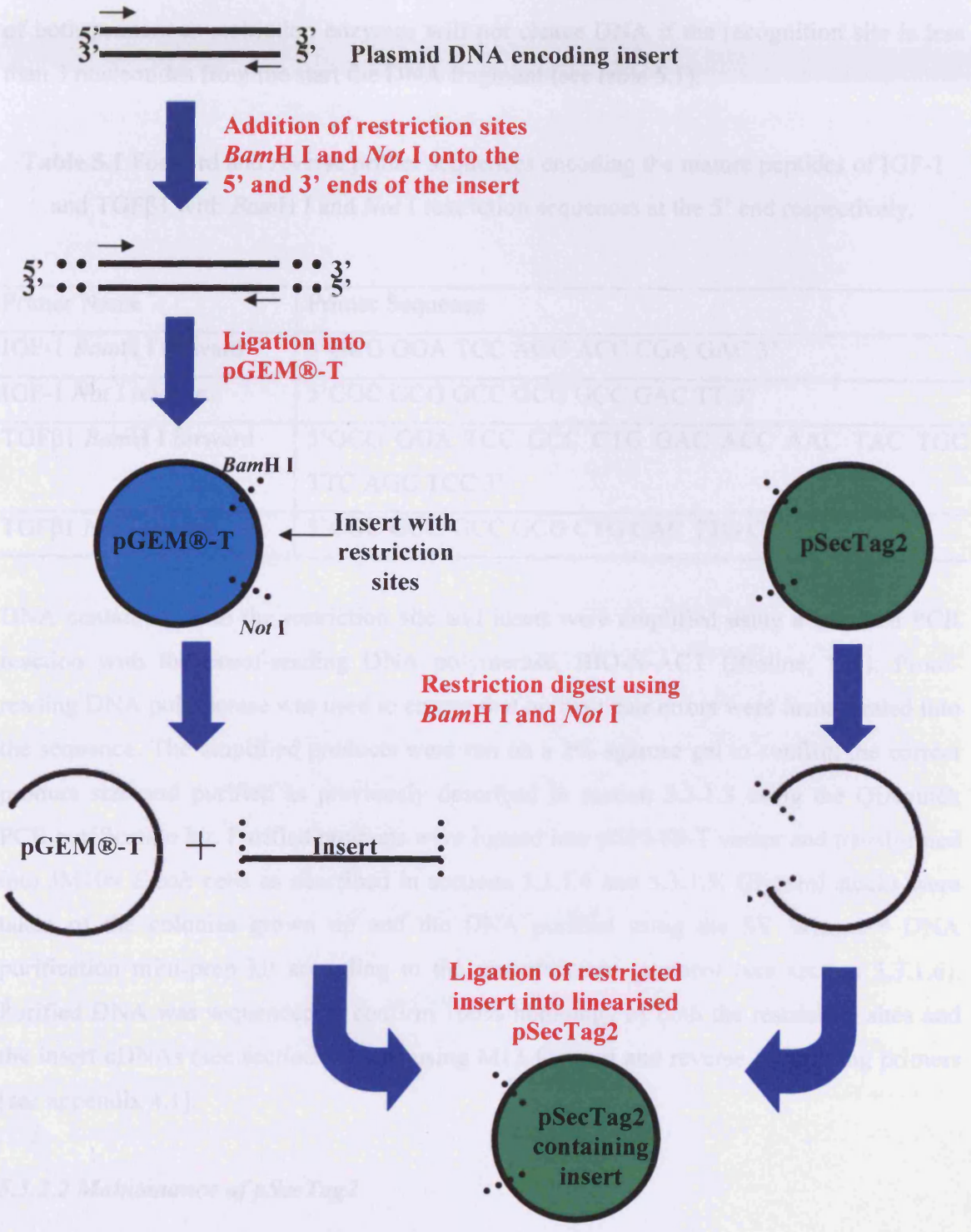


Fig.5.2 Summary of the procedures carried out for the generation of constructs encoding inserts for IGF-1 and TGFβ1 for the expression of these growth factors in articular chondrocytes

IGF-1 and TGFβ1 were altered to include a *Bam*H I site on the forward primer and a *Not* I site on the reverse (MWG Biotech, UK). Extra nucleotides were incorporated at the 5' end of both primers as restriction enzymes will not cleave DNA if the recognition site is less than 3 nucleotides from the start the DNA fragment (see table 5.1).

Table 5.1 Forward and reverse primer sequences encoding the mature peptides of IGF-1 and TGFβ1 with *Bam*H I and *Not* I restriction sequences at the 5' end respectively.

Primer Name	Primer Sequence
IGF-1 *Bam*H I forward	5'GCG GGA TCC AGG ACC CGA GAC 3'
IGF-1 *Not* I reverse	5'CGC GCG GCC GCG GCC GAC TT 3'
TGFβ1 *Bam*H I forward	5'GCG GGA TCC GCC CTG GAC ACC AAC TAC TGC TTC AGC TCC 3'
TGFβ1 *Not* I reverse	5'CGC GCG GCC GCG CTG CAC TTG CAG G 3'

DNA containing both the restriction site and insert were amplified using a standard PCR reaction with the proof-reading DNA polymerase, BIO-X-ACT (Bioline, UK). Proof-reading DNA polymerase was used to ensure that no base pair errors were incorporated into the sequence. The amplified products were run on a 2% agarose gel to confirm the correct product size and purified as previously described in section 5.3.1.3 using the QIAquick PCR purification kit. Purified products were ligated into pGEM®-T vector and transformed into JM109 *E.coli* cells as described in sections 5.3.1.4 and 5.3.1.5. Glycerol stocks were taken of the colonies grown up and the DNA purified using the SV Wizard™ DNA purification mini-prep kit according to the manufacturers protocol (see section 5.3.1.6). Purified DNA was sequenced to confirm 100% homology of both the restriction sites and the insert cDNAs (see section 5.3.1.9) using M13 forward and reverse sequencing primers [see appendix 4.1].

5.3.2.2 Maintenance of pSecTag2

In order to maintain long-term stocks of the pSecTag2 expression vector, the lyophilised vector was resuspended in DNase-free water to a concentration of 1μg/ml. The

reconstituted vector (2µl) was transformed into JM109 cells as previously described in section 5.3.1.5, and plated onto LB agar containing 100µg/ml ampicillin. Colonies were picked and transferred to 5ml of LB broth for growing up overnight at 37°C. Glycerol stocks were taken from the bacterial culture for storage at -80°C. DNA from the remaining culture was purified using the SV Wizard™ DNA purification mini-prep kit according the manufacturers protocol (see section 5.3.1.6).

5.3.2.3 Purification of Plasmid DNA

To achieve a higher yield of plasmid DNA the Qiagen HiSpeed® Midi Plasmid Purification Kit was used (see fig. 5.3). As per the manufacturer's instructions (Qiagen, 2005b), a swab from the glycerol stock of the bacterial culture, confirmed by sequencing to contain the correct insert with 100% homology, was taken and placed in a 5ml culture of LB broth containing 100µg/ml ampicillin. The culture was incubated at 37°C overnight with agitation at 225 RPM. From the resulting broth culture, 100µl was transferred into 50ml of LB broth and incubated for a further 16 hours at 37°C and at 150 RPM. The resulting 50ml culture was centrifuged at 3000 RPM for 15 minutes. The supernatant was disposed of and the pellet resuspended in 6ml of buffer P1 (containing the supplied RNase A solution) by pipetting up and down. An equal volume of buffer P2, a cell lysis buffer, was added to the suspension and mixed gently by inversion of the tube. The suspension was incubated for 5 minutes at room temperature to lyse the cells. A further 6ml of chilled buffer P3 was added to the lysate and mixed by inversion of the tube and subsequently decanted into the barrel of the QIAfilter cartridge. The cell lysate was incubated in the cartridge for 10 minutes at room temperature. This allows a precipitate containing proteins, genomic DNA and detergent to form a layer on the surface of the solution and allows efficient filtration through the QIAfilter cartridge. The cell lysate was filtered through the cartridge into a pre-equilibrated HiSpeed® tip (equilibrated with buffer QBT) allowing the DNA to bind to the resin. The tip was subsequently washed with 20 ml of buffer QC and the DNA eluted in 5ml of buffer QF.

The eluted DNA was precipitated by adding 3.5ml of isopropanol, mixing by inversion and incubating at room temperature for 5 minutes. The eluate/isopropanol mixture was transferred to a syringe barrel attached to a QIAprecipitator midi module and passed

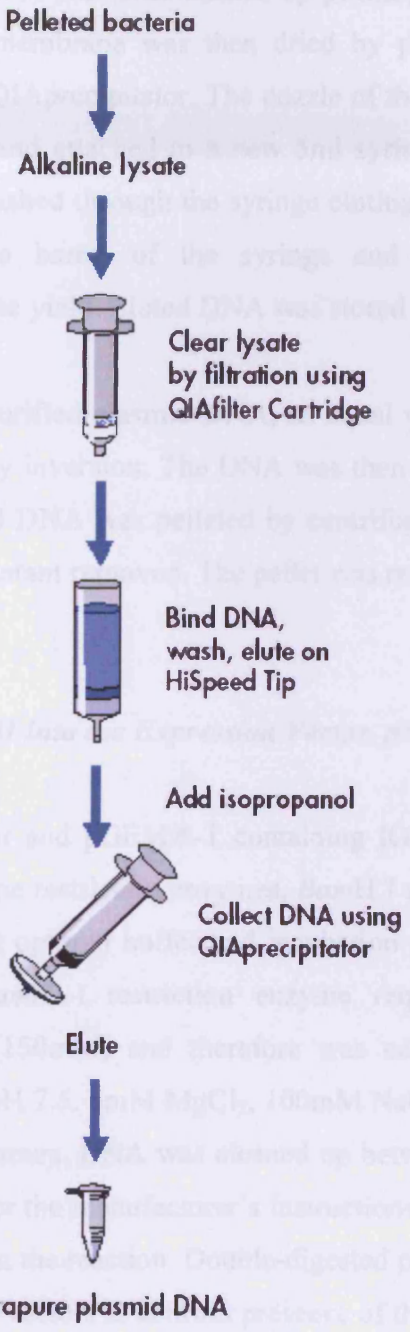


Fig.5.3 Summary of the HiSpeed® plasmid purification procedure (reproduced from Qiagen, 2005b)

through the QIAprecipitator. The DNA was then washed by pushing 2ml of 70% ethanol through the QIAprecipitator. The membrane was then dried by pushing air repeatedly through the syringe attached to the QIAprecipitator. The nozzle of the QIAprecipitator was dried to prevent ethanol carryover and attached to a new 5ml syringe containing 1ml of DNase-free water. The water was pushed through the syringe eluting the DNA. The eluate was then transferred back to the barrel of the syringe and pushed through the QIAprecipitator again to maximise the yield. Eluted DNA was stored at -20°C.

In order to further concentrate the purified plasmid DNA, an equal volume of isopropanol was added and the solution mixed by inversion. The DNA was then allowed to precipitate overnight at -20°C. The precipitated DNA was pelleted by centrifugation at 12,000 RPM for 10 minutes at 4°C and the supernatant removed. The pellet was resuspended in 100µl of DNase-free water.

5.3.2.4 Cloning of IGF-1 and TGFβ1 into the Expression Vector, pSecTag2

Purified pSecTag2 (A and B) vector and pGEM®-T containing IGF-1 or TGFβ1 cDNA inserts were double-digested using the restriction enzymes, *Bam*H I and *Not* I. Each digest was carried out separately using the optimal buffer and incubation conditions in order to achieve an effective cut. The *Bam*H I restriction enzyme requires a lower NaCl concentration (100mM) to *Not* I (150mM) and therefore was carried out first in the provided buffer E (6mM Tris-HCl, pH 7.5, 6mM $MgCl_2$, 100mM NaCl, 1mM DTT) . Each digest was incubated at 37°C for 2 hours. DNA was cleaned up between digests using the QIAquick PCR purification kit as per the manufacturer's instructions (see section 5.2.1.3). This removes enzymes and salts from the reaction. Double-digested products were run on a 2% agarose gel alongside undigested vectors to confirm presence of the insert.

5.3.2.4.1 Gel Extraction and Purification of the Restricted Insert

The digested pSecTag2 (A and B) vector and correctly sized insert from the IGF-1 and TGFβ1 containing plasmids were extracted from the agarose gel using the QIAEX II purification kit (Qiagen, UK) according to the manufacturer's instructions (see fig.5.4).

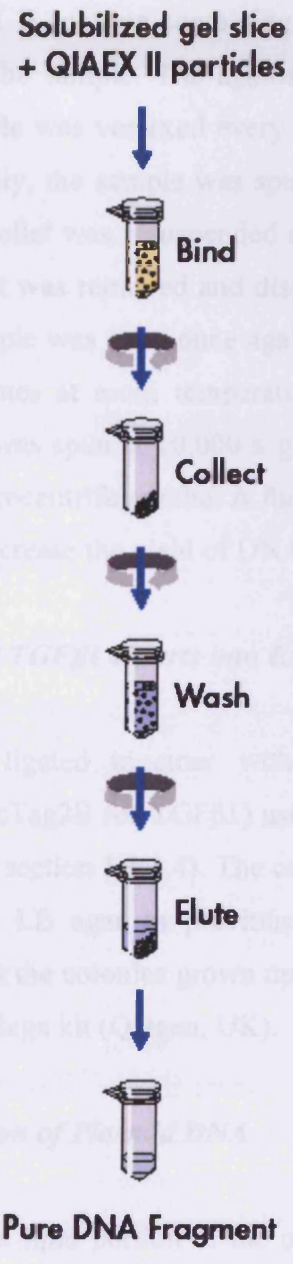


Fig.5.4 Summary of the QIAEX II protocol for DNA extraction from agarose gels (reproduced from Qiagen, 1999)

Briefly, the band was excised from the gel using a clean scalpel and transferred to a sterile 1.5ml microcentrifuge tube. The gel was weighed and 3 volumes of buffer QX1 were added to 1 volume of gel. The QIAEX II solution containing silica particles was resuspended by vortexing, and 30µl added to the sample. The agarose was solubilised by incubation at 50°C for 10 minutes. The sample was vortexed every 2 minutes to prevent settling of the QIAEX II particles. Subsequently, the sample was spun for 30 seconds at 10,000 x g and the supernatant removed. The pellet was resuspended and washed in 500µl of buffer QX1 and spun again. The supernatant was removed and disposed of and the pellet resuspended in 500µl of buffer PE. The sample was spun once again and this step repeated. The pellet was then air-dried for 30 minutes at room temperature before resuspending in 20µl of DNase-free water. The sample was spun at 10,000 x g for 30 seconds and the supernatant transferred to a new, sterile microcentrifuge tube. A further 20µl of water was added to the pellet and the step repeated to increase the yield of DNA.

5.3.2.4.2 Ligation of IGF-1 and TGFβ1 Inserts into Expression Vector

The gel excised DNA was ligated together with the linearised pSecTag2 vector (pSecTag2A for IGF-1 and pSecTag2B for TGFβ1) using T4 DNA ligase according to the manufacturer's instructions (see section 5.3.1.4). The constructs were then transformed into JM109 *E.coli* and plated onto LB agar as previously described (see section 5.3.1.5). Glycerol stocks were taken from the colonies grown up in LB broth, and the plasmid DNA purified using the Endo-free® Mega kit (Qiagen, UK).

5.3.2.4.3 Endo-free® Purification of Plasmid DNA

Endotoxins are found within the lipid portion of the outer layer of the cell membrane of gram-negative bacteria such as *E.coli* (see fig.5.5). Each endotoxin, or lipopolysaccharide (LPS), contains both hydrophobic and hydrophilic moieties as well as charged regions with the potential to interact with numerous other molecules. These endotoxins are shed during growth of the bacterium and during lysis. Large quantities of these toxins are released during plasmid preparations into the cell lysates. The presence of endotoxins drastically affects transfection efficiencies of plasmid DNA into both primary cells and sensitive

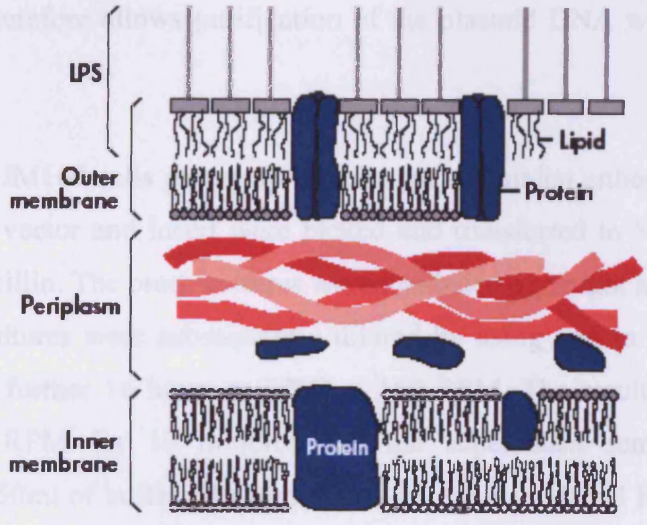


Fig.5.5 Schematic diagram illustrating the envelope of *E.coli* (reproduced from Qiagen, 2005a)

cultured cells. Large levels of endotoxins dramatically decrease the level of transfection achieved by competing with the plasmid DNA for transfection reagent. In order to achieve the highest level of transfection efficiency possible, plasmid DNA containing the inserts IGF-1 or TGFβ1 and the pSecTag2 (A or B) vector alone were purified using the Endo-free® Mega kit (Qiagen, UK) as per the manufacturer's instructions (see fig.5.6). This method incorporates the use of a QIAfilter cartridge as used in the HiSpeed® Midi Plasmid Purification Kit, but also includes an incubation step on ice, in the presence of an endotoxin removal buffer. This prevents the binding of the endotoxins to the resin within the filter cartridge and therefore allows purification of the plasmid DNA without contamination by the endotoxins.

