The role of connective tissue growth factor (CTGF) in articular cartilage

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Abstract

Work from our group has identified an important role for the pericellular matrix (PCM) of cartilage in mechanotransduction. This region of the matrix sequesters regulatory molecules such as fibroblastic growth factor 2 (FGF2) and releases them upon mechanical stimulation. By carrying out a proteomic analysis of PCM proteins we identified an additional regulatory molecule, connective tissue growth factor (CTGF). CTGF was of interest to us as this protein is up-regulated in osteoarthritis but its function in chondrocytes is unknown.

I confirmed the pericellular localisation of CTGF in cartilage and showed that it binds to heparan sulphate perlecan and like FGF2, CTGF is rapidly released from the PCM upon cartilage injury.

In order to determine its function in chondrocytes, I expressed recombinant CTGF and performed a microarray study in isolated human chondrocytes. Four genes were up-regulated by CTGF and all were known to be transforming growth factor beta (TGFβ) inducible. Recombinant CTGF was able to activate SMAD2 in porcine chondrocytes indicating that it can activate the canonical TGFβ signalling pathway. Both CTGF induced gene expression and SMAD2 phosphorylation was abrogated by TGFβ neutralising antibody and the TGFβ receptor inhibitor SB431542, suggesting that the activity of CTGF is TGFβ dependent. Adding exogenous CTGF to rested porcine cartilage increased TGFβ protein accumulation without affecting TGFβ mRNA levels, suggesting that CTGF may control TGFβ protein's bioavailability. Injured porcine cartilage caused rapid release of endogenous CTGF as well as a delayed accumulation of TGFβ in the conditioned medium (CM).

TGFβ proteins are synthesised and secreted in a latent complex associated with the latency-associated peptide (LAP). When I analysed the explantation CM under non-reducing conditions, CTGF resolved at a high molecular weight (~150kDa), and a high molecular weight fraction of this CM contained SMAD2 activity suggesting that CTGF may be in complex with latent TGFβ. This was supported by the identification of LAP in CTGF immunoprecipitates. Injured cartilage from CTGF knockout mice released reduced levels of CTGF but apparently normal levels of TGFβ suggesting that CTGF is not necessary for release of latent TGFβ. However, SMAD2-phosphorylating ability of the medium was compromised indicating that CTGF controls TGFβ bioavailability principally by facilitating activation of latent complex at the surface of the chondrocyte. These data unveil a novel role for CTGF in cartilage through controlling TGFβ bioavailability.

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Statement of Originality

I hereby certify that all the experimental work presented in this thesis has been performed by me. Any ideas, techniques, quotations, or any other material from the work of other people included in my thesis are fully acknowledged in accordance with the standard referencing practices.

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Abbreviations

ACVR	Activin receptor
ADAM	A disintegrin and metalloproteinase
ADAMTS	A Disintegrin And Metalloproteinase with Thrombospondin Motifs
ALK	Activin receptor like kinase
BMP	Bone morphogenetic protein
BMPR	BMP receptor
BSA	Bovine Serum Albumin
CCN	Cyr61, CTGF, Nov
CHX	Cycloheximide
CM	Conditioned medium
COL2	Type II collagen
COMP	Cartilage oligomeric matrix protein
COX2	Cyclooxygenase 2
CS	Chondroitin sulfate
CT	C terminal
CTGF	Connective tissue growth factor
DMEM	Dulbecco's Modified Eagle's Medium
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ERK	Extracellular signal regulated kinase
FCS	Fetal bovine serum
FGF2	Fibroblast growth factor 2
FGFR	FGF receptor
FT	Flow through
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HAC	Human articular chondrocyte
HEK293	Human embryonic kidney 293 cell line
HGF	Hepatocyte growth factor
HS	Heparan sulfate
HSPG2	Heparan sulfate proteoglycan 2
HUVEC	Human umbilical vein endothelial cell
IGF	Insulin like growth factor
IGFBP	Insulin like growth factor binding protein
IL1	Interleukin 1
IL1RI	IL1 signalling receptor
IL1RII	IL1 decoy receptor
IP	Immunoprecipitation
ITM	Interterritorial matrix
KS	Keratan sulfate
LAP	Latency associated peptide

LLC	Large latent complex
LPS	Lipopolysaccharide
LRP	Low density lipoprotein receptor related protein
LTBP2	Latent TGF beta binding protein
M-CSF	Macrophage colony-stimulating factor
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
mTORC	Mammalian target of rapamycin
N-CAM	Neural cell adhesion molecule
NF κB	Nuclear factor kappa B
NHDF	Normal human dermal fibroblast
NRK	Normal Rat Kidney
OA	Osteoarthritis
PBS	Phosphate buffered saline
PCM	Pericellular matrix
PDGF	Platelet derived growth factor
PI	Propidium iodide
PI3K	Phosphoinositide 3 kinase
PMEPA1	Prostate transmembrane androgen induced protein 1
PRELP	Proline/arginine rich end leucine rich repeat protein
pSMAD	Phospho-SMAD
PVDF	Polyvinylidene fluoride
RT-PCR	Reverse transcription polymerase chain reaction
RUNX	Runt related transcription factor 1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siCTGF	CTGF siRNA oligo
siSCR	Scrambled siRNA oligo
SLC	Small latent complex
SM	Starting material
TGFβ	Transforming growth factor beta
TGFRII	TGF beta type II receptor
TIMP	Tissue inhibitor of metalloproteinase
TM	Territorial matrix
TNFα	Tumor necrosis factor alpha
TRAF6	Transforming tumor necrosis factor inducible gene 6
TrKA	Neurotrophic tyrosine kinase receptor type 1
TSG6	Tumor necrosis factor receptor associated factor 6
TSP1	Thrombospondin 1
UBCre	Ubiquitin Cre
VEGF	Vascular endothelial growth factor
VWC	Von Willebrand factor type C repeat

Chapter 1:

Introduction

1.1 Background

Articular cartilage is an avascular tissue, consisting of articular chondrocytes isolated by an extensive extracellular matrix (ECM), rich in collagen, proteoglycans and water. One common disease associated with cartilage is osteoarthritis (OA), which is characterised by the loss of ECM and clustering of chondrocytes in enlarged lacunae, resulting in the degeneration of the tissue and exposure of the bone (see later for details). Mechanical injury on cartilage such as cutting or wear and tear predisposes the joint to OA, but how tissues respond to these mechanical insults is largely unknown.

I will start the thesis by giving an introduction to cartilage, its development and structure and how it is turned over in health and disease.

1.2 Articular cartilage

Cartilage is classified in the three groups: elastic cartilage, hyaline cartilage and fibrocartilage. Articular cartilage is hyaline cartilage. It is the smooth and white tissue covers the ends of bones where they meet together to form joints. Its principle role is to provide a lubricated surface for articulation and to facilitate the transmission of loads with a low friction coefficient. Articular cartilage is devoid of blood vessels, lymphatics, and nerves and is thought to have a limited capacity for intrinsic healing and repair (Pearle *et al.* 2005). In this regard, preservation of the integrity and health of articular cartilage is important for long term joint health.

1.2.1 Development of articular cartilage

Cartilage is formed by a process called chondrogenesis from condensed mesenchyme tissue that differentiates into clusters of cartilage cells called chondrocytes that begin secreting the molecules that form the extracellular matrix. Multipotential mesenchymal cells that are derived from the mesoderm germ layer receive signals that determine the shape, size and number of mesenchymal condensations. These signals are initiated by cell-cell and cell-matrix interactions. Adhesion molecules, neural cell adhesion molecule (N-CAM) and N-cadherin, are thought to have important roles in this process (DeLise *et al.* 2000; Goldring *et al*. 2006). The condensation process is followed by differentiation, a process controlled by a number of factors including the transcription factors Sox5, 6 and 9 and runt-related transcription factor 1 (RUNX1) and growth factors including fibroblast growth factors (FGFs) and transforming growth factor beta (TGFβ) (Cole 2011; Bi *et al*. 1999). Differentiated chondrocytes then undergo a process of proliferation and maturation which results in the production of an ECM characterised by the cartilaginous molecules type II collagen and aggrecan (Archer, 1994). The fate of developing chondrocytes varies depending on their location and origin. Some cells survive and remain as active and functional articular chondrocytes whereas those that comprise the epiphyseal growth plates go through a

proliferative phase and eventually become hypertrophic (Pacifici *et al.*, 2000). Hypertrophic chondrocytes then undergo terminal differentiation which results in cell death, followed by growth of osteoblasts and blood vessels, and formation of a mineralised matrix. The process by which the cartilage template is gradually replaced with a calcified bone matrix through the invasion of osteoblasts is termed endochondral ossification (Shimizu *et al.*, 2007).

1.2.2 Components and structure of articular cartilage

Articular cartilage is composed of chondrocytes embedded in a highly ordered extracellular matrix of collagen, proteoglycans and non-collagenous proteins. Its highly organised structure allows it to function as a frictionless gliding surface that cushions compressive, tensile and shearing forces from articulation of synovial joints. Articular cartilage is typically depicted in four zones which vary in matrix composition and cell morphology (Figure 1.1).