Colonies of the JM109 cells grown up on LB agar containing either the pSecTag2 (A or B) vector alone or vector and insert were picked and transferred to 5ml LB broth containing 100μg/ml ampicillin. The broth cultures were incubated overnight at 37°C and at 225 RPM. These starter cultures were subsequently diluted by using 1ml in 500ml of LB broth and incubated for a further 16 hours at 37°C at 150 RPM. The resulting bacterial cells were spun at 3000 RPM for 10 minutes and the supernatant removed. The pellet was resuspended in 50ml of buffer P1 (50mM Tris-Cl, pH 8.0, 10mM EDTA, 100μg/ml RNase A). An equal volume of buffer P2 (200mM NaOH, 1% SDS (w/v) was added to the suspension, mixed by inversion and incubated at room temperature for 5 minutes to lyse the cells. A further 50ml of chilled buffer P3 (3M potassium acetate, pH 5.5) was added to the lysate and mixed thoroughly by inversion before pouring into the QIAfilter mega cartridge and incubating at room temperature for 10 minutes. This allows the formation of a precipitate layer on the surface of the solution, containing proteins, detergent and genomic DNA, and prevents interference of the filtration process. The cartridge was connected to a vacuum source and the solution filtered through. The precipitate left in the cartridge was washed in 50ml of buffer FWB2 (1M potassium acetate, pH 5.0) before allowing the vacuum to pull through the buffer. The endotoxin removal buffer (buffer ER), 12.5ml, was added to the filtered lysate, mixed by inversion and incubated on ice for 30 minutes. During this incubation step a QIAGEN-TIP 2500 was equilibriated by adding 35ml of buffer QBT (750mM NaCl, 50mM MOPS [4-Morpholinepropanesulfonic acid], pH 7.0, 15% isopropanol (v/v), 0.15% Triton® X 100), allowing the solution to flow through by gravity.

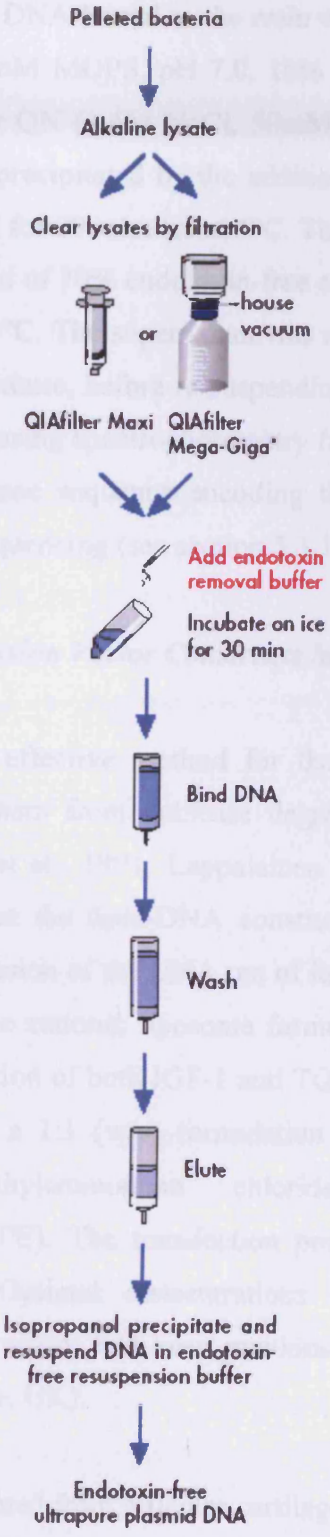


Fig.5.6 Summary of the Mega Endo-free® plasmid purification system (reproduced from Qiagen, 2005a)

The filtered lysate was transferred to the pre-equilibriated QIAGEN-TIP and allowed to flow through by gravity. The DNA bound to the resin within the tip was washed in 200ml of buffer QC (1M NaCl, 50mM MOPS, pH 7.0, 15% isopropanol (v/v)). The DNA was then eluted in 35ml of buffer QN (1.6M NaCl, 50mM MOPS, pH 7.0, 15% isopropanol (v/v)). The eluted DNA was precipitated by the addition of 24.5ml of isopropanol, mixed and centrifuged at 4,000 RPM for 30 minutes at 4°C. The supernatant was removed and the DNA pellet was washed in 7ml of 70% endotoxin-free ethanol and centrifuged for a further 10 minutes at 4,000 RPM at 4°C. The supernatant was removed and the pellet air-dried for 10-20 minutes at room temperature, before re-suspending in 1ml of DNase-free water. The purified DNA was quantified using spectrophotometry (as described in section 5.3.1.8) and sequence homology to the gene sequence encoding the mature peptides of IGF-1 and TGFβ1 confirmed by DNA sequencing (see section 5.3.1.9).

5.3.2.5 Transfection of Expression Vector Constructs into Articular Chondrocytes

Cationic liposomes form an effective method for the delivery of DNA constructs to eukaryotic cells, protecting them from nuclease degradation and enhancing both their uptake and potency (Chiang et al., 1991; Lappalainen et al., 1994; Zelphati and Szoka, 1996b). It is now thought that the lipid-DNA construct is internalised by endocytosis, ultimately resulting in the diffusion of the DNA out of its lipid carrier (Zelphati and Szoka, 1996a). In this experiment, the cationic liposome formula, Lipofectin® (Invitrogen Ltd., UK) was used for the transfection of both IGF-1 and TGFβ1 DNA constructs into primary chondrocytes. Lipofectin® is a 1:1 (w/v) formulation of the cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) and dioleoyl phophotidylethanolamine (DOPE). The transfection process was carried out as per the manufacturer's instructions. Optimal concentrations of the reagent and DNA for transfection of primary chondrocytes had been previously determined by Drs. S. Gilbert and E. Blain (Cardiff University, UK).

Bovine chondrocytes were isolated from articular cartilage of both 7 day old and 18 month old animals as previously described in section 2.3.2. Cells were seeded onto 8 well Lab Tek® II glass chamber slides (Nalge (Europe) Ltd., UK) at a concentration of 1 x 10^6 cells/ml in basal media and left to settle overnight. The transfection reagent, Lipofectin®,

was prepared in basal media (with no antibiotics present) at a 5.6% (v/v) ratio and incubated at 37°C for 40 minutes. DNA to be transfected was also diluted in basal media (with no antibiotics) to a concentration of 1μg/100μl and incubated for the same time period. Lipofectin® reagent and DNA were combined in a 1:1 ratio and mixed well by pipetting up and down. The mixture was subsequently incubated for a further 10 minutes at 37°C. The media from the cells was removed and disposed of and replaced with 200μl of either Lipofectin® alone, or Lipofectin® combined with the pSecTag2 vector or Lipofectin® combined with pSecTag2 containing insert. The media was made up to 500μl with the addition of basal media (containing no antibiotics). Cells were incubated for 18 hours in the presence of the transfection reagent before its removal and replaced with 500μl of basal media. The cells were incubated for a further 24 hours before the addition of 0.1μM monensin (Sigma Chemicals, UK). Monensin blocks the secretion of proteins from the Golgi apparatus by preventing the production of secretory vesicles (Moore et al., 1987). This allows the accumulation and concentration of proteins within the ER and Golgi apparatus for detection by immunohistochemistry. The chondrocytes were treated with monensin for 18 hours. In a similar setup, chondrocytes were seeded at 1 x 10^6 cells/ml onto 24 well plates and transfected as described above. After 18 hours the transfected cells were lysed in 1ml of Trizol® for extraction of RNA.

5.3.2.6 Confirmation of Protein Over-Expression of IGF-1 and TGFβ1

To confirm successful transfection and protein over-expression of the inserted DNA, immunohistochemistry was performed using the monensin treated chondrocytes seeded onto glass chamber slides. Within the C-terminal fusion peptide of the pSecTag2 vector there is a c-myc epitope. Immunohistochemistry using a monoclonal antibody raised against this epitope (Clontech, UK) was used to localize the expression of transcribed c-myc tagged protein within the transfected cells. Media from the monensin treated chondrocytes was removed and the cells were fixed in chilled 95% ethanol for 10 minutes. Ethanol preserves the structure of the cells as well as the secondary structure of intracellular proteins. Alcohol also acts to permeabilise the cell membrane, exposing epitopes for the antibody to act on. The fixed cells were washed twice in PBS (pH 7.4) for 10 minutes and then blocked in whole sheep serum (1:20) diluted in PBS/0.001% Tween20® for 30 minutes. The serum was removed and replaced with the c-myc primary antibody (1:500)

diluted in PBS/0.001% Tween20®. The primary antibody was incubated overnight at 4°C in a humidified chamber. Incubation with PBS/0.001% Tween20® instead of the primary antibody served as a negative control. All slides were washed with PBS/0.001% Tween20® for 10 minutes after overnight incubation with either the primary antibody or PBS/0.001% Tween20®, and subsequently incubated in a FITC-conjugated sheep anti-mouse secondary antibody (1:10,000) for 1 hour at room temperature. The slides were washed in PBS/0.001% Tween20® for 10 minutes before mounting under a coverslip using Vectashield® mountant.

Labelled chondrocytes were viewed and analysed by confocal laser scanning microscopy, using a Leica TCS SP2 AOBS spectral confocal microscope (Leica, Heidelberg, Germany). Chondrocytes were scanned with 63x/40x oil immersion objective lenses using appropriate settings for either single channel fluorescence recordings of FITC alone, or dual channel fluorescence of FITC and propidium iodide in combination (see table 5.1). Selected images are presented as single confocal optical sections and maximum intensity type reconstructions that were made using Leica Confocal Software (Leica, Heidelberg, Germany).

Table 5.2 Excitation and emission wavelengths for the fluorochromes, FITC and propidium iodide, as detected by scanning laser confocal microscopy.

Fluorochrome	Laser Excitation Line (nm)	Emissions Detected (nm)
FITC	488	500-540
Propidium iodide	546	590-680

5.3.2.7 Confirmation of mRNA Over-expression of IGF-1 and TGFβ1

Transfected cells were lysed in 1ml of Trizol® and RNA was extracted as detailed in sections 4.3.6.1-4.3.6.5. cDNA was generated using oligo dT primers (see section 4.3.6.6) and GAPDH (see table 4.3) was used as a house keeping gene for PCR. IGF-1, TGFβ1 and GAPDH primers (see table 4.3) were used in a 30 cycle PCR reaction (see section 4.3.6.7).

PCR products from both control (Lipofectin® only and pSecTag2 vector only treated cells) and treatment groups (either IGF-1 or TGFβ1 transfected cells) were run on a 2% agarose gel to confirm over-expression of mRNA within the treatment groups.

5.3.2.8 Comparing the Migration of Transfected Chondrocytes with Untreated Controls

Transfected chondrocytes (Lipofectin® and vector only controls and IGF-1 and TGFβ1 treatment groups) from both age groups of cartilage were detached from their 24 well plates using 0.05% trypsin EDTA (Invitrogen Ltd., UK) for 5 minutes with agitation at 37°C. Resuspended cells were diluted into basal media and counted using a haemocytometer (see section 2.3.2 for constituents of basal media). Resuspended cells were seeded onto Boyden chamber membranes previously coated with chondroitinase ABC treated A1D1 aggrecan at a concentration of 1 x 10^5 cells/well. The cells were allowed to migrate to the underside of the membrane over 24 hours before the remaining cells on the uppermost side of the membrane were removed. Migrated cells were stained with crystal violet dye and counted (see section 2.3.5.3.3). The mean + SEM was calculated for each treatment group and all data was assessed for normality using the Anderson-Darling test ($P>0.05$). Statistical analysis was performed using a 2 sample T-test.

5.4 Results and Discussion

5.4.1 Design and Preparation of Vector Constructs Encoding IGF-1 and TGFβ1 for the Transfection into Articular Chondrocytes

5.4.1.1 Isolation of mRNA encoding the Mature Peptides of IGF-1 and TGFβ1 from bovine cDNA.

A clear band observed at 210bp on a 2% agarose gel (see fig.5.7a) demonstrated the presence of the sequence encoding the mature peptide of IGF-1 mRNA on amplification of bovine cDNA generated from young (7 day old) calf liver RNA Liver cDNA was chosen for this initial amplification as IGF-1 is highly expressed within this organ (Lindberg et al., 2005). The sequence encoding the mature peptide of bovine TGFβ1 was also successfully amplified from young bovine chondrocyte cDNA. This was viewed as a 336bp band on a 2% agarose gel (see fig.5.7b). Both PCR products were purified and ligated into pGEM®-T vector for confirmation of 100% sequence homology by sequencing.

5.4.1.2 Confirmation of Sequence Identity and 100% Homology

The purified PCR products for IGF-1 and TGFβ1 cDNA were ligated into pGEM®-T vector and transformed into the competent bacterial cell line, JM109. The resulting bacterial plasmid DNA encoding IGF-1 or TGFβ1 was purified for sequencing. Sequence identity and homology was confirmed by sequencing using M13 forward and reverse primers. These primers sit outside of the MCS and can be used to amplify sequences within it. Sequencing data were analysed and the identity of the IGF-1 and TGFβ1 cDNA was confirmed with 100% homology to the known bovine sequences (see figs 5.8 and 5.9).

5.4.1.3 Restriction Digestion of Plasmid DNA to Confirm Expression of Correctly Sized Inserts

Purified plasmid DNA containing inserts coding for the IGF-1 and TGFβ1 mature peptides were digested using the restriction enzymes *Nco*1 and *Sal*1, both found within the MCS of

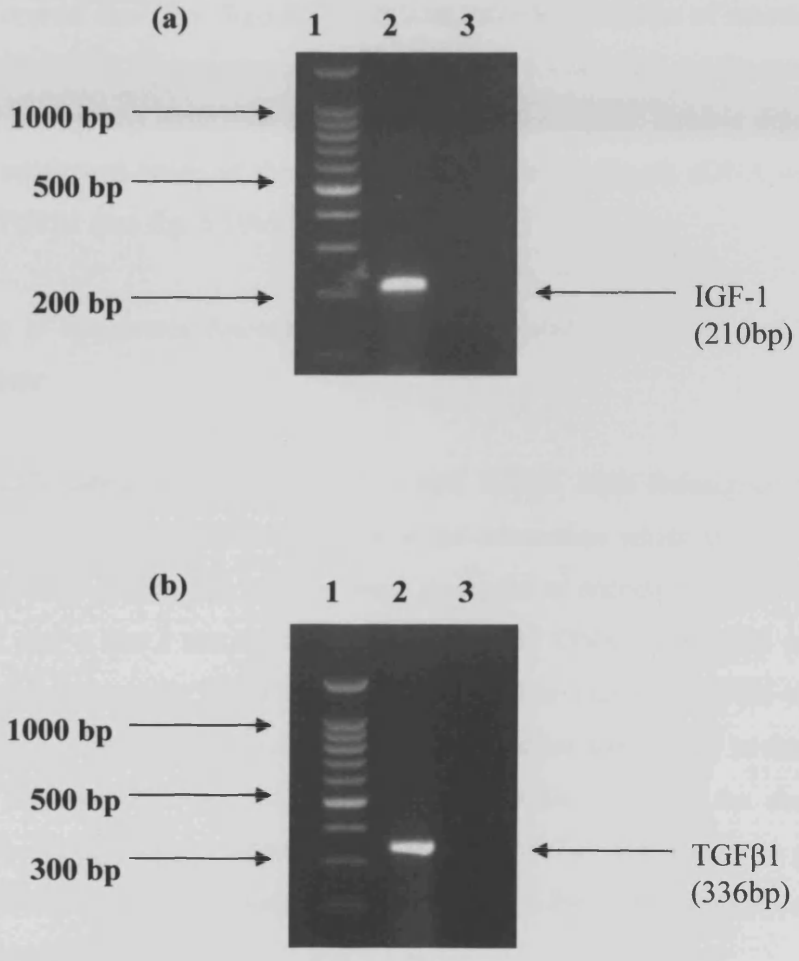


Fig.5.7 Amplification of bovine (a) IGF-1 and (b) TGFβ1 mRNA. Lane 1: 100bp ladder, lane 2: (a) IGF-1 amplified from bovine liver cDNA (b) TGFβ1 amplified from bovine chondrocyte cDNA, lane 3: water negative control.

the pGEM®-T vector. To ensure complete digestion by *Nco*1 and *Sal*1, and to confirm a product of the correct size after digestion with both enzymes, a series of reactions were set up and run together on a 2% agarose gel. As seen in figs 5.10a and b, undigested and single digested samples were run alongside the double digested samples. Double digestion of the plasmid DNA confirmed bands of the correct size for the full length cDNA of IGF-1 (see fig.5.10a) and TGFβ1 (see fig. 5.10b).

5.4.1.4 Cloning of Sequences Encoding the Mature Peptides of IGF-1 and TGFβ1 into Expression Vector

Primers previously designed to encode IGF-1 and TGFβ1 were redesigned to encode a restriction site at the 5' end. In order to control the orientation which the insert would sit within the expression vector, the primers were designed to encode a *Bam*H I site on the forward primer and a *Not* I site on the reverse. Plasmid DNA, previously confirmed to contain the correct insert with 100% homology was used as a template in the ensuing PCR reaction in order to generate PCR products with the restriction sites added to them. A proof-reading DNA polymerase was used to ensure no mutations within the sequence (see fig.5.11a and b). As previously described, the resulting cDNA could then be purified and ligated into pGEM®-T to confirm sequence homology and the correct restriction sequences using a T7 forward primer (see fig.5.12 and 5.13).

Restriction digests using the enzymes whose recognition sequences had been cloned into the insert were also performed confirming an insert of the correct size and also that the recognition sequences were of the correct homology for the enzymes to cut efficiently (see fig.5.14a and b). The insert bands were then gel extracted and the DNA purified for ligation into the expression vector chosen – pSecTag2A for IGF-1 and pSecTag2B for TGFβ1. Both these expression vectors have an Immuoglobulin (Ig)-leader sequence for secretion of the generated product and a c-myc tag at the 3' end for detection. Once within the expression vector the construct was transformed into JM109 cells once again to produce the required plasmid DNA. Purified plasmid DNA with the expression vector could then be sequenced once again to confirm sequence homology and in frame expression before large scale plasmid DNA preparation. The resulting vector insert construct sequence is shown in figs.5.15 and 5.16.

```
Query   1:    GGACCCGAGACCCTCTGCGGGGCTGAGTTGGTGGATGCTCTCCAGTTCGTGTGCGGAGAC  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 403:    GGACCCGAGACCCTCTGCGGGGCTGAGTTGGTGGATGCTCTCCAGTTCGTGTGCGGAGAC  462
       1:      G   P   E   T   L   C   G   A   E   L   V   D   A   L   Q   F   R   C   G   D

Query  61:    AGGGGCTTTTATTTCAACAAGCCCACGGGGTATGGCTCGAGCAGTCGGAGGGCGCCCCAG  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 463:    AGGGGCTTTTATTTCAACAAGCCCACGGGGTATGGCTCGAGCAGTCGGAGGGCGCCCCAG  522
       21:     R   G   F   Y   F   N   K   P   T   G   Y   G   S   S   S   R   R   A   P   Q

Query 121:    ACAGGAATCGTGGATGAGTGCTGCTTCCGGAGCTGTGATCTGAGGAGGCTGGAGATGTAC  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 523:    ACAGGAATCGTGGATGAGTGCTGCTTCCGGAGCTGTGATCTGAGGAGGCTGGAGATGTAC  582
       41:     T   G   I   V   D   E   C   C   F   R   S   C   D   L   R   R   L   E   M   Y

Query 181:    TGCGCGCCTCTCAAGCCCGCCAAGTCGGCC  210
              |||||||||||||||||||||||||||||
Sbjct 583:    TGCGCGCCTCTCAAGCCCGCCAAGTCGGCC  612
       61:     C   A   P   L   K   P   A   K   S   A
```

Fig.5.8 Comparing the mRNA sequence of the pGEM®-T vector containing the bovine IGF-1 insert (query) for 100% homology with the known mRNA sequence for the bovine IGF-1 mature peptide (subject). The amino acids encoded have been included below the nucleotide sequences.

```
Query   1: GCCCTGGACACCAACTACTGCTTCAGCTCCACAGAAAAGAACTGCTGTGTTCGTCAGCTC 60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 612: GCCCTGGACACCAACTACTGCTTCAGCTCCACAGAAAAGAACTGCTGTGTTCGTCAGCTC 671
        1:  A  L  D  T  N  Y  C  F  S  S  T  E  K  N  C  C  V  R  Q  L

Query  61: TACATTGACTTCCGGAAGGACCTGGGCTGGAAGTGGATTCATGAACCCAAGGGGTACCAC 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 672: TACATTGACTTCCGGAAGGACCTGGGCTGGAAGTGGATTCATGAACCCAAGGGGTACCAC 731
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Query 121: GCCAATTTCTGCCTGGGGCCCTGCCCTTACATCTGGAGCCTGGATACACAGTACAGCAAG 180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query 181: GTCCTGGCCCTGTACAACCAGCACAACCCGGGCGCTTCGGCGGCGCCGTGCTGCGTGCCT 240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 792: GTCCTGGCCCTGTACAACCAGCACAACCCGGGCGCTTCGGCGGCGCCGTGCTGCGTGCCT 851
       61:  V  L  A  L  Y  N  Q  H  N  P  G  A  S  A  A  P  C  C  V  P

Query 241: CAGGCGCTGGAGCCCCTGCCCATCGTGTACTACGTGGGCCGCAAGCCCAAGGTGGAGCAG
300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 852: CAGGCGCTGGAGCCCCTGCCCATCGTGTACTACGTGGGCCGCAAGCCCAAGGTGGAGCAG 911
       81:  Q  A  L  E  P  L  P  I  V  Y  Y  V  G  R  K  P  K  V  E  Q

Query 301: TTGTCCAACATGATCGTGCGCTCCTGCAAGTGCAGC  336
           |||||||||||||||||||||||||||||||||
Sbjct 912: TTGTCCAACATGATCGTGCGCTCCTGCAAGTGCAGC  947
      101:  L  S  N  M  I  V  R  S  C  K  C  S
```

Fig. 5.9 Comparing the mRNA sequence of the pGEM®-T vector containing the bovine TGFβ1 insert (query) for 100% homology with the known mRNA sequence for the bovine TGFβ1 mature peptide (subject). The amino acids encoded have been included below the nucleotide sequences.

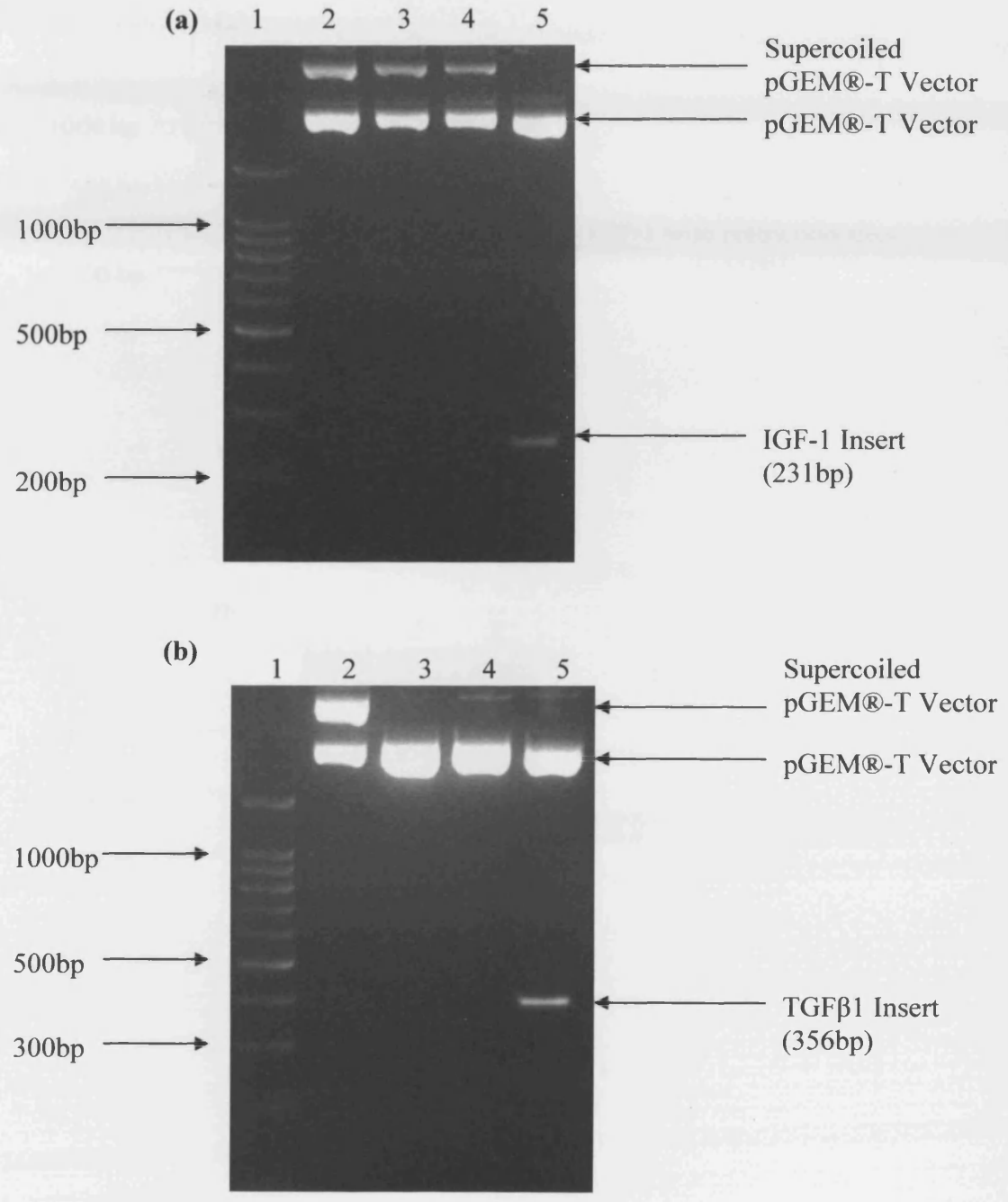


Fig. 5.10 Restriction digest of (a) pGEM®-T–IGF-1 and (b) pGEM®-T-TGFβ1 plasmid DNA using the restriction enzymes *Nco* I and *Sal* I. Lane 1: 100bp ladder, lane 2: undigested plasmid DNA, lane 3: *Nco* I digested plasmid DNA, lane 4: *Sal* I digested plasmid DNA and lane 5: *Nco* I and *Sal* I digested plasmid DNA. Lane 5 demonstrates a correctly sized band for (a) IGF-1 and (b) TGFβ1 DNA.

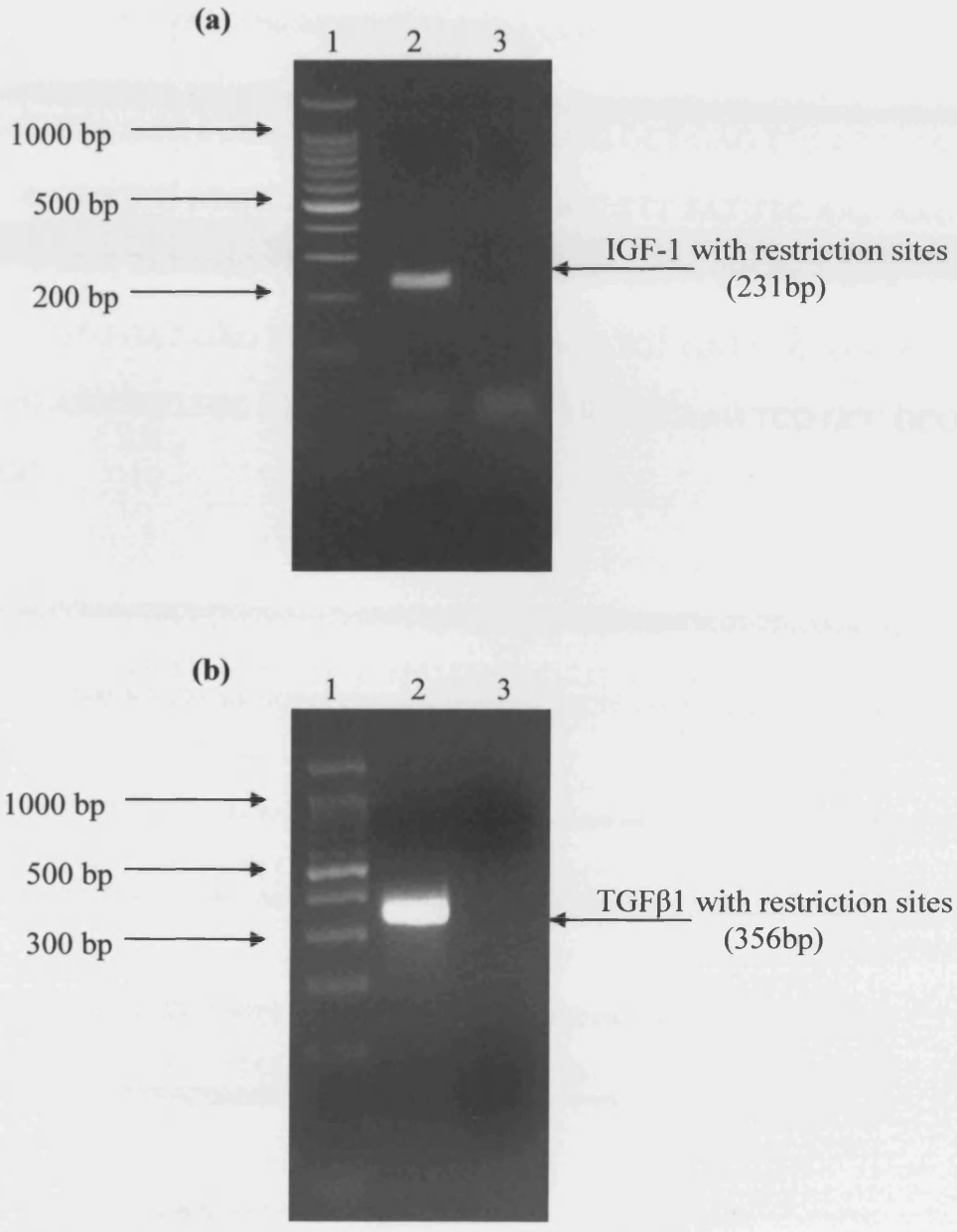


Fig. 5.11 Amplification of plasmid DNA encoding (a) IGF-1/(b) TGFβ1 and the *Bam*H I and *Not* I restriction sites. Lane 1: 100bp ladder, lane 2: (a) IGF-1 and the restriction sites for *Bam*H I and *Not* I, (b) TGFβ1 and the restriction sites for *Bam*H I and *Not* I, lane 3: water negative control.

(a)

TCG GAT CCA GGA CCC GAG ACC CTC TGC GGG GCT GAG TTG GTG GAT

GCT CTC CAG TTC GTG TGC GGA GAC AGG GGC TTT TAT TTC AAC AAG

CCC ACG GGG TAT GGC TCG AGC AGT CGG AGG GCG CCC CAG ACA

GGA ATC GTG GAT GAG TGC TGC TTC CGG AGC TGT GAT CTG AGG AGG

CTG GAG ATG TAC TGC GCG CCT CTC AAG CCC GCC AAG TCG GCC GCG

GCC CGC

(b)

```
Query  11 GGACCCGAGACCCTCTGCGGGGCTGAGTTGGTGGATGCTCTCCAGTTCGTGTGCGGAGAC
70
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 145 GGACCCGAGACCCTCTGCGGGGCTGAGTTGGTGGATGCTCTCCAGTTCGTGTGCGGAGAC
204

Query 71  AGGGGCTTTTATTTCAACAAGCCCACGGGGTATGGCTCGAGCAGTCGGAGGGCGCCCCAG
130
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 205 AGGGGCTTTTATTTCAACAAGCCCACGGGGTATGGCTCGAGCAGTCGGAGGGCGCCCCAG
264

Query 131 ACAGGAATCGTGGATGAGTGCTGCTTCCGGAGCTGTGATCTGAGGAGGCTGGAGATGTAC
190
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 265 ACAGGAATCGTGGATGAGTGCTGCTTCCGGAGCTGTGATCTGAGGAGGCTGGAGATGTAC
324


Query 191 TGCGCGCCTCTCAAGCCCGCCAAGTCGGCC 220
          ||||||||||||||||||||||||||||||
Sbjct 325 TGCGCGCCTCTCAAGCCCGCCAAGTCGGCC 354
```

Fig.5.12 Sequencing data confirming (a) in frame expression (red) and (b) homology with IGF-1 within pSecTag2A vector. Figure (a) also confirms the correct recognition sequences for the restriction enzymes *Bam*H I (blue) and *Not* I (green).