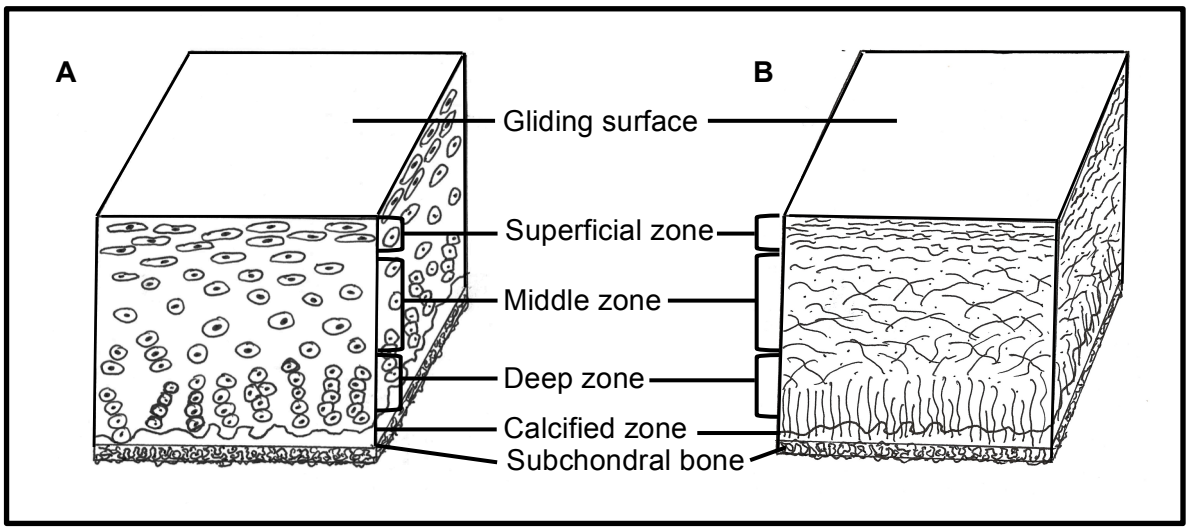


Figure 1.1 Schematic, cross-sectional diagram of normal articular cartilage (hand drawn, inspired by an image from Jazrawi *et al*. 2011). A: Cellular organisation in the zones of articular cartilage. B: Collagen fibres architecture.

The superficial zone includes a cell-free gliding surface that adjoins a layer consisting of elongated chondrocytes and densely packed collagen fibrils. These cells and fibrils have a highly organised alignment that is parallel to the articular surface. The middle zone (also

called the transitional zone) consists of a less organised arrangement of the collagen fibrils and the chondrocytes in this zone are mostly rounded. The deep zone (or radial zone) consists of large diameter collagen fibrils orientated perpendicular to the articular surface. This layer contains the highest proteoglycan and lowest water content. The chondrocytes are typically arranged in columnar fashion parallel to the collagen fibrils and perpendicular to the joint line. The calcified zone contains cells expressing a hypertrophic phenotype that are able to synthesise a calcified matrix that provides an interface between uncalcified cartilage and the underlying subchondral bone (Akizuki *et al*. 1986; Kempson *et al.* 1973; Poole *et al*. 2001).

1.2.3 Articular chondrocytes

Chondrocytes are normally morphologically rounded or polygonal except at tissue boundaries where they may be flattened such as within the superficial layer of articular cartilage (Villar-Suárez *et al*. 2004). Chondrocytes are not capable of cell division, however, they are metabolically active to synthesise and turn over a large volume of the extracellular matrix (Archer and Francis-West 2003). Articular chondrocytes are embedded in an extensive extracellular matrix with an extremely long diffusion distance from the nourishing arteries. As a result, chondrocytes must reply on diffusion from articular surface to obtain nutrients and metabolites and adapt highly effective mechanisms to the hypoxia environments (Distler *et al*. 2004). The majority of the cell's energy requirements come from glycolysis that occurs in the cytoplasm and, as a result, chondrocytes normally do not contain abundant mitochondria (Archer and Francis-West 2003).

1.2.4 The extracellular matrix

The extracellular matrix (ECM) of articular cartilage consists of collagen (predominately type II collagen), proteoglycan aggregates containing glycosaminoglycans (GAGs), and multiadhesive glycoproteins (noncollagenous proteins).

Collagens are the most abundant protein in the ECM and play important biological and mechanical roles in articular cartilage. Type II, IX and XI collagens form a fibrillar network that provides tensile strength throughout the ECM (Buckwalter and Mankin 1998; Yingst *et al.* 2009). Type VI collagen is thought to form hexagonal networks in close proximity to the chondrocytes (pericellular matrix, see later), enabling the cells to attach to the ECM via integrins (on cell membrane) and biglycan (in the ECM) (Buckwalter and Mankin 1998), and acting as a transducer of biomechanical and biochemical signals in the articular cartilage (Guilak *et al*. 2006; Roughley and Lee 1994).

Embedded in the collagen mesh are large aggregated proteoglycans (aggrecan attached to hyaluronan through link protein), which together give cartilage its stiffness and resilience for its long-term durability (Kiani *et al*. 2002; Roughley and Lee 1994). Aggrecan is the most abundant proteoglycan that exhibits a bottlebrush structure, in which chondroitin sulphate (CS) and keratan sulphate (KS) chains are attached to an extended protein core (Fosang *et al*. 1992). These CS and KS chains are negatively charged GAGs that interact with positive sodium ions thus attracting water molecules (60% to 80% of wet weight of articular cartilage) through osmosis (Pearle *et al.* 2005). The resulting hydrated aggrecan provides lubrication within the joint and contributes to the load-bearing capacity of articular cartilage. Small proteoglycans in cartilage include decorin (Krusius and Ruoslahti 1986), fibromodulin (Antonsson *et al.* 1991), biglycan (Fisher *et al.* 1989), proline/arginine-rich end leucine-rich repeat protein (PRELP) (E Bengtsson *et al*. 1995) and chondroadherin (Neame *et al*. 1994). Decorin and fibromodulin both interact with the type II collagen fibrils in the matrix and participate in fibrillogenesis and interfibril interactions. Biglycan is mainly found in the immediate surroundings of the chondrocytes, where it may interact with type VI collagen (Roughley and Lee 1994; Buckwalter and Mankin 1998). PRELP and chondroadherin are able to facilitate adhesion of chondrocytes to matrix proteins by binding to the transmembrane receptor proteins syndecans and integrins (Beauvais and Rapraeger 2004).

Other non-collagenous proteins in articular cartilage such as cartilage oligomeric matrix protein (COMP) (thrombospondin family protein) are markers of ECM turnover and degeneration (Di Cesare *et al*. 1996). Thrombospondin-1, tenascin and fibronectin influence interactions between the chondrocytes and the ECM (Buckwalter and Mankin 1998; Burton-Wurster *et al.* 1997). Some of the key fibrillar and non-fibrillar collagens, proteoglycans, and non-collagenous proteins present in the ECM of cartilage are illustrated in figure 1.2.

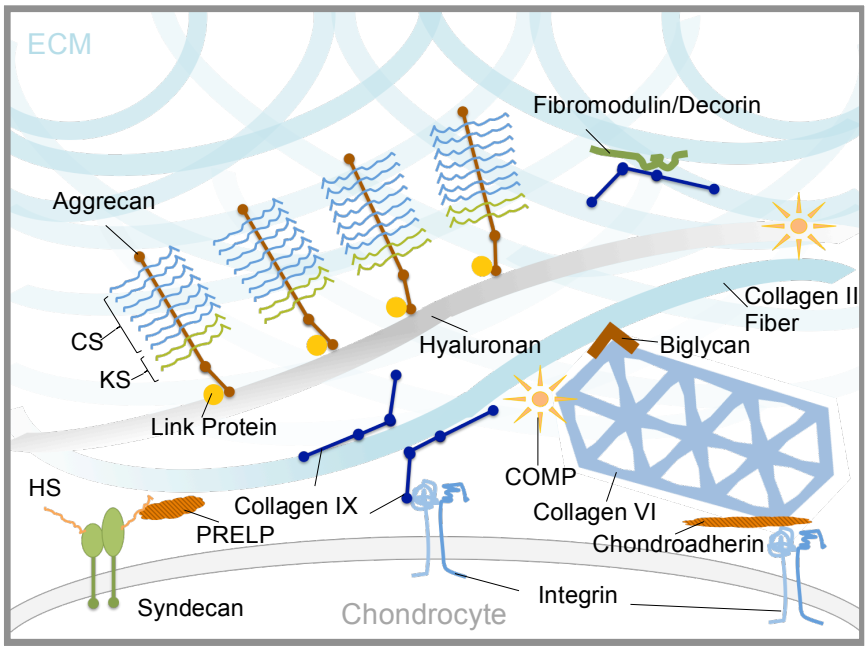


Figure 1.2 Molecular composition of the ECM of articular cartilage. The schematic diagram shows the major collagenous and non-collagenous components of cartilage ECM.