CG GGA TCC GCC CTG GAC ACC AAC TAC TGC TTC AGC TCC ACA GAA

AAG AAC TGC TGT GTT CGT CAG CTC TAC ATT GAC TTC CGG AAG GAC

CTG GGC TGG AAG TGG ATT CAT GAA CCC AAG GGG TAC CAC GCC AAT

TTC TGC CTG GGG CCC TGC CCT TAC ATC TGG AGC CTG GAT ACA CAG

TAC AGC AAG GTC CTG GCC CTG TAC AAC CAG CAC AAC CCG GGC GCT

TCG GCG GCG CCG TGC TGC GTG CCT CAG GCG CTG GAG CCC CTG CCC

ATC GTG TAC TAC GTG GGC CGC AAG CCC AAG GTG GAG CAG TTG TCC

AAC ATG ATC GTG CGC TCC TGC AAG TGC AGC GCG GCC GCG CG

```
Query   9 GCCCTGGACACCAACTACTGCTTCAGCTCCACAGAAAAGAACTGCTGTGTTCGTCAGCTC   68
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 612 GCCCTGGACACCAACTACTGCTTCAGCTCCACAGAAAAGAACTGCTGTGTTCGTCAGCTC  671

Query  69 TACATTGACTTCCGGAAGGACCTGGGCTGGAAGTGGATTCATGAACCCAAGGGGTACCAC  128
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 672 TACATTGACTTCCGGAAGGACCTGGGCTGGAAGTGGATTCATGAACCCAAGGGGTACCAC  731

Query 129 GCCAATTTCTGCCTGGGGCCCTGCCCTTACATCTGGAGCCTGGATACACAGTACAGCAAG  188
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 732 GCCAATTTCTGCCTGGGGCCCTGCCCTTACATCTGGAGCCTGGATACACAGTACAGCAAG  791

Query 189 GTCCTGGCCCTGTACAACCAGCACAACCCGGGCGCTTCGGCGGCGCCGTGCTGCGTGCCT  248
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 792 GTCCTGGCCCTGTACAACCAGCACAACCCGGGCGCTTCGGCGGCGCCGTGCTGCGTGCCT  851

Query 249 CAGGCGCTGGAGCCCCTGCCCATCGTGTACTACGTGGGCCGCAAGCCCAAGGTGGAGCAG  308
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 852 CAGGCGCTGGAGCCCCTGCCCATCGTGTACTACGTGGGCCGCAAGCCCAAGGTGGAGCAG  911

Query 309 TTGTCCAACATGATCGTGCGCTCCTGCAAGTGCAGC  344
          ||||||||||||||||||||||||||||||||||||
Sbjct 912 TTGTCCAACATGATCGTGCGCTCCTGCAAGTGCAGC  947
```

Fig.5.13 Sequencing data confirming (a) in frame expression (red) and (b) homology with TGFβ1 within pSecTag2B vector. Figure (a) also confirms the correct recognition sequences for the restriction enzymes *Bam*H I (blue) and *Not* I (green).

5.4.2 Transfection of IGF-1 and TGFβ1 into Bovine Articular Chondrocytes

Subsequent to confirmation of in frame expression of the inserts and their homology with the gene of interest, the DNA was amplified using a large scale preparation and endotoxins from the bacterial cells removed before transfection of the DNA into mammalian cells.

5.4.2.1 Confirmation of Over-expression of IGF-1 and TGFβ1 mRNA

Bovine chondrocytes isolated from both young and skeletally mature cartilage were transfected with either IGF-1, TGFβ1 or a vector control. Lipofectin® alone was also used as a control group. Transfected cells were lysed in Trizol® and their RNA was extracted. cDNA generated from these transfected cells was amplified using standard PCR reaction using the primers originally designed encoding the mature peptides of both of these growth factors. Over-expression of IGF-1 and TGFβ1 was confirmed within the sense transfected treatment groups with intense bands demonstrating expression of growth factor when compared to the controls used (see fig.5.17a-h). This confirmed successful transfection of the constructs within the treatment groups. As both IGF-1 and TGFβ1 would be found within both the control and treatment groups, protein levels of the c-myc tag were investigated as this will only be present within transfected cells and not the lipofectin controls. Western blotting methods using a monoclonal antibody against c-myc did not demonstrate the presence of bands within the treatment groups (data not shown). This is probably due to the low levels of protein for detection within the cells and culture media. In the light of this information, transfected chondrocytes were treated with monensin. Monensin prevents the secretion of protein from the ER and Golgi apparatus but does not affect protein synthesis. Treatment with this ionophore allows concentration of the protein produced within the cell and therefore detection by immunohistochemistry using monoclonal antibodies to the c-myc tag.

Treatment of the cells with monensin increased the size of the cell. Immunohistochemistry for c-myc using a FITC conjugated secondary antibody demonstrated definite patterns of staining within transfected chondrocytes. Staining was exclusively cytoplasmic and punctate foci were seen around the edge of the cell close to the cell membrane as well as

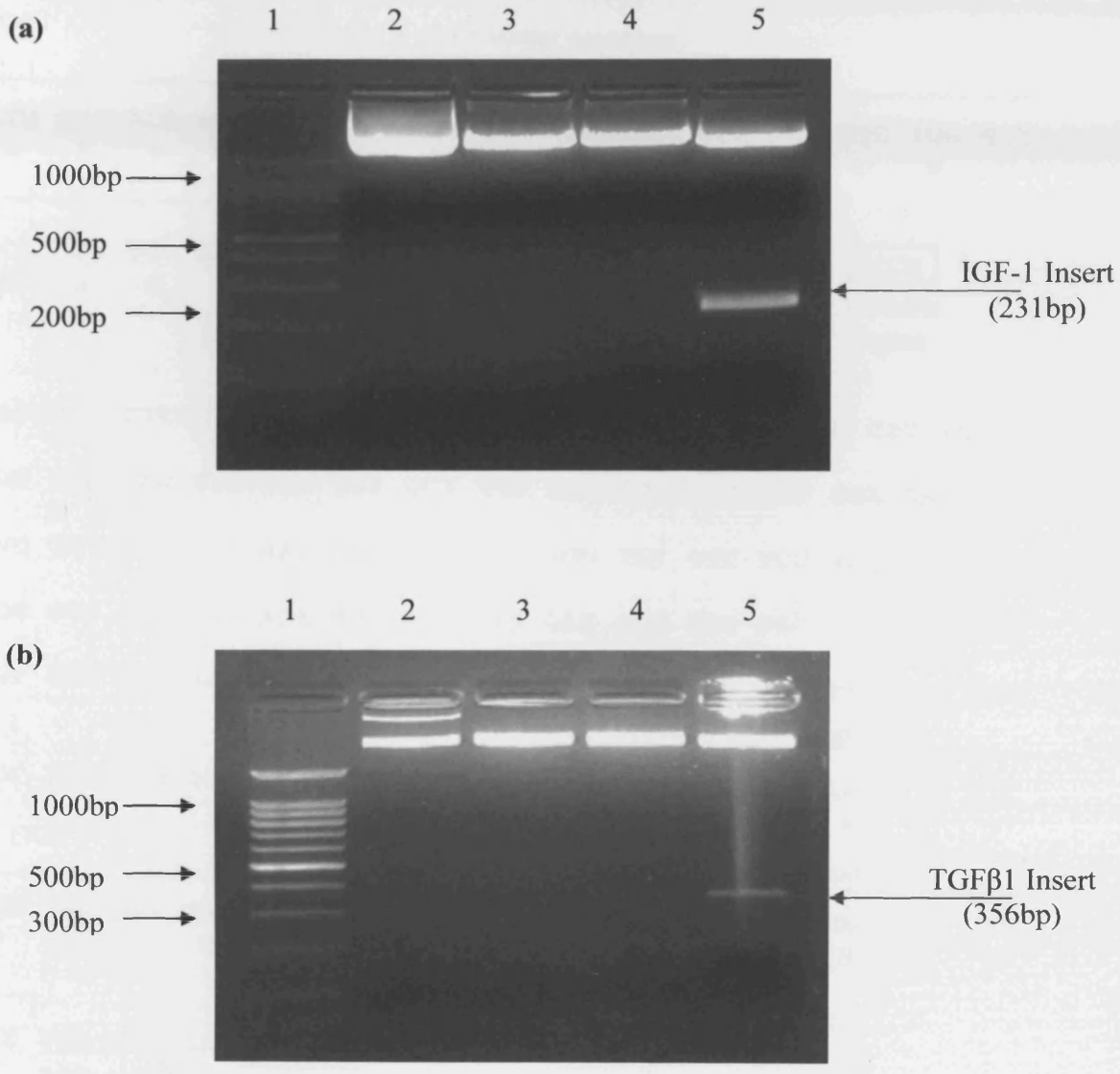


Fig. 5.14 Restriction digest of pSecTag2 vector encoding the mature peptide of (a) IGF-1 and (b) TGFβ1 using the restriction enzymes *Bam*H I and *Not* I. Lane 1: 100bp ladder, lane 2: undigested plasmid DNA, lane 3: *Bam*H I digested plasmid DNA, lane 4: *Not* I digested plasmid DNA and lane 5: *Bam*H I and *Not* I digested plasmid DNA. Lane 5 demonstrates a correctly sized band for the plasmid insert.

intense 'hot spots' close to the Golgi and ER (see fig.5.18 and 5.19 a, b and c). By taking sections through transfected cells using confocal microscopy it was possible to develop a 3D image of the cell and define exactly where the transfected protein was residing. Figure 5.20c and f demonstrated specific staining in TGFβ1 transfected cells with no staining seen in Lipofectin® controls (see fig.5.20a and d). Similar results were seen with the IGF-1 transfected chondrocytes.

5.4.2.2 Evaluating the Transfection Efficiency of Chondrocytes Using Lipofectin®

Using images at x 40 magnification, generated by confocal laser scanning microscopy, it was possible to calculate an efficiency rate of transfection for both the chondrocytes isolated from young and mature cartilage. No difference in transfection efficiency between the age groups was seen, with an average of 22% of chondrocytes isolated from the young tissue successfully expressing the c-myc epitope and 24% from the mature cartilage.

Cells transfected with the pSecTag2 expression vector can be selected for using the Zeocin™ antibiotic resistance gene encoding the *Sh ble* protein (Dumas et al., 1994; Gatignol et al., 1988). Unfortunately the effectiveness of this antibiotic is dependent on the high division rate of the treated cells. As chondrocytes have a low rate of division Zeocin™ does not exert a toxic effect and is therefore not effective as a selective agent in this cell type. As a result of this successfully transfected chondrocytes were not able to be selected for and isolated from the non-transfected cells. This must be taken into account when interpreting the results obtained in section 5.4.3 where the ability of chondrocytes transfected with IGF-1 and TGFβ1 to migrate is discussed.

5.4.3 Does the Transfection of Chondrocytes with IGF-1 or TGFβ1 Alter Their Ability to Migrate?

Chondrocytes transfected with IGF-1 or TGFβ1 were seeded onto Boyden chamber membranes coated with 50μg/ml of chondroitinase ABC treated aggrecan. As previously described for the experiments within chapter 2, the cells were allowed to migrate from the upper side of the membrane to the lower side over a period of 24 hours. After this incubation period the number of migrated cells was counted. As previously mentioned, the

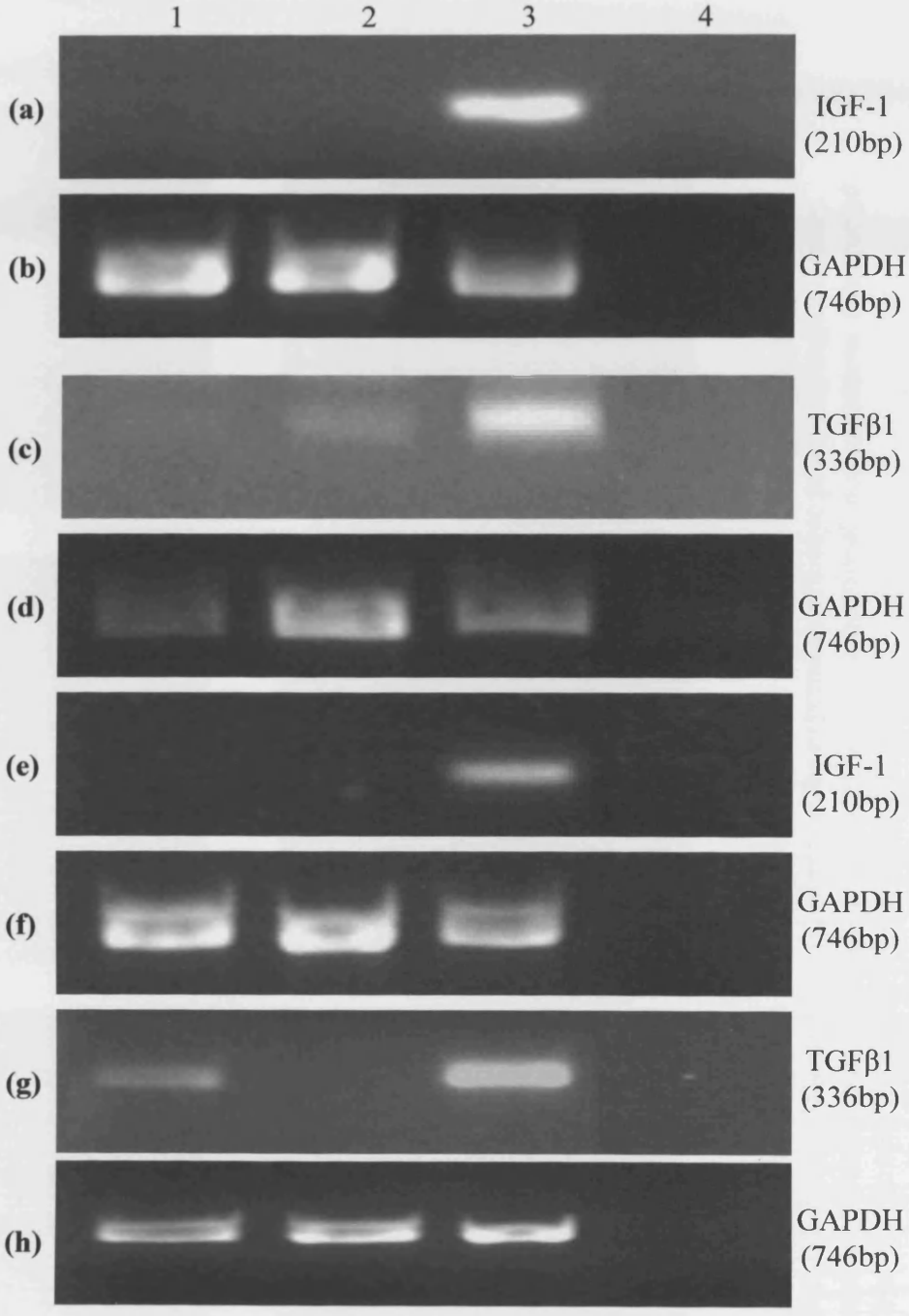


Fig. 5.17 Comparing the expression of (a and e) IGF-1 and (c and g) TGFβ1 mRNA between: lane 1 Lipofectin® only, lane 2 vector only controls and lane 3 IGF-1/TGFβ1 over-expressing chondrocytes. Lane 4 water negative control. Rows (a-d) mRNA from chondrocytes isolated from young articular cartilage and (e-h) mRNA from chondrocytes isolated from mature articular cartilage. GAPDH was used as a housekeeping gene.