As there is little cell-cell contact within cartilage, communications between cells occurs mainly through the ECM (Mitrovic *et al.*, 1983; Poole *et al.*, 2001). The ECM of articular cartilage can be defined according to its spatial relationship to the chondrocyte. There are two broad regions recognised: the pericellular matrix (PCM), which surrounds individual chondrocytes throughout the tissue, and the further removed matrix, which is divided into the territorial matrix (TM) and interterritorial matrices (ITM). The later two make up most of the volume of the mature articular cartilage. One of our major interests is the role of the PCM in articular cartilage; therefore the PCM will be discussed further.

1.2.5 The pericellular matrix and its structure

The pericellular matrix (PCM) is described as a lucent zone that surrounds individual chondrocytes throughout articular cartilage. A unit comprising a chondrocyte and its surrounding PCM is termed the 'chondron', which was first identified by Benninghoff in a range of hyaline cartilages (Poole 1997). Little research about the PCM was done until the development of mechanical and enzymatic methods to isolate intact chondrons by Poole's group (Alexopoulos *et al*. 2003). These methods have allowed a number of biological and biomechanical assays for studying the composition and structure of the PCM. The PCM is distinct from the type II collagen rich TM and it is made up of type VI collagen and other non-collagenous proteins such as perlecan (Melrose *et al*. 2006), laminin (Dürr *et al*. 1996), fibrillin (Keene *et al*. 1997) and previously mentioned ECM components including biglycan, hyaluronan, link protein, fibronectin, and decorin. Our group has previously studied the structure of the PCM and organisation of some of the major components of the PCM using electron microscopy (Figure 1.3A and B) and confocal microscopy (Figure 1.3C).

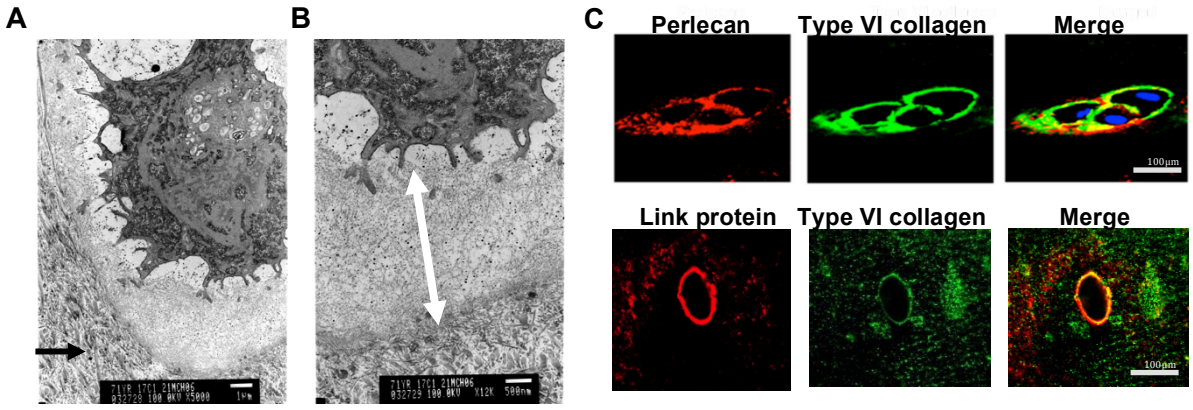


Figure 1.3 The pericellular matrix by electronic microscopy (A 5000X and B 12000X) and confocal microscopy (C 60X). Courtesy of Celia McLean (PhD 2009)

In the electron microscopic images, the PCM (white arrow, Figure 1.3B) is the lucent matrix layer that surrounds the chondrocyte and separates the cell from the TM. The TM is denser due to the presence of type II collagen fibers (black arrow, Figure 1.3A), whereas the PCM is

devoid of these fibrils. Cell processes can be seen extending from the chondrocytes into the PCM where they presumably mediate interactions between the cell and the matrix. Fluorescent images from confocal microscopy show the localisation of perlecan, type VI collagen and link protein within the PCM (Figure 1.3C).

1.2.5.1 Perlecan and its binding regulatory molecules

Perlecan, also known as heparan sulfate proteoglycan 2, is localised exclusively within the PCM of articular cartilage. To the protein core of perlecan three long chains of GAG are attached. These can be heparan sulphate (HS) or chondroitin sulphate (CS) (Melrose *et al*. 2006; Noonan *et al*. 1991), although our unpublished work has shown that perlecan in adult articular cartilage is almost exclusively substituted with HS, and it is the major HS proteoglycan of articular cartilage (Vincent *et al*. 2007). Perlecan binds to a number of matrix proteins such as type VI collagen, fibronectin and PRELP (Bengtsson *et al*. 2002; Melrose *et al.* 2006; Hopf *et al.* 1999). Perlecan also functions as a ligand reservoir for numerous regulatory molecules through attachments to its protein core or GAG chains. The matrix metalloproteinases (MMP) 2, 7, 9 and 13 have been shown to bind HS in the extracellular space and are released in culture upon heparitinase or heparin treatment (Yu and Woessner 2000). Tissue inhibitor of metalloproteinase (TIMP) 3 is sequestered in the pericellular environment by perlecan (Langton *et al*. 1998). Perlecan also sequesters a large number of heparin binding growth factors including FGF2 (Whitelock *et al.* 1996), vascular endothelial growth factor (VEGF) (Savore *et al*. 2005) and connective tissue growth factor (CTGF) (Nishida *et al*. 2003). These findings suggest that perlecan acts as a docking station for these matrix-modelling molecules and may play a prominent role in controlling their actions in cartilage. The interaction of FGFs and HS chains of perlecan has been shown to be important in regulating presentation of FGFs to FGF receptors (FGFRs) and their subsequent oligomerisation and activation. The HS chains of perlecan act as co-receptors for FGF18 signalling in articular cartilage (Melrose *et al*. 2006). There are also reports showing the interaction of perlecan and growth factors through its protein core. Mongiat *et al*

(2000) reported that FGF7 binds to perlecan's protein core, and influences the effect of FGF7 on cancer growth and tissue remodelling (Mongiat *et al*. 2000). Smith *et al* (2007) observed that in addition to the HS chains, FGF18 also binds to perlecan via its protein core, which altered its mitogenic effect in growth plate chondrocytes (Smith *et al.* 2007). Work from our group shows that perlecan sequesters FGF2, and allows it to be liberated upon injury and mechanical loading (Vincent *et al*. 2007; Vincent *et al*. 2002). The released FGF2 activates the mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) pathway and induces the expression of chondroprotective genes including activin A, tumor necrosis factor-inducible gene 6 (TSG6) and TIMP1 (Chong *et al*. 2013). Activin A is a TGFβ family protein that has anti-catabolic effects *in vitro* and plays roles in inflammation and tissue repair (see later). TSG6 plays anti-inflammatory role *in vivo* and TIMP1 is a natural inhibitor of MMPs that involved in degradation of the ECM. We showed that FGF2 was chondroprotective as FGF2 null mice developed accelerated OA following joint destabilisation (Chia *et al.* 2009). In FGF2 null mice, those FGF2 dependent chondroprotective genes (such as activin A and TIMP1) were suppressed in the knee tissue following joint destabilisation (Chong *et al*. 2013).

1.2.6 Matrix maintenance and turnover

The integrity of articular cartilage in normal and healthy adult is maintained through a steady state between synthesis of the ECM by chondrocytes and the rate of cartilage degradation. Cartilage is constantly remodelled, which is critical for development and normal physiology. Any change in this homeostatic steady state could rapidly affect the normal function of the cartilage and may lead to excessive degradation, which is characteristic of the pathogenesis of osteoarthritis (Tetlow *et al.* 2001; Walter *et al*. 1998). ECM turnover depends on the ability of chondrocytes to detect changes in the microenvironment. These changes include alternations in matrix composition such as degraded matrix molecules, presence of inflammatory cytokines and other 'danger' sensors. Spontaneous matrix degradation results in the release of matrix macromolecules and associated regulatory molecules, which trigger

responses from chondrocytes. For example, insulin like growth factor 1 (IGF1) is constitutively sequestered in the ECM by binding to its IGF-binding protein (IGFBP) and fibronection. Release of IGF1 as a result of matrix degradation increases local bioavailability of IGF1, which stimulates chondrocyte anabolic activity (Martin and Buckwalter 2000). FGF2 release from the PCM of articular cartilage upon mechanical compression appears to occur in a protease independent fashion and results in a rapid (<30sec) activation of the chondrocytes (Vincent *et al*. 2007). The exact role of these presumed anabolic regulatory molecules will be discussed later in this chapter. ECM degradation is also accompanied by the release of catabolic molecules. MMPs for example, may be sequestered in the PCM, are released and activated upon injurious signals (Gepstein *et al*. 2002). Conversely, TIMP3, the principle metalloproteinase inhibitor is also heparin sulphate bound in the PCM (Troeberg *et al*. 2014).

Mechanical loading is arguably the most important pathological as well as physiological stimulus in cartilage. Mechanical loading creates mechanical, electrical, and physicochemical signals that help to direct the synthesis and degradation of cartilage (Mobasheri *et al*. 2002). Prolonged and severe decrease in joint use leads to alterations in the composition of the ECM and eventually tissue atrophy and changes to its specific biomechanical properties. On the other hand, normal physical strain stimulates the synthesising activity of chondrocytes and possibly internal tissue remodelling (Joseph *et al.* 2004; Maffulli and King 1992). In this context, the ECM acts as a signal transducer for chondrocytes (Millward-Sadler and Salter 2004).

Although articular cartilage is able to tolerate a significant amount of intensive and repetitive physical stress, it shows limited ability to heal in the event of even a minor injury (Newman 1998; Martin *et al*. 2004). This makes joints particularly sensitive to degenerative processes. Furthermore, aging also leads to alterations in the ECM composition and alters the activity of chondrocytes, including their ability to respond to a variety of stimuli such as growth factors

(Ralphs and Benjamin 1994; Hudelmaier *et al.* 2001). All these alterations increase the probability of cartilage degeneration (Sarzi-Puttini *et al.* 2005; Poole 1999), which may eventually result in OA.

1.3 Osteoarthritis

Osteoarthritis (OA) is a common disease of joints causing pain and loss of function. It is characterised by cartilage degeneration and osteosclerosis with formation of bone cysts and osteophytes (Conaghan *et al.* 2008). In the early stage of disease the cartilage surface becomes fibrillated, which then gradually extends into the middle and eventually the deep zones of the cartilage. This process involves degradation of cartilage ECM molecules including proteoglycans and collagens. In advanced stages, the organisation of the cartilage becomes completely disordered and may be replaced by scar-like fibrocartilage. In some weight bearing surfaces of the joint, cartilage may be lost down to the subchondral bone. Where tissue remains, cells are arranged in clusters in enlarged lacunae. Sometimes, these lacunae are surrounded by regions of intense metachromatic staining indicating increased proteoglycan formation, suggesting attempted repair of the damaged cartilage (Pearle *et al.* 2005; Martel-Pelletier *et al.* 2008).

1.3.1 Risk factors in osteoarthritis

The development of OA is dependent on interactions between several factors and thus this process may be considered the product of a combination of risk factors including advancing age, genetics, trauma, knee malalignment, increased biomechanical loading of joints through obesity, augmented bone density and an imbalance in physiological processes (Heidari 2011). The pathogenesis of OA at the molecular and cellular level is not understood. Once considered a simple disease of wear and tear, it is now regarded as being due to active degrading processes associated with the regulation and activity of specific proteinases. There is evidence of up-regulation of MMP1 and MMP13 transcripts (Aigner *et al.* 2001) and increased collagenase (Billinghurst *et al.*, 1997) and aggrecanase (Malfait *et al*.

2002) activity in late OA cartilage. Although a number of enzymes are able to degrade aggrecan *in vitro* (e.g. MMP3), the principal enzymes that drive this process in disease are members of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family (especially ADAMTS4 and 5). What drives these enzymes *in vivo* is not known, although our group has shown that mechanical factors per se are able to drive protease expression in the articular cartilage following surgical induction of OA (McNamee *et al*. 2010; Burleigh *et al*. 2012). These enzymes are strongly induced by cytokines *in vitro*, raising the possibility that inflammatory cytokines could be important in disease. Proinflammatory cytokines such as interleukin 1 (IL1) and tumor necrosis factor alpha (TNFα) induce chondrocytes, through the activation of nuclear factor kappa B (NF-κB) pathway, to express degrading enzymes such as MMPs and cyclooxygenase 2 (COX2) (Shakibaei *et al*. 2007). Dysregulation of anabolic signals such as TGFβ, which amongst other things is able to inhibit IL1 induced degradation of cartilage, is also observed in late OA (Finnson *et al*. 2010; Parker *et al.* 2007). The combination of accelerated catabolic activity along with breach of the cartilage homeostatic balance results in the development of OA.

1.3.2 Growth factor expression in OA tissue

In early OA, chondrocytes proliferate and synthesise enhanced amounts of matrix molecules possibly through the action of known anabolic factors including IGF1, TGFβ and FGF2 in an attempt to repair damaged matrix (Sandell and Aigner 2001). IGF1 has been shown to increase expression of collagen and proteoglycan *in vitro* in human cartilage and cultured chondrocytes (Franchimont and Bassleer 1991), and antagonise the catabolic effects of IL1 pathway by up-regulating the IL1 decoy/type II receptor (IL1RII) (Wang *et al.* 2003a). Expression of IGF1 and its receptors are increased in OA cartilage compared with normal human cartilage (Wang *et al.* 2003b), but so too are the IGF binding proteins which are able to inhibit IGF by competing with it binding to its receptor (Olney *et al.* 1996).

TGFβ and bone morphogenetic proteins (BMPs) have demonstrable anabolic effects on chondrocytes acting in a paracrine manner, where TGFβ family ligands signal through a pair of transmembrane serine/threonine kinase receptors, transmitting an intracellular signal through a cascade of SMAD pathways (canonical) to induce the synthesis of a range of ECM molecules (Finnson *et al.* 2010). The SMAD pathways have been found to play a critical role during OA development. A single-nucleotide polymorphism in the intron region of human smad3 gene has been linked to the incidence of hip and knee OA in a 527 European patient cohort (Valdes *et al*. 2010). Mice deficient for genes encoding any of the three TGFβ isoforms (TGFβ1, 2 and 3) show embryonic lethality, and loss of TGFβ2 or TGFβ3 genes leads to numerous bone defects affecting limbs and craniofacial bones, suggesting that TGFβ plays an important role in skeletogenesis (Dünker and Krieglstein 2000). TGFβ can also counteract the actions of IL1 by inhibiting the synthesis of IL1 signalling/type I receptor (IL1RI) (Redini *et al*. 1993). The expression of TGFβ is increased in early OA perhaps in an attempt to repair the damaged matrix, but TGFβ transcript levels are low in late OA cartilage (Blaney Davidson, et al. 2006). *In vivo* chondroprotection by TGFβ has also been suggested by Blaney Davidson *et al* (Blaney Davidson *et al.* 2007).

FGF2 is an abundant matrix bound growth factor in articular cartilage that is released following mechanical loading and injury (Vincent *et al*. 2007; Vincent *et al*. 2002). FGF2 knockout mice exhibited accelerated spontaneous and surgically induced OA (Chia *et al.* 2009). FGF2 was able to inhibit IL1 dependent degradation of cartilage *in vitro*, possibly through the induction of activin A. Activin A is another TGFβ family protein and increased significantly in OA cartilage (Hermansson *et al*. 2004). However its molecular role in cartilage is not clear. TGFβ family proteins and CTGF will be introduced in more detail later in this chapter.

1.4 Connective tissue growth factor

1.4.1 CTGF structure and binding

CTGF is the second member of Cyr61, CTGF, Nov (CCN) protein family (also known as CCN2). These proteins are characterised by 4 homologous modules: the insulin like growth factor binding protein (IGFBP) like, von Willebrand factor type C repeat (VWC) with conservation of 38 cysteine residues, thrombospondin type I repeat (TSP1) and the C-terminal (CT) module (Brigstock *et al.* 1997; Lau and Lam 1999; Moussad and Brigstock 2000; Nakanishi *et al*. 1997a) (Figure 1.4). Each of these modules interacts with a range of molecules involved in a number of different cellular activities (Figure 1.4 lower part). CTGF was once classified as a low density IGF binding protein (IGFBP) superfamily protein. The interaction between CTGF and IGF has been proposed to modulate the activity of IGF in promoting normal and neoplastic cell growth (Kim *et al*. 1997). The VWC module is thought to mediate interactions with TGFβ and BMP4, and controls their activity in Mv1Lu (mink lung epithelial) and 10T1/2 (mouse embryo) cell lines respectively (Abreu *et al*., 2002). The TSP1 module has been implicated in enabling binding of CTGF to ECM proteins, MMPs and integrins (Bornstein 2001; Lau and Lam 1999). In certain cell types, the interaction of CTGF with integrins promotes cell survival, adhesion and migration (Babic *et al.* 1999). This module is also involved in binding to heparin, heparan sulphate proteoglycan and low-density lipoprotein receptor related protein (LRP). CTGF has been reported to be internalised and degraded in the endosome through the LRP receptor in a heparin dependent manner (Gao and Brigstock 2004). Our observations show that addition of heparin to cells in culture leads to increased CTGF protein in the medium. Heparin can block the normal LRP-mediated re-uptake mechanism, as has been described for LRP-mediated uptake of TIMP3 (Troeberg *et al.* 2008). CTGF has been reported to co-localise with perlecan in the PCM of the chondrosarcoma cell line, HCS-2/8 (Nishida *et al*. 2003). The TSP1 module also interacts with VEGF in control of angiogenesis through the assistance of

the CT module (Ivkovic *et al*. 2003). The CT module has been implicated in heparin and integrin binding (Ball *et al*. 2003) and dimerization (Bork 1993).