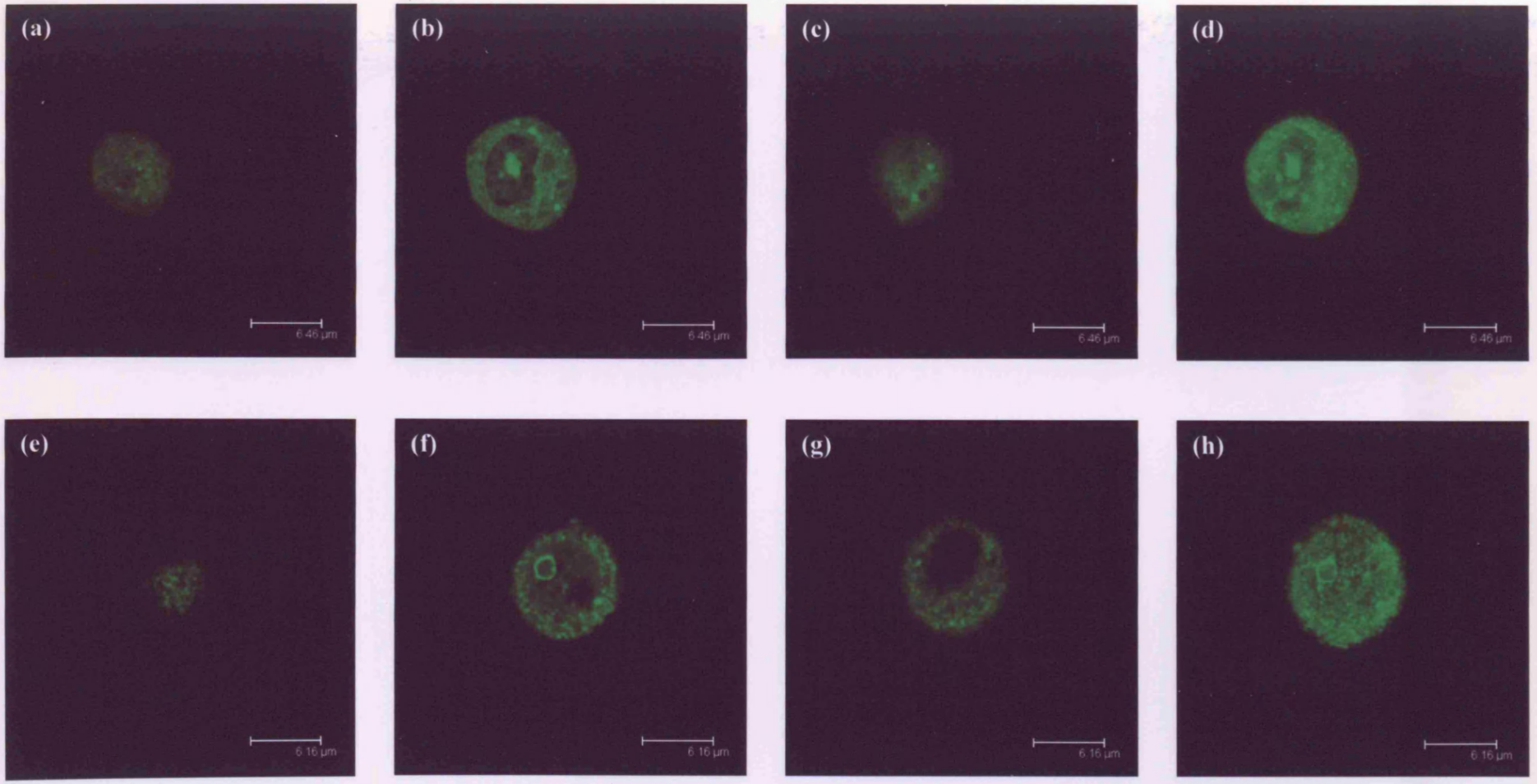


Fig. 5.18 Confocal microscopy demonstrating the expression of c-myc (labelled green) in mature bovine chondrocytes over-expressing the pSecTag2A vector (a-d) and IGF-1 (e-h). (a-c) and (e-g) illustrate the expression of c-myc protein through different optical sections within the cell and its localisation in punctate foci. Fig.5.18 (d) and (h) are 3 dimensional reconstructions constructed from all the optical sections taken throughout the cell.

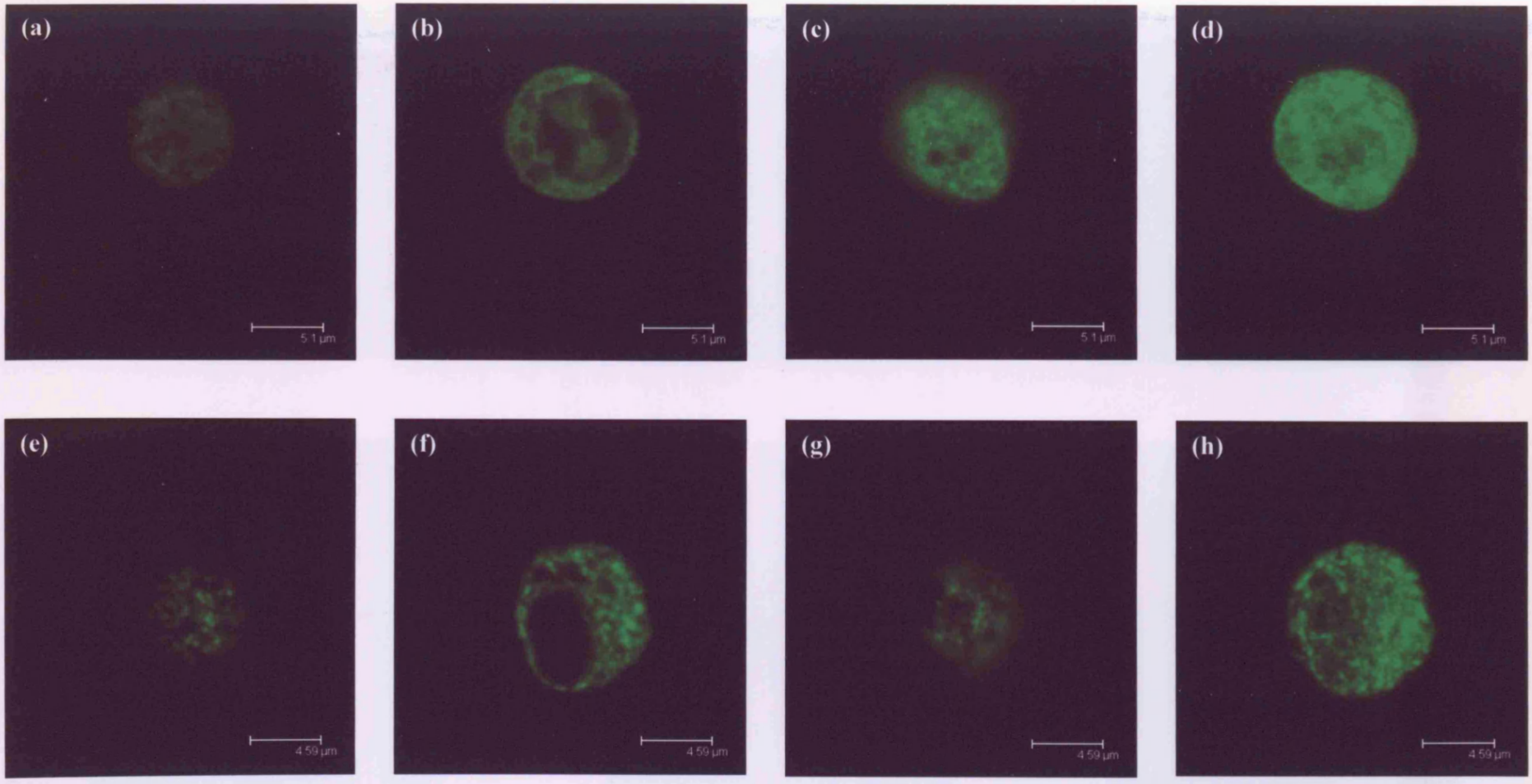


Fig. 5.19 Confocal microscopy demonstrating the expression of c-myc (labelled green) in young bovine chondrocytes over-expressing the pSecTag2A vector (a-d) and IGF-1 (e-h). (a-c) and (e-g) illustrate the expression of c-myc protein through different optical sections within the cell and its localisation in punctate foci. Fig.5.19 (d) and (h) are 3 dimensional reconstructions constructed from all the optical sections taken throughout the cell.

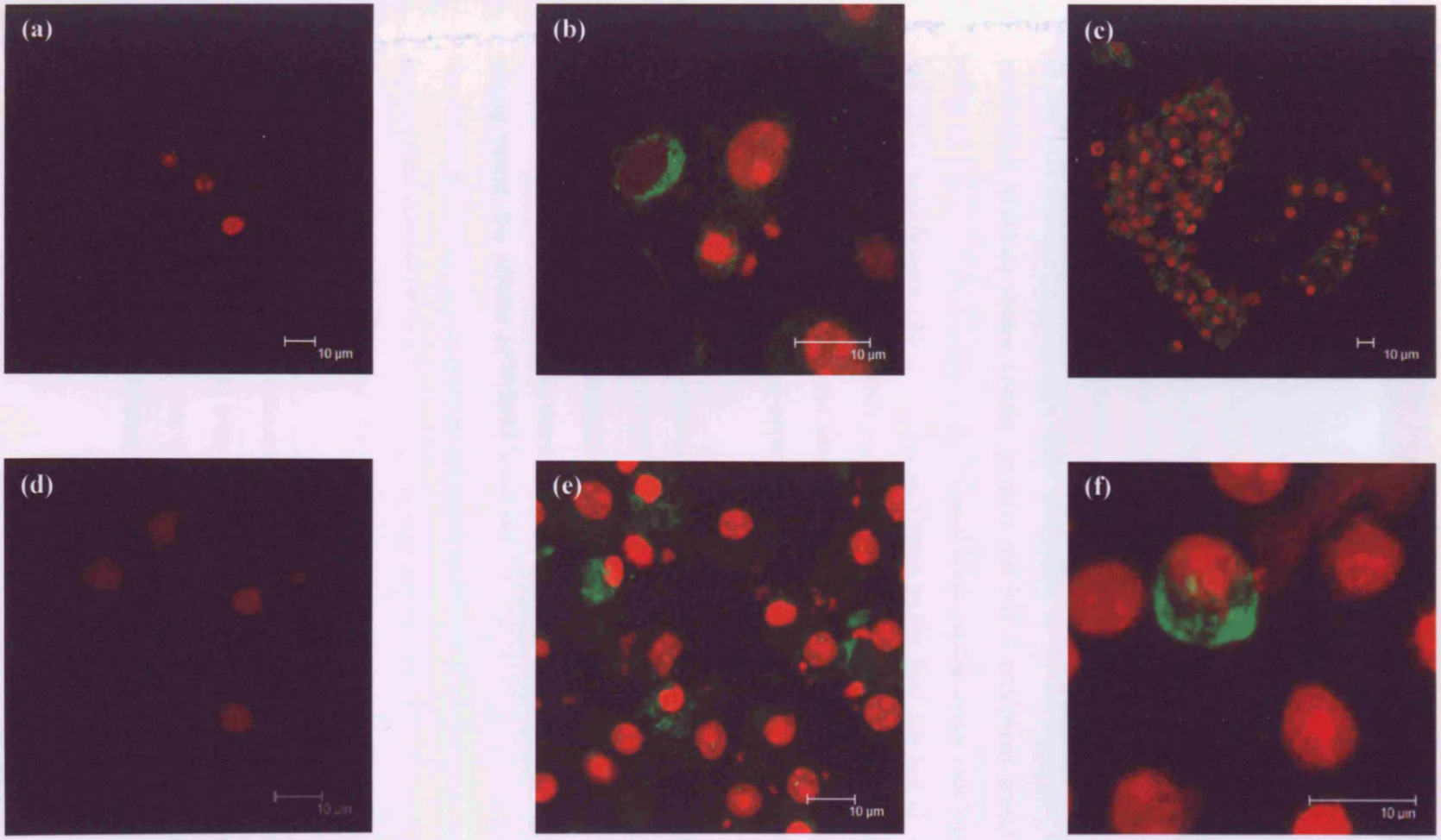


Fig. 5.20 Confocal microscopy demonstrating the expression of c-myc protein (labelled green) in young (a-c) and mature (d-f) transfected chondrocytes. Cell nuclei are stained red with propidium iodide. Figures (a) and (d) are Lipofectin® only controls, (b) and (e) illustrate the cytoplasmic expression of c-myc in pSecTag2B vector only controls, (c) and (f) illustrate transfection with TGFβ1.

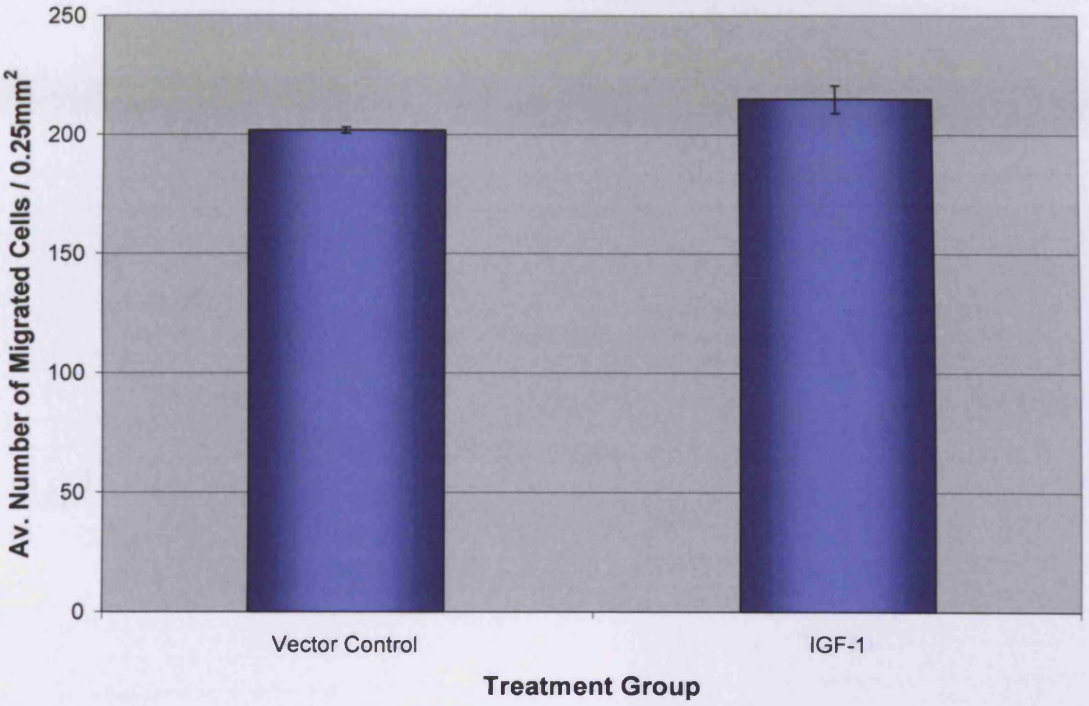
transfection efficiency of chondrocytes isolated from both young and mature articular cartilage is approximately 22-24% and due to the low division rate of these primary cells transfected cells were unable to be selected from non-transfected chondrocytes using the Zeocin™ resistance gene.

Both vector control and IGF-1 transfected chondrocytes isolated from both age groups of articular cartilage were seen to migrate over 24 hours on chondroitinase ABC treated aggrecan (see fig.5.21a and b). Although no significant differences between vector control and IGF-1 treatment groups were seen with either age group of cells, definite trends were apparent. Chondrocytes from both age groups demonstrated increased trends of migration in the IGF-1 transfected group when compared to vector controls. Two sample T-tests performed between vector control groups and IGF-1 treatment groups demonstrated a P value of 0.083 for the chondrocytes isolated from young tissue (see fig.5.21a). The lack of statistical significance can in part be attributed to the fact that not all of the chondrocytes seeded onto the Boyden chamber membranes were in fact transfected with either the vector or IGF-1 construct. This would also explain variability between the wells, although the standard error bars are not high between samples.

Treatment with TGFβ1 also demonstrated an increasing trend of migration in both age groups when compared to the vector controls (see fig.5.22a and b). Again these differences were not significant when compared using a 2 sample T-test however. In contrast to the IGF-1 treatment where trends were more apparent with the chondrocytes isolated from young tissue, the effects of TGFβ1 were the reverse. A P value of 0.051 was achieved for the increased levels of migrating TGFβ1 transfected chondrocytes isolated from mature tissue when compared to vector controls (see fig.5.22b). Higher error bars in the younger age group resulting in a P value of only 0.530 (see fig.5.22a).