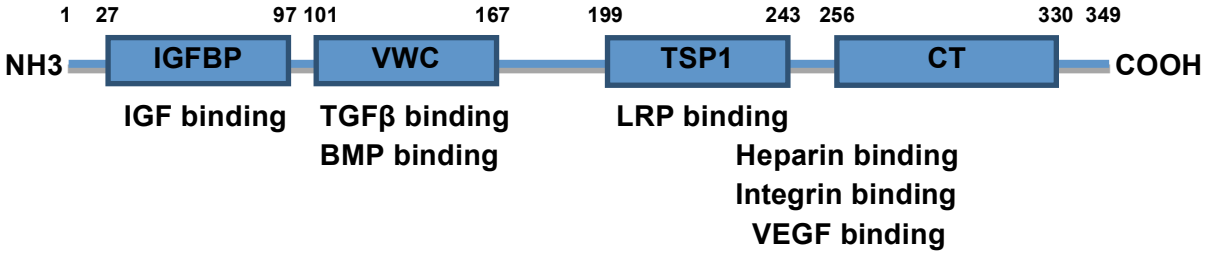


Figure 1.4 The modular structure of CTGF protein and its binding molecules. Upper part of the figure shows the four modules in the structure of human CTGF protein. IGFBP: insulin like growth factor binding protein, VWC: von Willebrand factor type C repeat, TSP1: thrombospondin type I repeat and CT: C- terminal. Lower part of the figure shows the molecules that bind to CTGF at each module of the structure respectively.

1.4.2 Role of CTGF

CTGF was first identified as a factor secreted by human umbilical vein endothelial cells (HUVEC) that competed with platelet derived growth factor (PDGF) binding to its cell surface receptor and had PDGF like biological activity (Bradham *et al*. 1991). A 38kDa cysteine rich secreted protein was shown to be the major PDGF related mitogen and termed connective tissue growth factor (Bradham *et al*. 1991). CTGF is highly expressed during embryonic development and during healing suggesting that CTGF is important in growth and regeneration of skeletal and vascular tissues (Ivkovic *et al*. 2003). The generation of CTGF knockout mice showed that deficiency of CTGF leads to lethal skeletal dysmorphisms including defects within the axial skeleton, shortening of sterna, deformation of limb cartilage and the craniofacial skeleton (Ivkovic *et al*. 2003). These defects are likely to be due to impaired chondrocyte proliferation and extracellular matrix composition within the hypertrophic zone (Ivkovic *et al*. 2003). CTGF has also been implicated in pathogenesis of fibrosis. Fibrotic diseases are characterised by scarring due to excessive production, deposition, and contraction of collagen (Leask and Abraham 2004). CTGF is constitutively overexpressed in dermal fibrotic lesions and its role in fibrosis is thought to be in mediating

downstream effects of TGFβ (Grotendorst 1997), which is considered a mediator of the elevated production of matrix proteins including collagen and fibronectin (Leask and Abraham, 2004).

In articular cartilage, we have observed that CTGF protein is up-regulated in OA cartilage (Hermansson *et al*. 2004). Administration of recombinant CTGF protein has been reported to promote repair of damaged cartilage in a murine model of OA (Nishida *et al*. 2004). Treatment of rabbit articular chondrocytes *in vitro* suggested that CTGF was able to stimulate the gene expression of type II collagen and aggrecan, providing evidence that CTGF may have a role in cartilage anabolism (Nakanishi *et al*. 2000). Transgenic mice that overexpress CTGF under the Col2a1 promoter showed increased staining for proteoglycan and reduced staining for type X collagen and MMP13, when compared with wild-type mice. Observation of up-regulation of Col1a1, ColXa1, Mmm13 and Mmp9 mRNA in wild-type chondrocytes under cyclic tension stress was not seen in the CTGF transgenic chondrocytes (Itoh *et al*. 2013). As type X collagen and MMP13 are up-regulated in OA (Van der Kraan and Van den Berg 2012), these findings suggest that CTGF may have a beneficial role in articular cartilage. However, the molecular response and exact mechanism by which CTGF signals remains unclear and the existing literature is complicated by conflicting reports of the effect of CTGF on mature cartilage.

There have also been a number of theories proposed about the mechanism by which CTGF mediates its cellular effect. Firstly that CTGF acts as a matricellular protein, which means that it does not activate the cell in a classical sense (signaling through a cell surface receptor) but helps the cell to interact with matrix proteins (Gao and Brigstock, 2004). Secondly, it acts as a cofactor for TGFβ family members, by binding directly to the TGFβ family ligand and affecting responses at the receptor (Abreu *et al*. 2002). Finally it may activate the cell via the tyrosine kinase receptor A (TrKA) (Wahab *et al.* 2005), LRP receptors or integrins (Gao and Brigstock 2003; Gao and Brigstock 2004). In order to understand by which mechanism

CTGF conducts its cellular response in articular chondrocytes, further *in vitro* investigation is required.

1.5 TGFβ family molecules

The transforming growth factor beta (TGFβ) superfamily is a large family of structurally related cell regulatory proteins that was named after its first member, TGFβ1, originally described in 1983 (Assoian *et al*. 1983). TGFβ family proteins include TGFβ, activin/inhibin, BMPs and myostatin (Vale *et al*. 1988). Most of TGFβ family proteins, if not all, are produced as latent complexes. The bioavailability of these factors is controlled through binding to ECM proteins. TGFβ pro-peptide dimerises through disulfide bonds during secretion. The pro-region of TGFβ (known to be a latency-associated peptide LAP) is cleaved by a furin-like protease and remains associated with the mature TGFβ through non-covalent interaction in a complex known as small latent complex (SLC). LAP forms disulfide bonds with latent TGF binding protein (LTBP1, 3 & 4). The resulting complexes (known as large latent complexes LLCs) bind to fibrillins and fibronectins in the ECM through LTBP. Figure 1.5 shows a schematic structure of SLC and LLC of TGFβ. TGFβ can be activated in a number of ways *in vitro* (see more detail in chapter 5), however, the study of its activation in articular cartilage is limited. There may be a different mechanism of activation in cartilage, which is suggested by the fact that the integrin alpha v, an important component of the activation complex in many cell types, is very little expressed in chondrocytes (Woods *et al*. 1994). Activin A is also produced in a complex with a latent pro-peptide and its biological activity may be modulated by the ECM. In Xenopus, activin A has been shown to act as a long range dorsalising signal to establish a concentration gradient, suggesting the potential associations of activin A with ECM components (McDowell *et al*. 1997). Li *et al* (2010) observed that recombinant activin A protein was able to bind to perlecan directly through its pro-region *in vitro* (Li *et al.* 2010). Heparitinase treatment of perlecan abolished the binding, suggesting that activin A binds to

perlecan through its heparan sulphate chains (Li *et al*., 2010). BMP2, 4, 7 and 10 also bind to fibrillin through their N terminal pro-region (Sengle *et al*. 2008).

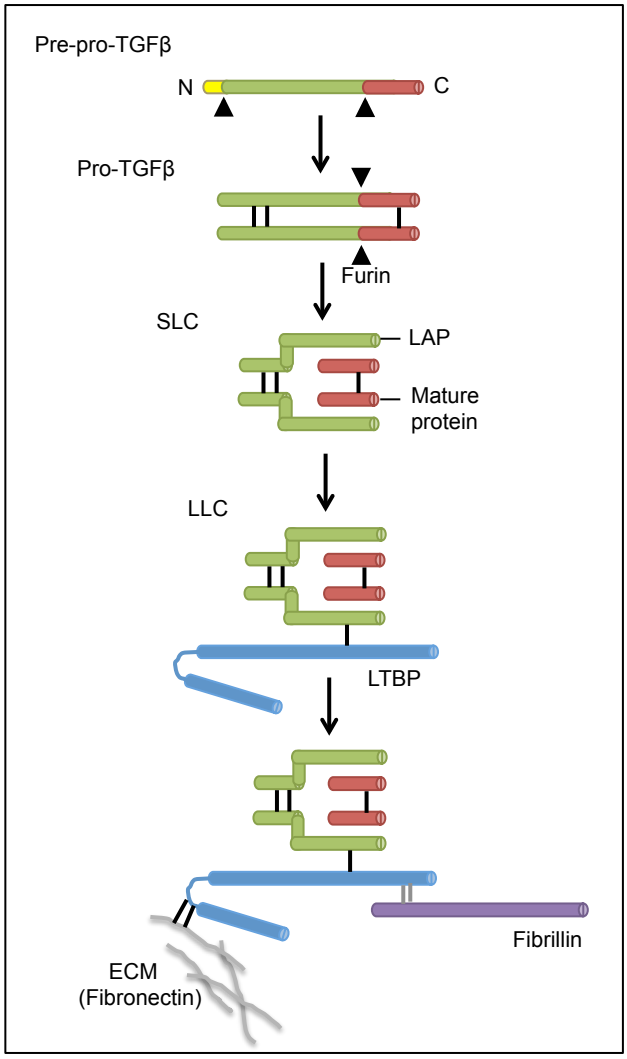


Figure 1.5 Schematic structures of pre-pro-peptide, SLC and LLC of TGFβ. TGFβ is synthesised as a pre-pro-peptide containing a N-terminal signal peptide (yellow) which is proteolytically processed in the rough endoplasmic reticulum. The pro-peptide form a dimer through disulfide bonds, which is cleaved by a furin-like protease. The pro-region of the peptide (LAP, green) remains associated with mature TGFβ (red) to form a SLC. LAP form disulfide bonds with LTBP (blue) to form LLC, which associates with fibrillin through non-covalent interaction and fibronectin through disulfide bond in the ECM. Inspired by an image from Dijke and Arthur 2007.