Both sets of data clearly demonstrate the ability of these growth factors to exert pro-migratory effects through a non-chemotactic mechanism. No studies to date have investigated the effects of over-expressing growth factors in chondrocytes on their migratory capabilities. In addition, the photomicrographs shown in fig.5.23 and 5.24

(a)



(b)

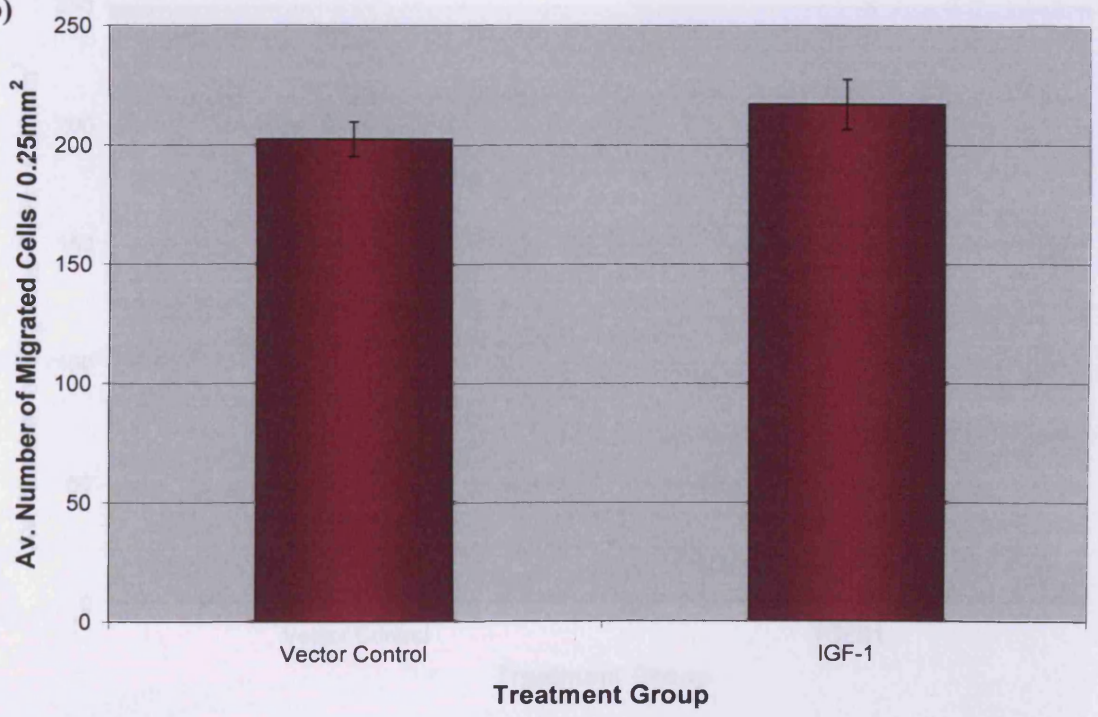


Fig. 5.21 The migration of chondrocytes transfected with pSecTag2A vector or IGF-1 isolated from (a) young [P=0.083] and (b) mature articular cartilage [P=0.150] over 24 hours. Results illustrate the mean number of migrating cells per 0.25mm^2 visual field with SEM bars and an n value of 3.

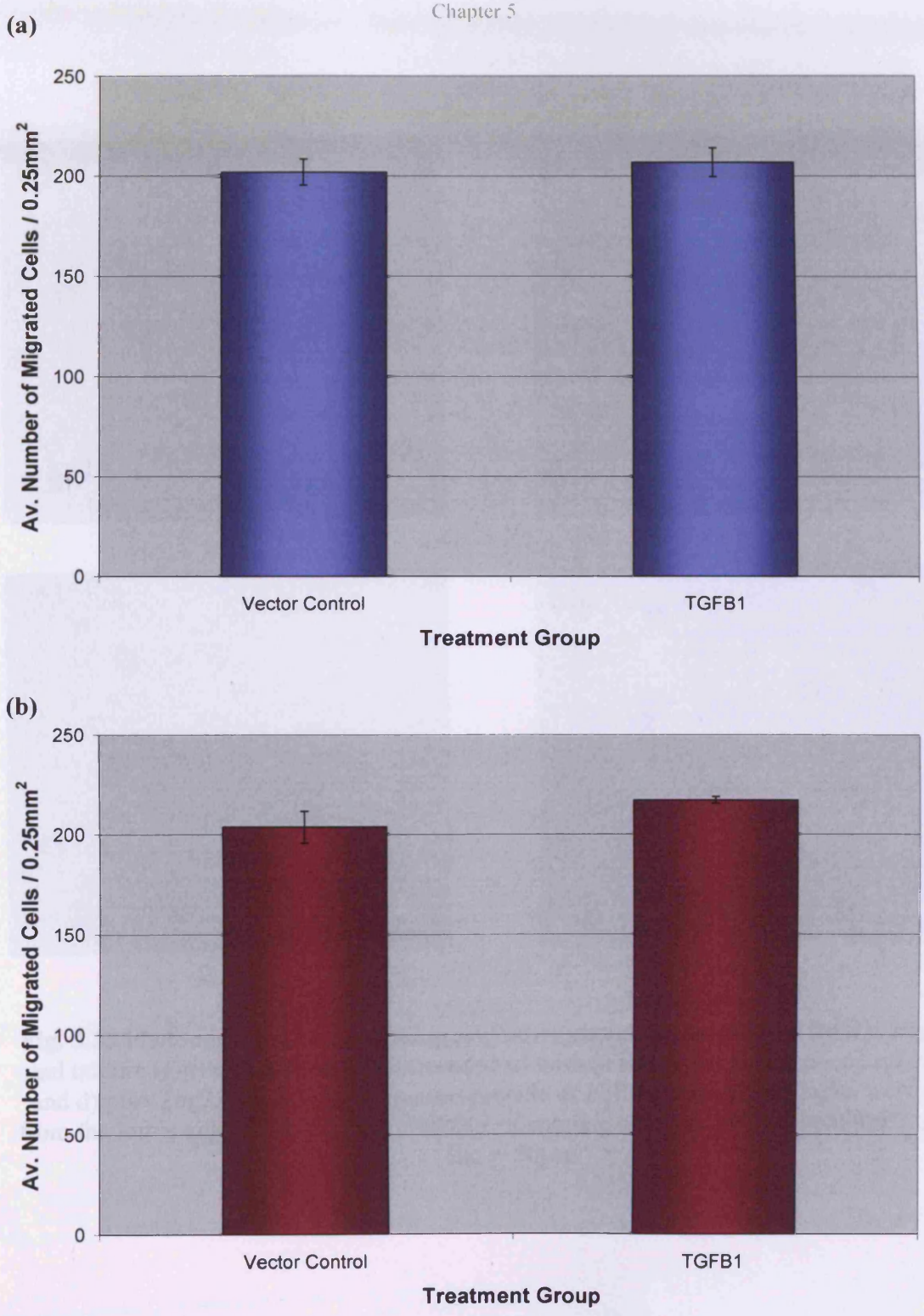


Fig. 5.22 The migration of chondrocytes transfected with pSecTag2B vector or TGFβ1 isolated from (a) young [P=0.530] and (b) mature [P=0.051] articular cartilage over 24 hours. Results illustrate the mean number of migrating cells per 0.25mm^2 visual field with SEM bars and an n value of 3.

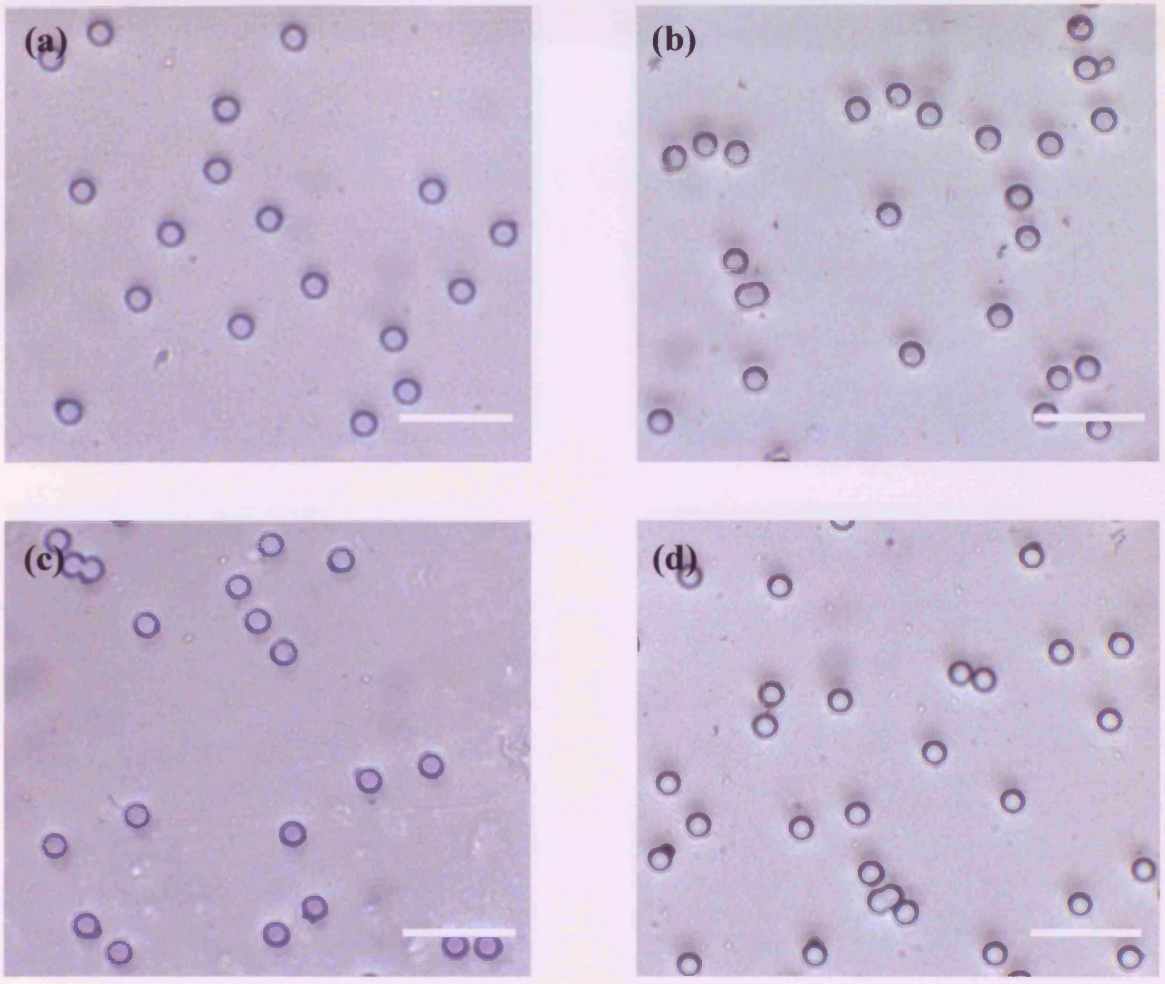


Fig. 5.23 Photomicrographs illustrating migratory chondrocytes isolated from young (a-b) and mature (c-d) articular cartilage transfected with (a and c) the vector pSecTag2A or (b and d) pSecTag2A encoding the mature peptide of IGF-1. Photomicrographs were taken from the lower side of the Boyden chamber membrane after the 24 hour incubation period. Bar = 50μm

demonstrate a definite chondrocytic cell morphology suggesting that these migrating cells are still expressing the chondrocyte phenotype. Over-expression of IGF-1 and TGFβ1 demonstrated definite trends in the up-regulation of chondrocyte migration in both age groups. Adaptation of the employed method to incorporate a selection step for the isolation of transfected cells from the non-transfected cells may provide a means to prove a statistically significant effect of over-expression of IGF-1 and TGFβ1 on the migration of chondrocytes isolated from both young and skeletally mature articular cartilage.

5.5 Conclusions

The aim of the work described within this chapter was to develop constructs for the over-expression of IGF-1 and TGFβ1 in articular chondrocytes. Work detailed in chapters 2 and 4 have demonstrated the positive effects that these 2 growth factors have on chondrocyte migration and matrix biosynthesis; however these effects are limited by the short half lives of IGF-1 and TGFβ1 once in their active form.

I have successfully developed constructs for the over-expression of IGF-1 and TGFβ1 in articular chondrocytes. Within this chapter, chondrocytes isolated from young and mature articular cartilage were transfected with the growth factors, and confirmation of their over-expression was achieved by PCR and immunohistochemistry using a c-myc tag antibody. The transfected chondrocytes were used in a Boyden chamber system to determine whether their ability to migrate was enhanced by over-expression of the growth factor. This experiment was limited by the inability to select for the successfully transfected cells. Results obtained from the migration assay did demonstrate a consistent trend of increased migration with the transfected chondrocytes from both age groups when compared to controls. The development of an appropriate method to select for the transfected cells may prove this difference in migratory capacity to be statistically significant. Due to time restrictions, only preliminary experiments were carried out using these growth factor constructs. It would be interesting to look at the longevity of the expression of these constructs within chondrocytes and also their effects on chondrocyte matrix biosynthesis in addition to migration. Further work could also be undertaken to look at the effects of over-expressing both these growth factors within the same cell.

In summary:

- Constructs were successfully developed for the over-expression of IGF-1 and TGFβ1 in chondrocytes.
- Confirmation of over-expression was achieved at the protein level by immunohistochemistry using a c-myc antibody, and at the mRNA level using primers designed against the region encoding the mature peptides of IGF-1 and TGFβ1.
- Transfected chondrocytes encoding IGF-1 or TGFβ1 demonstrated increased trends of migration on chondroitinase ABC aggrecan.
- These results suggest that IGF-1 and TGFβ1 may be able to increase chondrocyte migration through a non-chemotactic mechanism.

Chapter 6

Final Discussion

6.1 Background

Articular cartilage is an aneural and avascular tissue which consequently demonstrates a low capacity to initiate a repair response once injured. The low density and entrapment of the endogenous chondrocytes within an extensive ECM further hinders the ability of articular cartilage to self-renew. Left untreated, articular cartilage defects lead to degeneration of the surrounding cartilage and the development of diseases such as OA. Injuries which have extended into the subchondral bone frequently lead to filling of the defect area with a fibrocartilaginous tissue generated by the stem cells transported into the defect site from the vasculature. This repair tissue frequently degenerates with time as it is unable to withstand the biomechanical forces exerted on the joint.

Significant advances in the area of cartilage repair have been made in the last twelve years. Development of autologous chondrocyte transplantation by Brittberg et al. (1994) demonstrated the ability of isolated chondrocytes to form new cartilage and fill defects caused by injury. Long term clinical studies into the durability of this repair tissue suggest that it is stable for up to 10 years (Peterson et al., 2003). Histological analysis has shown that this repair tissue is rarely integrated with the endogenous cartilage however, and the compressive and shearing forces associated with use of the joint frequently lead to fibrillation of this repair tissue with subsequent degeneration in later years. Recent research into cell-based cartilage repair strategies has focussed on the generation of a hyaline-like repair tissue that is able to withstand biomechanical loading, and the enhancement of integration with the endogenous, surrounding cartilage.

Findings from both *in vitro* and *in vivo* studies have demonstrated 2 main areas needing to be addressed in order to improve the quality of cartilage repair techniques for the future. These areas are: the need for integration between newly synthesised cartilage and the endogenous tissue, and the generation of a biomechanically stable repair tissue, with the correct composition of collagen and proteoglycan molecules for both tensile and compressive force resistance. This led to the hypothesis that integration between injured articular cartilage surfaces can be enhanced by the addition and stimulation of isolated chondrocytes to the interface regions.

The aim of this project was primarily to devise novel strategies for the enhancement of integration of transplanted chondrocytes and newly synthesised tissue with pre-existing cartilage. Work in the study has focussed on investigating the innate migratory capacities of both chondrocytes isolated from young and skeletally mature articular cartilage and ways that this can be enhanced by the use of the growth factors, IGF-1 and TGFβ1. The ability of these cells to migrate on different matrix components has also been investigated. Few studies have been published looking at the migratory capacity of articular chondrocytes. It is generally accepted that these cells do not migrate. A recent publication by Chang et al., (2003) demonstrated the migration of chondrocytes isolated from newborn calves on fibronectin. No studies to date have investigated the migration of chondrocytes isolated from skeletally mature bovine articular cartilage. Studies within this project have used an *in vitro* model of cartilage injury to establish the role of the GAG, CS in limiting the migration of transplanted chondrocytes into intact articular cartilage. It has been previously demonstrated that the presence of proteoglycans at the wound edge can inhibit the adhesion of transplanted chondrocytes onto the endogenous cartilage (Lee et al., 2000). The effects of IGF-1 and TGFβ1 on chondrocyte biosynthesis of collagen and proteoglycans have also been investigated. Both these growth factors have been demonstrated within my studies to have anabolic effects on articular chondrocytes. Previous studies using TGFβ1 have conflicting results, with both anabolic and catabolic responses being reported. This is thought to be dependent on the cell cycle stage of the treated cells (Trippel, 1995; Vivien et al., 1992; Vivien et al., 1990). The data from my studies demonstrated the potential value of IGF-1 and TGFβ1 in enhancing cartilage repair and led to the development of constructs for the transfection of IGF-1 or TGFβ1 into articular chondrocytes. Transfected chondrocytes were used to look at the over-expression of IGF-1 and TGFβ1 on the migration of articular chondrocytes. No previous studies have looked at the effects of transfecting IGF-1 and TGFβ1 on chondrocyte migration. The overall objective of this project was therefore to combine stimulation of migration of chondrocytes into pre-existing cartilage with enhanced biosynthesis of matrix to produce an integrated and biomechanically stable repair tissue.

6.2 The Migratory Capacities of Chondrocytes Isolated from Young and Mature Bovine Articular Cartilage

Initially the differences in gross morphology of young and skeletally mature articular cartilage was characterised by general histological methods. The number of chondrocytes decreased within the mature cartilage when compared to the younger tissue and consequently the cells were more isolated from each other in this older cartilage. The localisation of the major ECM components, proteoglycans and collagen, were comparable between age groups.

The ability of both chondrocytes isolated from young and skeletally mature articular cartilage to migrate was investigated on a number of ECM macromolecules. This study demonstrated that chondrocytes isolated from mature articular cartilage can migrate when released from their ECM. The choice of matrix protein was shown to affect both the degree of migration and the cellular response to any supplemented growth factors. The migration of chondrocytes isolated from young articular cartilage was inhibited by the anti-cell adhesive proteoglycan aggrecan, but enhanced on the pro-adhesive glycoprotein fibronectin when compared to BSA controls. Treatment of the native monomeric aggrecan substrate with chondroitinase ABC allowed increased migration of chondrocytes isolated from young articular cartilage compared to native aggrecan. Matrix protein did not appear to effect chondrocytes isolated from mature cartilage, but as seen with the younger cells a trend of decreased migration on native aggrecan when compared to chondroitinase ABC treated aggrecan was demonstrated. Chemotactic effects by the growth factors IGF-1 and TGFβ1 to enhance the migration of both age groups of chondrocytes only occurred when the cells were seeded onto a fibronectin substrate. Further to this, a significant increase in the migration of chondrocytes isolated from mature articular cartilage was only seen when both growth factors were used in combination.

In summary these experiments demonstrated the ability of isolated chondrocytes from both young and skeletally mature articular cartilage to migrate and the role of CS in inhibiting this migratory process. Data from these experiments also demonstrated that IGF-1 and TGFβ1 only have chemotactic effects on chondrocytes when the cells are seeded onto a fibronectin substrate.

6.3 Removal of Wound Surface Chondroitin Sulphate to Enhance Migration of Chondrocytes into Articular Cartilage Explants

In light of the findings summarised in section 6.2, the role of CS in inhibiting cell adhesion, migration and integration into wounded articular cartilage was investigated. Many studies have demonstrated the up-regulation of proteoglycan synthesis after articular cartilage injury (Jeffrey et al., 1997; Walker et al., 2000). These proteoglycans have been targeted as one of the reasons for poor integration in cartilage repair. An *in vitro* model of cartilage injury was designed and wound surface CS chains were digested using the eliminase, chondroitinase ABC prior to transplantation with isolated chondrocytes. The region of digestion was detected using monoclonal antibodies, 2B6 and 1B5, which recognise the chondroitinase ABC generated CS-stubs C-4-S and C-0-S respectively. The migration of fluorescently labelled transplanted chondrocytes was tracked into the digested tissue over periods of up to 28 days. Cells from both age groups migrated into the digested tissue, with increasing numbers of labelled chondrocytes found within all zones of the cartilage with time in culture. Depth of chondroitinase ABC digestion of the tissue was a limiting factor for the migration of both age groups of chondrocytes. Controls with undigested explants saw no migration of either age group of cells within the first 7 days. These results correlated well with those discussed in section 6.2. Later time points demonstrated a small number of cells migrating into the undigested explants. This is probably due to the leaching of proteoglycans from the explants with time in culture. Work published by Caterson and Lowther (1976) demonstrated that if cartilage is not under compressive force, proteoglycans will leach out of the tissue with time.

These experiments have demonstrated the ability of chondrocytes from both age groups of cartilage to migrate through the native cartilage ECM if the CS chains from the proteoglycans found within the tissue have been removed. Sufficient digestion of the injured tissue is fundamental to the adequate migration of transplanted cells into the matrix.

6.4 The Effects of IGF-1 and TGFβ1 on the Biosynthesis of Matrix Macromolecules

Work detailed in chapters 2 and 3 has emphasised the importance of chondrocyte migration and the role of matrix macromolecules, as well as chemoattractants, in enhancing this process. In addition to their chemotactic effects, IGF-1 and TGFβ1 have both been demonstrated to have anabolic effects on chondrocytes (Bonassar et al., 1997; Glansbeek et al., 1998). The effects, therefore, of IGF-1 and TGFβ1 on the biosynthesis of the matrix macromolecules, proteoglycans and collagens, and cellular proliferation were investigated, as well as the ability of these growth factors to maintain the chondrocytic phenotype.

IGF-1 and TGFβ1 demonstrated the ability to significantly increase the biosynthesis of sGAGs and collagen in chondrocytes isolated from both age groups of cartilage. The increase in these matrix macromolecules was firstly seen within the culture media removed from the cells after a 1 day incubation period. Significant levels were later seen at day 3 within the cell lysates and their cell associated material. These results strongly suggest deposition of the newly synthesised molecules into matrix. No significant differences in sGAG or collagen levels were seen between treatment groups and the controls by day 7 however, suggesting an up-regulation of matrix degradation returning levels back to normal. Experiments set up to investigate the levels of degradation with IGF-1 and TGFβ1 treatment unfortunately did not result in any meaningful data which could be analysed.

The work within this chapter also addresses the effects of IGF-1 and TGFβ1 on the chondrocytic phenotype. IGF-1 has previously been reported to maintain the chondrocytic phenotype (Guerne et al., 1994; Tyler, 1989) and TGFβ1 has recently been documented to aid in the re-differentiation of chondrocytic cells grown in monolayer culture for ACT techniques (Goldberg et al., 2005). As the chondrocytes used in this study were being grown in a monolayer system, their ability to maintain the chondrocytic phenotype on exposure to both IGF-1 and TGFβ1 was investigated by Western blotting using a type II collagen antibody as well as looking at the mRNA levels of the transcription factor Sox 9 and type II collagen gene. Western blotting conclusively demonstrated the presence of type II pro-collagen in cell lysates and associated material from both age groups of chondrocytes. PCR analysis also demonstrated the consistent expression of both Sox 9 and

type IIB collagen (mature form) throughout time in culture. These data confirm the maintenance of the chondrocytic phenotype throughout the culture period.

The effects of IGF-1 and TGFβ1 on the gene expression of these growth factors, as well as their corresponding receptors were determined. With time in culture message levels of both the growth factors and their receptors decreased. Exposure of chondrocytes to these growth factors exogenously did not affect the message levels of IGF-1 or TGFβ1. Exposure to IGF-1 did up-regulate its own cell surface receptor however, with visible differences in expression between IGF-1 treatment groups and controls at the initial sampling point of 10 minutes and again at days 1 and 3. Conversely exposure to TGFβ1 down-regulated its cell surface receptor with a decrease in expression seen at the 10 minutes, 1 and 3 day time points. The results from these PCR based experiments must be treated with caution and would need to be confirmed by quantitative PCR.

In addition to the previously documented effects of IGF-1 and TGFβ1 on chondrocyte migration, these results confirm the anabolic stimulation they have on this cell type at the concentration they were used at (10ng/ml). PCR data demonstrate that a consequence of exposure to these growth factors is a differential regulation of the specific growth factor's cell surface receptor. These effects were seen whilst still maintaining the characteristic type II collagen and Sox 9 expressing chondrocyte phenotype.

6.5 The Over-expression of IGF-1 and TGFβ1 in Articular Chondrocytes

This chapter brought together the findings and ideas generated from the previous studies and sought to address the limitations and obstacles uncovered; chapter 2 demonstrated the chemotactic effects of both IGF-1 and TGFβ1 on chondrocyte migration but also confirmed the fact that this only occurred if the cells were seeded onto a fibronectin substrate. The effects of these growth factors on chondrocyte migration and matrix biosynthesis, as discussed in chapters 2 and 4 were limited by the short half lives of these growth factors. Once within their active state both IGF-1 and TGFβ1 have a half life of minutes. In order to have a sustained effect on chondrocytes, these growth factors need to be over-expressed within the cell. This chapter documents the development of constructs encoding for the mature peptides of bovine IGF-1 and TGFβ1.

Constructs were developed and initially expressed using the pGEM®-T vector within competent E-coli (JM109). Bacterial plasmid was produced and the correct sequence homology was confirmed as well as in frame expression. Sequences encoding *Bam*H I and *Not* I restriction sites were added to the 5' ends of the forward and reverse primers respectively of IGF-1 and TGFβ1 ensuring the correct orientation of the inserted sequences into the mammalian expression vector, pSecTag2.

Chondrocytes were transfected with the plasmids using a liposomal method. This allows transient over-expression of the growth factors within the chondrocytes. Due to the low replication rate of this cell type, transfected cells could not be selected for using the selective antibiotic Zeocin®. Over-expression of the constructs and a good transfection efficiency (22% and 24% of chondrocytes isolated from young and mature cartilage respectively) was confirmed by immunohistochemistry using an antibody raised against the c-myc epitope. PCRs also demonstrated an increased level of mRNA for IGF-1 and TGFβ1 in the transfected cells with the respective growth factor when compared to vector only controls.

The beneficial effects of removing CS chains in enhancing migration were demonstrated in chapters 2 and 3. Transfected chondrocytes were seeded onto a chondroitinase ABC treated aggrecan substrate to investigate whether these growth factors could further increase cell migration. Data from this experiment suggests that this may be the case with a consistent trend of increased migration with cells transfected with either IGF-1 or TGFβ1 when compared to vector only controls. Due to the inability to select over-expressing cells from those non-transfected, these effects were not significant. Development of a method to select transfected chondrocytes from the mixed population may confirm this increased migratory effect.

The data from this chapter uses the information gathered from previous work to demonstrate the use of over-expression of these anabolic growth factors with a cartilage repair system. By transiently over-expressing IGF-1 or TGFβ1 there is the possibility of increasing both cell migration and matrix biosynthesis within the initial repair period and then returning the cells back to their normal status. My data confirm that chondrocytes isolated from young and mature articular cartilage can be successfully transfected and that

increased expression of these growth factors exerts a positive effect on chondrocyte cell migration.

6.6 Implications for Cartilage Repair

These experiments bring together the ideas of enhanced integration for the development of stable repair tissue for cartilage repair. Data from the migration and biosynthesis experiments, and the generation of transfected chondrocytes over-expressing an anabolic growth factor such as IGF-1 or TGFβ1, suggests that the phenotype of chondrocytes can be manipulated such that they may enhance cartilage repair.

As discussed in section 1.13.2, enhanced migration will not improve cartilage repair on its own. Enhanced migration will provide a scaffold for integrated repair but the strength and stability of the repair tissue also needs to be considered. Fibrocartilaginous repair tissue; containing a large proportion of type I collagen does not possess the same biomechanical characteristics as native articular cartilage. Stabilisation of the chondrocytic phenotype is essential for the development of a stable hyaline cartilage repair tissue. A culmination of enhanced migration and the formation of integrated hyaline cartilage repair tissue may provide an advance in cell-based cartilage repair strategies currently available. Fibrocartilaginous repair tissue seen with monolayer expanded chondrocytes is most likely due to the fragility of the chondrocyte phenotype and the dedifferentiation of the cells into fibroblasts. Over-expression of a growth factor such as IGF-1 or TGFβ1, which potentially stabilises and protects the chondrocytic phenotype, and the ability to produce characteristic type II collagen, may be important during the necessary monolayer expansion of isolated chondrocytes required for the ACT procedure. As discussed in section 5.1 exogenous provision of growth factors is not effective due to their short half-lives. Transient over-expression of the gene within the target cell may help overcome these problems.

6.7 Future Avenues for Investigation

Experiments from this project have confirmed that chondrocytes isolated from both young and mature articular cartilage have the ability to migrate. This has not previously been reported in cells from skeletally mature, bovine articular cartilage. The Boyden chambers

used for these experiments are a simple and effective way of quantifying cell migration, however it would be interesting and highly informative to visualise the migratory process of these cells using time-lapse video-microscopy. This method would not only allow the migration of chondrocytes to be measured using a different system but also provide a means to look at the morphology of the cell and rearrangement of the cytoskeleton during motility. As discussed in section 2.5, attempts were made to investigate chondrocyte migration using time-lapse video-microscopy. Ideally, using an automated microscope stage, comparative analysis of chondrocyte migration on different ECM substrates, using the same batch of cells, could have been achieved. The lack of adhesion of the cells to the substrates resulted in the detachment of the cells from the plate surface as the microscope stage moved. Further optimisation regarding the attachment period is required to resolve this issue. It is possible to overcome this problem by the use of a static microscope stage; however this would mean that it would not be possible to use the same batch of primary cells for each substrate to be investigated.

Data in chapter 2 documented the enhanced migration of chondrocytes in the presence of IGF-1 and TGFβ1. This effect was substrate dependent and was only seen when the cells were seeded onto fibronectin. Chemotactic effects can also be calculated using a Dunn chamber. This system uses time-lapse video-microscopy to visualise the movement of cells towards a source of chemoattractants. It is possible using this method to calculate whether directional movement and therefore a true chemotactic response is being seen.

Work in chapter 3 developed the ideas from the previous migration work and used an *in vitro* model of cartilage injury to visualise the migration and integration of transplanted chondrocytes into an articular cartilage explant. The tissue had been previously digested with chondroitinase ABC, and it was reported that CS inhibited the migration of these isolated cells into the cartilage matrix. Removal of this GAG resulted in increased migration of both age groups of cells into the explant. As discussed in chapter 3, migrating chondrocytes appeared to demonstrate the correct morphology for the zone of the cartilage explant that they had migrated into, and were also situated within lacunae. It would be interesting to investigate this finding further, possibly through the transfection of chondrocytes with a type VI collagen expressing vector and GFP tag. This would

demonstrate whether the migrating chondrocytes are moving in and occupying pre-existing lacunae, or rather are generating their own.

Furthering this work, it would be possible to adapt this model to look at the migration of isolated chondrocytes into opposing cartilage explants digested with chondroitinase ABC, and visualise over time, the possible integration of the explants by the transplanted chondrocytes. Their increased migration and continued matrix biosynthesis may provide a well integrated repair tissue. The mechanical integrity of the integrated tissue needs also to be investigated. Techniques, such as the single-lap shear test, for the quantification of mechanical integration between opposing cartilage explants have been previously described (DiMicco and Sah, 2001; Giurea et al., 2002; McGowan and Sah, 2005).

In addition, it would be beneficial to investigate whether other GAG chains such as KS within the cartilage ECM have such an inhibitory effect on chondrocyte migration. Using the previously established model, it would be possible to use specific keratanase enzymes to digest the matrix of this GAG chain and look at chondrocyte migration into the digested matrix. Valuable information could also be gathered from further understanding of the interaction between CS with the chondrocyte in exerting its inhibitory effects. Knowing the structure of CS it would be feasible to generate fragments of the GAG which could be used as substrates for further migration studies, possibly elucidating the inhibitory component.

Further work investigated the effects of the growth factors, IGF-1 and TGFβ1, on chondrocytes from young and mature cartilage. The data illustrated the anabolic effects of these growth factors on matrix biosynthesis, but it would have been greatly beneficial to have been able to compare these biosynthesis data to the amount of degradation occurring within the cells, and to have looked at whether this had also been affected by the growth factors. IGF-1 has been previously shown to decrease MMP expression and increase the levels of TIMPs (Hui et al., 2001a; Hui et al., 2001b). In addition, as seen with TGFβ1, this growth factor can also counteract the degradative effects of pro-inflammatory cytokines such as IL-1 (Tyler, 1989; van Beuningen et al., 1993; van Beuningen et al., 1994). A cold chase experiment running alongside the biosynthesis experiment would have given an indication as to the amount of degradation of the newly synthesised material over the

culture period. Such an experiment was set up for inclusion in this thesis; however the data obtained was insufficient for analysis. Optimisation of the procedure would be required.

These biosynthesis experiments used exogenous growth factors. Using the constructs developed for the transient over-expression of IGF-1 and TGFβ1 it would now be possible to investigate the effects of long-term exposure to these growth factors on both matrix biosynthesis and degradation. Time limitations only allowed preliminary experiments to be performed using the transfected cells. It would be interesting to further characterise the longevity of the transfected construct and also its effects on protein expression of the encoded growth factor. These transfected cells were used in a preliminary experiment to investigate whether over-expression of IGF-1 or TGFβ1 enhanced the migration of isolated chondrocytes. Development of an appropriate selection technique would have proved these results to be significant, although without such conditions in place it was encouraging to see a consistent trend of increased migration in the growth factor over-expressing cells when compared to controls. It would also be interesting to investigate the effects of over-expressing both growth factors within articular chondrocytes, in terms of their migratory capacity and biosynthetic activities. Further experiments, using both the time-lapse method previously discussed with different substrates as before, and the model developed for cartilage injury would investigate any differences in migratory capacities further.

All the experiments within this project have compared chondrocytes isolated from young and skeletally mature bovine cartilage. This work gives proof of principle to methods for enhancing cartilage repair. Further research is required to demonstrate that similar effects are seen within human chondrocytes. Clinical studies investigating ways to enhance cartilage repair using methods such as ACT have started to encompass older patients and those suffering from degenerative diseases such as OA. The results described in this thesis have focussed on young and skeletally mature cartilage, but not old. Further work is therefore needed to investigate whether chondrocytes isolated from old cartilage behave in the same manner to those isolated from the skeletally mature cartilage.