1.5.1 Role of TGFβ

TGFβ exists in at least three isoforms i.e. TGFβ1, TGFβ2 and TGFβ3, which show a 70% to 80% structural homology (Hall *et al*. 2013). TGFβ is secreted by many cell types and has been extensively reported to play numerous roles in different cell types including regulation

of early development, differentiation, extracellular matrix formation, hematopoiesis, angiogenesis, chemotaxis, immune response, and the induction of apoptosis. TGFβ mediated growth inhibition and apoptosis can be correlated with its function as a tumor suppressor (Schuster and Krieglstein 2002). TGFβ exerts tumor-suppressive effects that cancer cells must elude for malignant evolution. Yet, paradoxically, TGFβ also modulates processes such as cell invasion, immune regulation, and microenvironment modification that cancer cells may exploit to their advantage (Massague 2008). In the immune system, TGFβ induces lymphocyte growth arrest and apoptosis via the SMAD pathway. TGFβ1 knockout mice die 2 to 3 weeks after birth and show massive inflammation (Kulkarni *et al*. 1993; Shull *et al*. 1992). Transgenic mice overexpressing a dominant negative mutant of the TGFβ type II receptor (TGFβRII), specifically on T cells, show a hyperplasia of T cells resulting in massive expansion of lymph nodes and spleen (Lucas *et al*. 2000).

Another important molecular activity of TGFβ is to induce the expression of matrix molecules. In many tissues, the release and activation of TGFβ upon injury stimulates the production of extracellular matrix proteins and inhibits the degradation of these matrix proteins. These actions of TGFβ contribute to tissue repair, which under ideal circumstances leads to the restoration of normal tissue architecture and may involve a component of tissue fibrosis. In many diseases, excessive TGFβ contributes to a pathologic excess of tissue fibrosis that compromises normal organ function (Branton and Kopp 1999). Excessive activity of TGFβ in Marfan syndrome occur as a result of misfolding of fibrillin1 so that it is unable to control the bioavailability of latent TGFβ in the ECM. This causes deleterious effects on lungs, heart valves and aorta leading to weaker connective tissues (Kainulainen *et al.* 1994). In articular cartilage, TGFβ likely plays a role in maintaining normal cartilage homeostasis by inducing expression of ECM molecules. Up-regulation of TGFβ mRNA observed in early OA implicates its role in attempted cartilage repair (Kenneth *et al.* 2012).

1.5.2 Role of activin A

Activin A was originally discovered as a molecule in ovarian follicular fluid that caused pituitary cells in culture to secrete follicle-stimulating hormone. Inhibins, which share the β chain with activin but form heterodimers with α chain subunits, were found to be inhibitors of this process (Werner and Alzheimer 2006). Activin A has been shown to be important in embryogenesis; activin A deficient mice die within 24 hours after birth and develop severe craniofacial abnormalities including lack of whiskers and lower incisors, and defects in the second palate (Matzuk *et al*. 1995a). Like some of the other TGFβ family proteins, activin A is also involved in tissue repair. It has been shown that activin A is strongly induced after skin injury and high levels persist following wounding (Matzuk *et al.* 1995b). This induction is thought to be caused by released serum growth factors such as epidermal growth factor (EGF) and PDGF during haemorrhage as well as pro-inflammatory cytokines derived from neutrophils and macrophages (Hubner and Werner 1996). Overexpression of activin A in mice leads to increased proliferation of the basal keratinocytes and significant enhancement of granulation tissue formation (Munz *et al*. 1999). Overexpression of its sequestering inhibitor follistatin in the basal epidermis of skin was found to delay wound healing in mice (Munz *et al*. 1999).

In cartilage injury, induction of activin A is FGF2 dependent (Alexander *et al*. 2007). Activin A is elevated in synovial fluid of patients with rheumatoid arthritis and osteoarthritis. It is induced by inflammatory stimuli such as IL1, TGFβ and bacterial lipopolysaccharide (LPS) in cultured synoviocytes and chondrocytes (Yu *et al.* 1998). Proteomic analysis of the proteins secreted by human articular cartilage *in vitro* showed activin A protein is up-regulated in OA compared to normal tissue (Hermansson *et al*. 2004). Activin A is up-regulated after cartilage injury through an ERK dependent pathway caused by release of FGF2 (Alexander *et al*. 2007; Vincent *et al*. 2002). Activin A may be chondroprotective as it inhibits IL1 induced cartilage degradation (Alexander *et al*. 2007), but its molecular activities in cartilage chondrocytes are poorly understood.

1.5.3 TGFβ family proteins activate SMAD signalling pathways

The TGFβ family signalling pathway is involved in many cellular activities, in both adult and embryo, including cell proliferation, differentiation, cellular homeostasis and apoptosis. TGFβ family ligands bind to their specific type II receptor (Figure 1.6), a serine/threonine receptor kinase, which recruits and phosphorylates the type I receptor (also called activin like kinase, ALK) (Miyazono *et al.* 2001). The activin type II receptor (ACVR2) binds to ALK 4, the BMP type II receptor (BMPR2) binds to ALK3 and 6 and the TGFβ type II receptor (TGFβR2) binds to ALK5. The type II and type I receptors form a heterotetrametric complex with the ligand and phosphorylate receptor regulated SMADs (collectively known as R-SMADs) (Massagué *et al.* 2005). TGFβs and activins activate SMAD2 and SMAD3, while BMPs activate SMAD1, SMAD5 and SMAD8. Precent studies on endothelial cells have identified ALK1 as a second type I TGF-beta receptor and have shown that ALK1 and ALK5 have opposing functions in these cells (Finnson *et al.* 2008). ALK1 activates SMAD1 and SMAD5 phosphorylation, which is dependent on the activity of ALK5 (Goumans *et al*. 2003). The phosphorylated R-SMADs have high affinity for the co-SMAD (SMAD4) with which they form a complex and enter the nucleus. Nuclear SMAD complexes control gene transcription by binding to regulatory elements on the target DNA. These include the DNA of an inhibitory SMAD (SMAD7), which negatively modulates the signal pathway by competing with R-SMAD from binding to type I receptor, which will then be degraded through ubiquitination (Massagué *et al.* 2005; Ishisaki *et al.* 1999). TGFβ can also activate other pathways including MAPK and phosphoinositide 3 kinase (PI3K) pathways, which are collectively referred to as 'non-canonical' TGFβ signalling and complement SMAD action. The biochemical links between TGFβ receptors and the MAPK and PI3K pathways involve tumor necrosis factor receptor-associated factor 6 (TRAF6), mammalian target of rapamycin (mTORC) and other mediators. However, the molecular mechanism is unclear due to a lack of structural information (Massagué 2012). In cartilage the non-canonical pathways have been reported to be necessary for proper cartilage and joint development (Gunnell *et al.*

2010). Figure 1.6 shows a schematic diagram of the canonical SMAD signalling pathway of TGFβ, activin A and BMPs.