6.8 Conclusions

In conclusion, this study demonstrates the ability of both chondrocytes isolated from young and skeletally mature articular cartilage to migrate when released from their ECM. The importance of the matrix protein in contact with the migrating cell has been demonstrated, with aggrecan seen to have an inhibitory effect. This inhibitory effect was partially attributed to the anti-cell adhesive sGAG, CS as demonstrated in both the Boyden chamber system and using an *in vitro* model of cartilage injury. Removal of this sGAG enhanced the migration of cells from both age groups into the ECM of articular cartilage. The substrate dependent chemotactic effects of IGF-1 and TGFβ1 on chondrocyte migration were also elucidated, with their enhancing effects only demonstrated when the cells were seeded onto a fibronectin substrate. Further work confirmed the anabolic effects of both these growth factors on chondrocyte matrix biosynthesis in addition to their chemotactic effects. The short half life of exogenous IGF-1 and TGFβ1 led to the development of constructs encoding these growth factors, used to transfect articular chondrocytes. Preliminary work suggests that over-expression of IGF-1 and TGFβ1 may help overcome their substrate dependent effects on enhanced cell migration. Further studies to characterise the effects of transient over-expression of these growth factors in articular cartilage chondrocytes may provide a means of delivering anabolic factors to a defect site and aid the process of chondrocyte migration and matrix production for the development of a biomechanically stable repair tissue that is well integrated with the pre-existing cartilage.

Chapter 7

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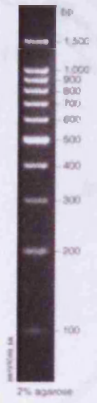
Appendices

Appendix 1

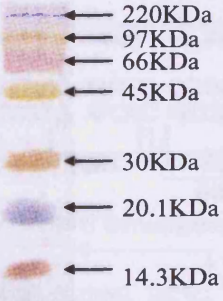
1.1 DNA Molecular Weight Standard

1.1.1 100bp Molecular Weight Ladder (Promega, UK)



1.2 Protein Molecular Weight Standard

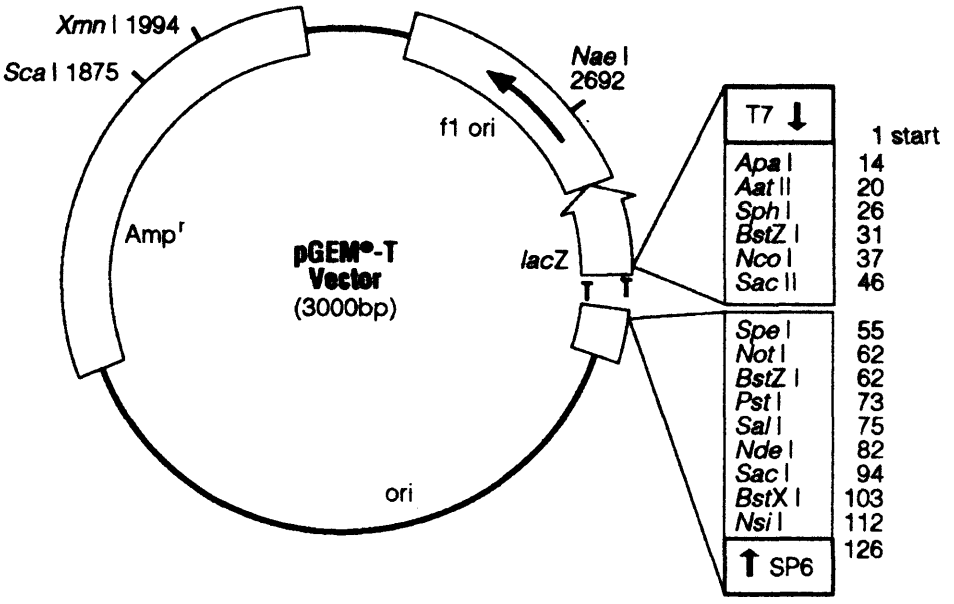
1.2.1 Rainbow Molecular Weight Ladder (GE Healthcare Ltd., UK)

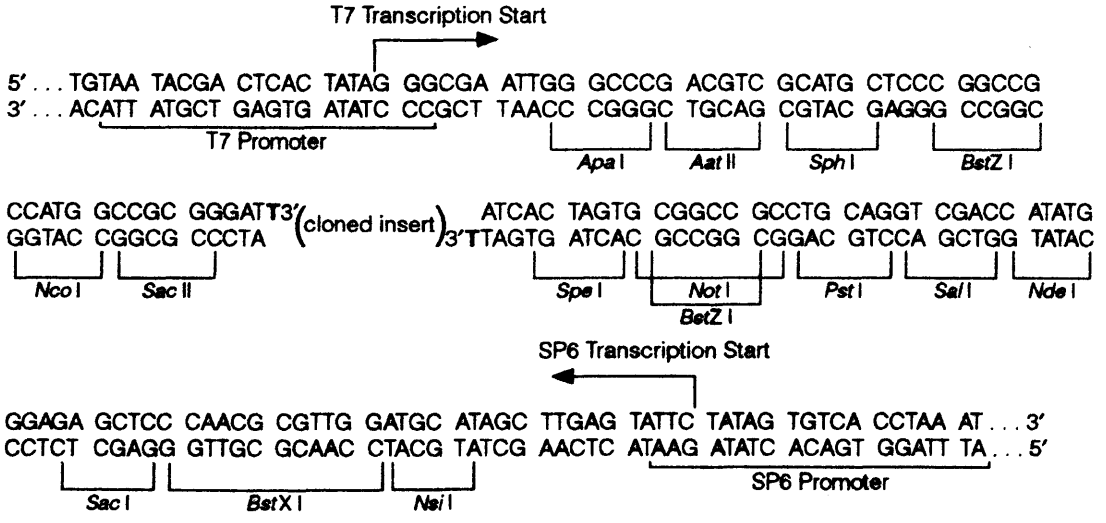


Appendix 2

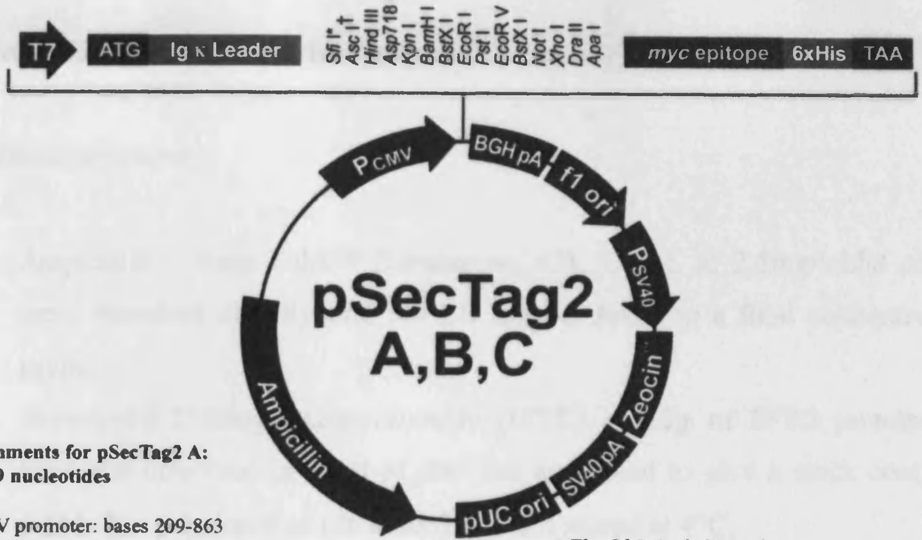
2.1 Vector Maps and Multiple Cloning Sites

2.1.1 pGEM®-T Vector (Promega, UK)





2.1.2 pSecTag2 Vector (Invitrogen Ltd., UK)



Comments for pSecTag2 A:
5159 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Murine Ig κ-chain V-J2-C signal peptide: bases 905-967
Multiple cloning site: bases 970-1081
c-myc epitope: bases 1082-1114
Polyhistidine tag: bases 1127-1144
BGH reverse priming site: bases 1167-1184
BGH polyadenylation sequence: bases 1166-1380
fl origin: bases 1443-1856
SV40 promoter and origin: bases 1924-2245
EM-7 promoter: bases 2261-2327
Zeocin™ resistance gene: bases 2328-2702
SV40 polyadenylation sequence: bases 2832-2961
pUC origin: bases 3345-4018
Ampicillin resistance gene: bases 4163-5023 (C)

* The *Sfi* I site is located before the variable region.

†Frame-dependent variations: *Asc* I is only found in version A.

```
                    enhancer region (3' end)
 699  AATGGGAGTT TGTTTTGGCA CCAAAATCAA CGGGACTTTC CAAAATGTCG TAACAACTCC

                  CAAT                                TATA       3' end of hCMV
 759  GCCCCATTGA CGCAAATGGG CGGTAGGCGT GTACGGTGGG AGGTCTATAT AAGCAGAGCT

                 putative transcriptional start        T7 promoter primer binding site
 819  CTCTGGCTAA CTAGAGAACC CACTGCTTAC TGGCTTATCG AAATTAATAC GACTCACTAT

                                        Ig κ-chain leader sequence
 879  AGGGAGACCC AAGCTGGCTA GCCACC ATG GAG ACA GAC ACA CTC CTG CTA TGG
                                   Met Glu Thr Asp Thr Leu Leu Leu Trp

                                                                   Sfi I
 932  GTA CTG CTG CTC TGG GTT CCA GGT TCC ACT GGT GAC GCG GCC CAGCCG
      Val Leu Leu Leu Trp Val Pro Gly Ser Thr Gly Asp
                                        Signal cleavage site

                           Hind III  Asp718 I  Kpn I   BamH I        BstX I*  EcoR I
 980  GCCAGGCGC [GCGCGCCG] TACGAAG CTTGGTACCG AGCTCGGATC CACTCCAGTG TGGTGGA

            Pst I  EcoR V     BstX I*  Not I    Xho I    Dra II  Apa I
1041  ATTCTGCAGA TATCCAGCAC AGTGGCGGCC GCTCGAGGAG GGCCC GAA CAA AAA CTC
                                                      Glu Gln Lys Leu

                 myc epitope                            Polyhistidine tag
1098  ATC TCA GAA GAG GAT CTG AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT
      Ile Ser Glu Glu Asp Leu Asn Ser Ala Val Asp His His His His His

                                   BGH reverse priming site
1146  CAT TGA GTTTAAACCC GCTGATCAGC CTCGACTGTG CCTTCTAGTT GCCAGCCATC
      His ***

1202  TGTTGTTTGC CCCTCCCCCG TGCCTTCCTT GACCTGGAAA GGTGCCACTC CCACTGTCCT

          BGH poly (A) addition site
1262  TTCCTAATAA AATGAGGAAA TTGCATCGCA TTGTCTGAGT AGGTGT
```

*Note that there are two *BstX* I sites in the polylinker.

Appendix 3

3.1 Media Recipes for Bacterial Growth

3.1.1 Stock Solutions

1. Ampicillin – Amp Tabs™ (Stratagene, CA, USA), at 2.5mg/tablet of ampicillin, were dissolved directly into the LB agar or broth to a final concentration of 100 μg/ml.
2. isopropyl-β-D-thiogalactopyranoside (IPTG) – 1.2g of IPTG powder (Promega, UK) was dissolved in 50ml of distilled water and to give a stock concentration of 0.1M. The solution was filter sterilised and stored at 4°C.
3. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) – 50 mg/ml stock concentration in N, N-dimethylformamide (DMF) was stored at -20°C (Promega, UK).
4. SOC media – 2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgS0_4$, 20mM glucose (Invitrogen Ltd., UK)

3.1.2 Luria Broth (LB) Agar Plates

One LB agar tablet was dissolved per 50ml of distilled water and autoclaved at 121°C for 11 minutes. The agar was allowed to cool to temperate before the addition of supplements. Plates were poured aseptically and allowed to set before storing at 4°C until use. Plates were allowed to warm to room temperature before use.

3.1.3 Supplements Added to LB Agar

pGEM®-T Vector Selection –	Ampicillin (100μg/ml)
	IPTG (0.5mM)
	X-Gal (80μg/ml)

3.1.4 LB Broth

LB broth tablets were dissolved, 1 per 50ml of distilled water and autoclaved at 121°C for 11 minutes. Both were allowed to cool to temperate before the addition of ampicillin (100μg/ml) and storage at 4°C. Broth was stored for up to 2 weeks at 4°C.

Appendix 4

4.1 Sequencing Oligonucleotide Primers

T7	5' TAA TAC GAC TCA CTA TAG GG 3'
M13 Forward	5' CGC CAG GGT TTT CCC AGT CAC GAC 3'
M13 Reverse	5' AGC GGA TAA CAA TTT CAC ACA GGA 3'
IGF-1 *Bam*H I Forward	5'GCG GGA TCC AGG ACC CGA GAC 3'
IGF-1 *Not* I Reverse	5'CGC GCG GCC GCG GCC GAC TT 3'
TGFβ1 *Bam*H I Forward	5'GCG GGA TCC GCC CTG GAC ACC AAC TAC TGC TTC AGC TCC 3'
TGFβ1 *Not* I Reverse	5'CGC GCG GCC GCG CTG CAC TTG CAG G 3'

Appendix 5

5.1 Buffer Recipes

5.1.1 Phosphate Buffered Saline (PBS) pH 7.4

A 10x stock was made up for storage at room temperature containing:

87g Sodium Chloride
2g Potassium Chloride
2.72g Potassium hydrogen phosphate
11.36g Sodium hydrogen phosphate

The 10x stock was diluted to 1 x using distilled water and the pH adjusted to 7.4 using hydrochloric acid before autoclaving at 121°C for 11 minutes and storing at room temperature.

5.1.2 PBS and Tween 20™

A 1x working concentration of PBS was made up and the pH adjusted as previously described. Tween 20™ was added to a working concentration of 0.001%.

5.1.3 Laemmli Buffer

A 10x stock was made up for storage at room temperature containing:

100g SDS
300g Tris
1440g Glycine

The above reagents were dissolved in 10 litres of distilled water.

Publications/Presentations

XXth Federation of the European Connective Tissue Societies (FECTS) Meeting,Oulu, Finland, 1-5th July 2006
"Comparing the Response of Chondrocytes from Young and Mature Articular Cartilage to IGF-1 and TGFβ1" LC Davies, Blain E, Caterson B, Duance V

British Society of Matrix Biology Autumn Meeting, Manchester, 12-13th September 2005
"The Role of Sulphated Glycosaminoglycans in Inhibiting Chondrocyte Migration"
LC Davies, Blain E, Caterson B, Duance V
International Journal of Experimental Pathology *In Press*

XIXth Federation of the European Connective Tissue Societies (FECTS) Meeting, Taormina-Giardini Naxos, Italy, 9-13th July 2004
"Enhancing Tissue Integration for Cartilage Repair: Comparing Young and Mature Chondrocytes" LC Davies, Blain E, Caterson B, Duance V

5th International Cartilage Repair Society (ICRS) Symposium, Ghent, Belgium 26-29th May 2004
"Chondroitinase Treatment Enhances Tissue Integration in an *in vitro* Model of Cartilage Repair" LC Davies, Blain E, Caterson B, Duance V

British Society of Matrix Biology and UK Tissue and Cell Engineering Society, Bristol, 13-14th September 2004
"Comparing the Migratory Capacities of Young and Mature Chondrocytes for the Enhancement of Cartilage Repair" LC Davies, Blain E, Caterson B, Duance V
International Journal of Experimental Pathology Vol. 86 No. 3 A11

British Society of Matrix Biology Autumn Meeting, London, 18-19th September 2003
"Novel Strategies for Enhancing Tissue Integration in Cartilage Repair"
LC Davies, Caterson B, Duance V
International Journal of Experimental Pathology Vol. 85 No. 1 February 2004 A21

Tissue and Cell Engineering Society, Cardiff, 8-10th September 2003
"Novel Strategies for Enhancing Tissue Integration in Cartilage Repair"
LC Davies, Caterson B, Duance V
European Cells and Materials Journal Vol. 6 Suppl. 2 Sept 2003 pg 32

Oral Presentations

Internal Seminar Series, Dental School, University Hospital, Wales, 1st July 2005
"The Role of Sulphated Glycosaminoglycans in Inhibiting Chondrocyte Migration"
LC Davies, Blain E, Caterson B, Duance V

XIXth Federation of the European Connective Tissue Societies (FECTS) Meeting, Taormina-Giardini Naxos, Italy, 9-13th July 2004
"Enhancing Tissue Integration for Cartilage Repair: Comparing Young and Mature Chondrocytes" LC Davies, Blain E, Caterson B, Duance V