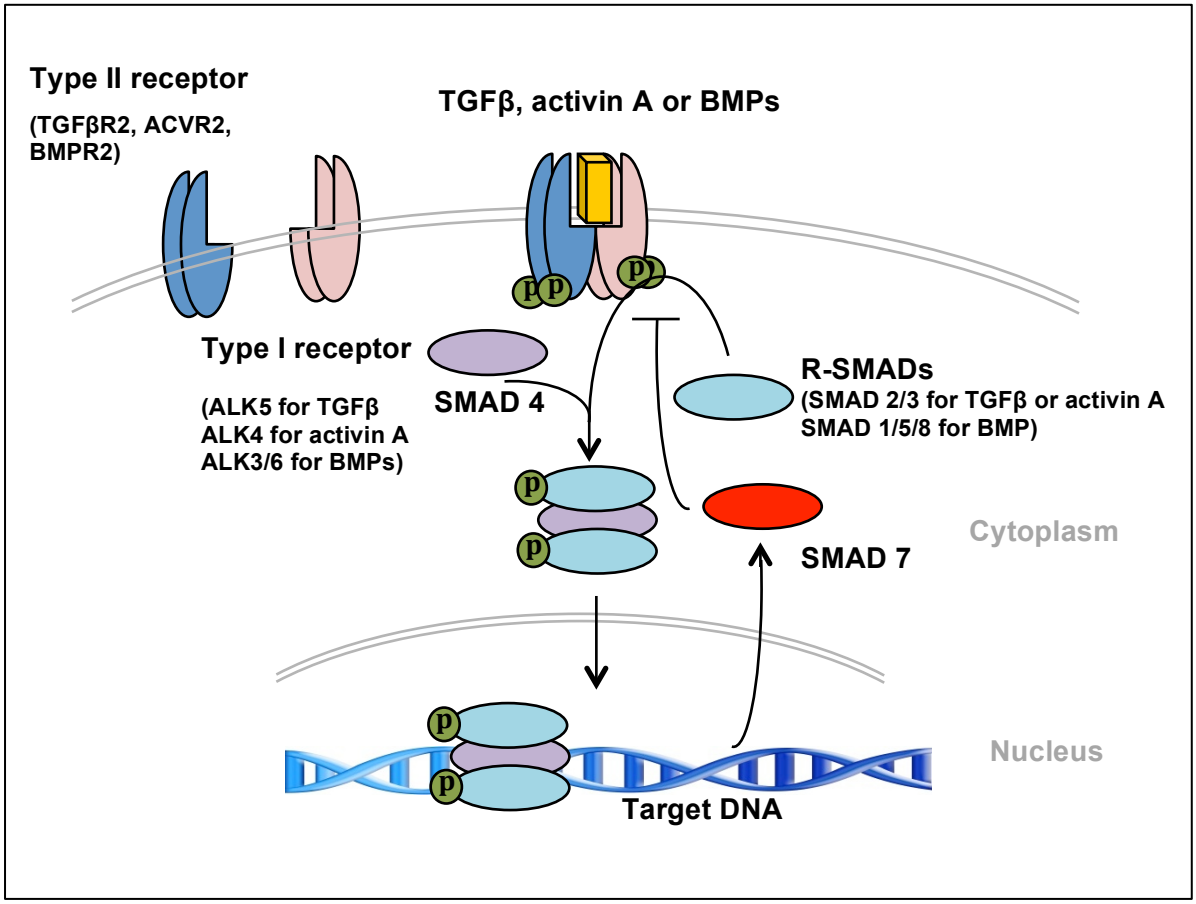


Figure 1.6 Schematic diagram of SMAD signalling pathway shared by TGFβ, activin A and BMPs.

1.6 Aims and objectives

Previously, Tonia Vincent found that cutting cartilage rapidly activated the ERK1 and 2 in chondrocytes, which was caused by the release of FGF2 from a sequestered extracellular pool bound to perlecan in the PCM (Vincent *et al*. 2002). In this context, the PCM acts as a mechano-transduction platform, transforming mechanical stimuli into a chemical signal (released FGF2) that leads to cellular responses. To extend these observations, the group sought to identify other PCM bound molecules whose activities could be controlled in a similar way. Celia McLean (a previous PhD student in our group) carried out a mass spectrometry study of PCM proteins using porcine articular cartilage. Amongst the known matrix components of the PCM, she identified CTGF. Previous work from our laboratory had shown that CTGF was highly expressed in articular cartilage and over expressed in OA (Hermansson *et al.* 2004). Little is known about the role of CTGF in mature articular cartilage, although it is essential for musculoskeletal development and the knockout animals demonstrate perinatal lethality and severe musculoskeletal defects (Ivkovic *et al*. 2003).

The aims of this project were to characterise CTGF as a candidate of pericellular protein in normal mature articular cartilage, and to investigate its role in articular cartilage in response to mechanical injury.

Chapter 2

Materials and Methods

2.1 Reagents and chemicals

2.1.1 General reagents: Materials were purchased from the following sources: Trizma® base, sodium chloride, beta glycerophosphate, sodium vanadate, sodium deoxycholate, IGEPAL CA-630, proteases inhibitor, Tris-HCl, sodium dodecyl sulphate (SDS), Tween 20, glycine, sodium thiosulphate, sodium pyrophosphate, Ammonium persulfate, ammonium acetate, tetramethyl-ethylenediamine and imidazole from Sigma-Aldrich Company Ltd (Dorset, UK); phosphate buffered saline (PBS), acetic acid, chloroform, ethylenediaminetetraacetic acid (EDTA), glycerol, methanol, formaldehyde, boric acid and acetone from VWR International (Leicestershire , UK); bovine serum albumin (BSA), trypsin-EDTA, fetal calf serum (FCS), penicillin/streptomycin, amphotericin B and HEPES from PAA (Les Mureaux, France); agarose and SYBR®-Safe DNA gel stain from Invitrogen (Paisley, UK); orange/blue DNA dye and 100bp DNA ladder from Promega (Southampton, UK); collagenase A from Roche (Mannheim, Germany); chondroitinase ABC and heparitinase from Seikagaku (Tokyo, Japan).

2.1.2 Table of recombinant proteins and chemicals used in cell/tissue culture:

Proteins/inhibitors	Specificity	Source
CTGF	Human protein	Purified in house
Activin A	Human protein	R&D systems. Minneapolis, USA
TGFβ1	Human protein	Peprotech EC Ltd. London, UK
BMP2	Human protein	Peprotech EC Ltd. London, UK
SB431542	ALK4 and ALK5 inhibitor	R&D systems
RAP	LRP inhibitor	Gift of Linda Troeberg
Heparin	Porcine glycosaminoglycan	Isolated in house
Cycloheximide	Protein synthesis inhibitor	Sigma-Aldrich

Table 1 Recombinant proteins and chemicals used in cell/tissue cultures

2.1.3 Table of antibodies used for western blotting, immunofluorescence, neutralising or immunoprecipitation

Antibody against	Use	Type	Source	Dilution
Perlecan	Immunofluorescence	Rat monoclonal	Chemicon (MAB1948)	1:1000
CTGF	Immunofluorescence Western blot Immunoprecipitation	Goat polyclonal	Santa Cruz (sc-14939)	1:500
FGF2	Immunofluorescence	Mouse monoclonal	Upstate (05-118)	1:1000
Type VI collagen	Immunofluorescence	Rabbit polyclonal	Abcam (ab6588)	1:1000
Activin A	Immunofluorescence Neutralisation	Goat polyclonal	R&D systems (AF338)	1:500 1µg/ml
LAP1	Western blotting Immunofluorescence	Goat polyclonal	R&D systems (AB-246-NA)	1:500
LAP1	Western blotting Immunoprecipitation	Mouse monoclonal	Santa Cruz (sc-34830)	1:500
TGFβ -1, -2, -3	Western blotting	Rabbit polyclonal	Cell signalling (3711)	1:1000
TGFβ -1, -2, -3	Neutralisation	Mouse monoclonal	R&D systems (MAB1835)	1µg/ml
LTBP1	Western blotting	Mouse monoclonal	Santa Cruz (sc-271140)	1:500
Protein G HRP	Western blotting	Bacterial protein	Bio-RAD (1706425)	1:1000

Phospho SMAD2	Western blotting	Rabbit polyclonal	Cell signalling (138D4)	1:1000
Phospho SMAD1/5	Western blotting	Rabbit polyclonal	Cell signalling (41D10)	1:1000
Tubulin	Western blotting (Loading control)	Rabbit polyclonal	Cell signalling (2144)	1:50000
Goat IgG HRP	Western blotting (Secondary)	Rabbit anti goat	DAKO (P0160)	1:2000
Mouse IgG HRP	Western blotting (Secondary)	Rabbit anti mouse	DAKO (P0218)	1:2000
Rabbit IgG HRP	Western blotting (Secondary)	Swine anti rabbit	DAKO (P0217)	1:2000
Rat IgG HRP	Western blotting (Secondary)	Rabbit anti rat	DAKO (P0450)	1:2000
Alexa 488-conjugated goat IgG	Immunohistochemistry (Secondary)	Rabbit anti goat	Invitrogen (A-11078)	1:2000
Alexa 647-conjugated mouse IgG	Immunohistochemistry (Secondary)	Rabbit anti mouse	Invitrogen (A-21239)	1:2000
Alexa 647-conjugated rabbit IgG	Immunohistochemistry (Secondary)	Goat anti rabbit	Invitrogen (A-31852)	1:2000
Alexa 647 conjugated rat IgG	Immunohistochemistry (Secondary)	Goat anti rat	Invitrogen (A-21247)	1:2000

Table 2 Antibodies used for western blot, immunohistochemistry, neutralising and immunoprecipitation.

2.2 Cell culture

2.2.1 Cell culture reagents: Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/l glucose and L-glutamine (Lonza, Verviers, Belgium). DMEM was always supplemented with 25mM HEPES buffer (pH7.0-7.6), 100U/ml penicillin, 100µg/ml streptomycin and 0.5µg/ml amphotericin B. Heat inactivated FCS was added at a final concentration of 10% when cells were cultured. All cell culture plastics were purchased from Beckton-Dickinson (Oxford, UK).

2.2.2 Normal human skin dermal fibroblasts (NHDFs): Normal human skin dermal fibroblasts (NHDFs) were obtained from American Type Culture Collection (ATCC) and cultured in DMEM with 10% (v/v) FCS. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C. All cell culture was carried out in class II laminar flow cabinets. For passaging, cells were washed three times with sterile PBS. Trypsin-EDTA was added to the cells, which were then incubated at 37°C for 5min. Once cells had detached they were centrifuged in an equal volume of DMEM at 235x g at room temperature for 5min. The cell pellet was then resuspended in DMEM prior plating. Cells were passaged for general maintenance every week by plating them at a density of 1 x 10^6 cells per 15cm cell culture plate (50% confluence). Cells were plated in 6 well plates at a density of 3 x 10^5 cells per well for siRNA oligo transfection and stimulation (50% confluence). NHDFs were passaged to a maximum of P15.

2.2.3 Primary human articular chondrocytes (HACs): Normal human knee cartilage was obtained from patients at the Royal National Orthopaedic Hospital (RNOH, Stanmore, UK) who were undergoing surgery for tumors involving the lower leg (not involving the knee joint). Samples were obtained from individuals of different ages ranging from 8-76 years and were used with consent from the local ethics committees (Riverside Research Ethics Committee and Joint RNOH institute of Orthopaedics and Musculoskeletal Science Research Ethics

Committee). Written informed consent was obtained from all patients. Dissected cartilage explants were incubated with 1mg/ml/g collagenase for 5-6hr at 37ºC. The digest was then strained and centrifuged at 235x g for 5min. Finally the cell pellet was washed 3 times and resuspended in 10% FCS containing DMEM. Cells were plated (50% confluence) and grown to confluence in 15cm culture plates. They were passaged 1 or 2 (P1 or P2) times before use.

2.2.4 Human chondrons: Human articular cartilage was dissected as described above and allowed to rest in serum free DMEM overnight at 37ºC. Chondrons were obtained using 0.3% collagenase and 0.2% dispase in Ca^{2+}- and Mg^{2+}- free PBS at 37 ºC with constant agitation for 4hr. The resulting chondrons suspension was filtered through a 70µm nylon cell strainer and centrifuged at 528x g for 15min. The resulting pellet was resuspended in 5ml PBS. 200µl was used for confocal microscopy to stain for perlecan to assess the maintenance of the pericellular matrix. The suspension was then centrifuged at 528x g for 5min to obtain the chondrons pellet (Lee *et al*. 1997).

2.2.5 Primary porcine articular chondrocytes: Porcine articular cartilage was dissected from the trotter metacarpophalangeal joints of 3-6 month old pigs obtained fresh from a local abattoir within 24hr of slaughter. For chondrocytes isolation, freshly dissected cartilage explants were digested in FCS DMEM containing 1mg/ml/g collagenase for 5hr at 37ºC with agitation. The digest was strained through a cell strainer (BD Falcon™, New Jersey, US) to remove debris and the flow-through was collected in a new 50ml falcon tube. Cells in the medium were pelleted by centrifugation at 235x g for 5min at room temperature and washed three times with fresh DMEM. After the last spin, the cell pellet was resuspended in certain amount of 10% FCS DMEM that gives desired cell density for cell culture experiment. Cells were plated in 12 well or 24 well plates at a density of approximately 2 million cells or 1 million cells per well respectively. Chondrocytes were serum starved overnight prior to stimulation.

2.2.6 Porcine articular cartilage: Porcine articular cartilage was obtained from trotter metacarpophalangeal joints as mentioned above. For tissue culture experiments, cartilage explants were cut into similar size by a biopsy punch (4mm) (Stiefel® Laboratories Ltd, Bucks, UK) in serum free DMEM. 5 explant pieces were incubated in 200µl of DMEM per well of a 48 well plate. After 48hr, the cartilage explantation conditioned medium was collected for protein analysis and fresh DMEM was replaced for further stimulation or recutting. In recutting experiment, each cartilage explant was further cut into 6 pieces using a No.15 disposable scalpel (Swann Morton, Sheffield, UK). The recutting conditioned media were then collected at different time courses for further analysis.

2.2.7 Mouse articular cartilage: CTGF floxed mice (Liu *et al.* 2011) were acquired from collaboration with Professor David Abraham, London and Professor George Bou-Gharios, Liverpool. Conditional CTGF knockout mice were generated by crossing the floxed mice with the Ubiquitin Cre ERT2 mice (purchased from The Jackson Laboratory). Litters were genotyped and at the age of 4 weeks treated with tamoxifen for 2 weeks to induce the deletion. At the age of 6 weeks, these mice were culled and their cartilages were obtained from hip joints. Two hip explants were obtained per mouse and cultured separately in 50µl of serum free DMEM in a 96 well plate. One of these rested hip explants was recut and the other remained intact as control. The injury-conditioned media were then collected for further analysis.

2.3 Confocal microscopy: Normal human or osteoarthritic cartilages were obtained from patients at the RNOH, Stanmore and Charing Cross Hospital, Hammersmith, respectively. Explants were washed in serum free DMEM and snap frozen using liquid nitrogen. All frozen tissue sections were processed and mounted in OCT (optimal cutting temperature compound) and 7µm frozen cryostat sections were cut onto Surgipath snowcoat Xtra slides, air dried for 1h and then left for a further hour at 60°C to ensure tissue adherence. Tissue sections were fixed in methanol for 10min and pretreated with chondroitinase ABC (10mu/ml)

for 120min at 37°C. The sections were blocked with 10% goat serum/PBS for 60min at room temperature, and then incubated with primary antibody for 1hr at room temperature or overnight at 4°C. Sections were washed three times with PBS and incubated (in dark) with secondary fluorescein-labelled antibodies for 1h at room temperature. Sections were further washed and mounted using 50% glycerol. Florescence signals were detected by Ultraview confocal microscopy (Perkin-Elmer, x60 oil immersion lens).

2.4 ELISA-like binding assay: ELISA plates (Corning 96 wells, Sigma, US) were coated with 0.5µg/ml of perlecan (purified in house) in PBS for 3hr at room temperature or overnight at 4°C. Plates were washed three times with PBS/Tween and then blocked with 1% BSA/PBS (100µl/well) for 1hr at room temperature. Plates were washed again with PBS and incubated with 0 to 10µg/ml of CTGF in PBS for further 3hr at room temperature. After washes, plates were incubated with primary antibody (perlecan or CTGF) in 1% BSA/PBS for 2hr at room temperature or overnight at 4°C, then secondary antibody in 1% BSA/PBS for 1h at room temperature. After washing, TMB peroxidase substrate (Microwell peroxidase substrate system, KPL, USA) was added to the plates to generate a colour change reaction, which was stopped by sulphuric acid (0.2M). The intensity of transmitted light through each well was detected by Spectramax fluorometer (Molecular Devices, USA) at 450nm. In some experiments plates were also treated with heparintinase or chondroitinase ABC (10mu/ml) for 2hr at 37°C prior to incubation with 0.5µg/ml CTGF.

2.5 Sample preparation, Gel electrophoresis, Western Blotting, silver staining and protein extraction from unstained SDS-PAGE gel.

2.5.1 Protein sample preparation from cell lysates: Cultured NHDFs and HACs were lysed with dissociative RIPA buffer consisting of 100 mM Trizma base (pH 7.4), 300 mM NaCl, 20mM NaF, 2mM EDTA, 0.2mM beta glycerophosphate, 2% (w/v) Na vanadate, 2% (w/v) Na deoxycholate, 0.5% SDS and 1% (v/v) IGEPAL. RIPA buffer was also supplemented with a cocktail of proteases inhibitors containing 1mM phenylmethlsulfonyl

fluoride (PMSF), 10μM E64, 2μg/ml pepstatin A, 10μg/ml aprotinin and 1μM microcystin with broad specificity for the inhibition of serine, cysteine, aspartic proteases and amino peptidases. The inhibitor cocktail was mixed with RIPA buffer at a 1:100 ratio (v/v) according to the manufacturer's instructions. For cell lysates, 100μl of ice-cold RIPA buffer containing protease inhibitors was added to the cell layer of each well. After 10min incubation at 4ºC the cell lysate in each well was collected into an eppendorf tube and spun at 16200x g at 4°C for 10min to remove cell debris. 80μl of supernatant was removed and mixed with 20μl of 5 x reducing SDS sample buffer containing 0.2M Tris HCl pH 6.8, 20% (v/v) glycerol, 10% (w/v) SDS, 10mM dithiothreitol (DTT) and 0.05% (w/v) bromophenol blue. After mixing, the samples were boiled for 10min prior to electrophoresis.

2.5.2 Protein sample preparation from cell/tissue medium: Medium from cultures was collected and processed with or without acetone precipitation. For precipitation, samples were mixed with ice-cold acetone at 1:5 ratio (v/v) at -20ºC. After 30min, the samples were centrifuged at 16200x g at 4°C for 10min and the supernatant was removed. The resulting protein pellet was dried in a vacuum dryer and then dissolved in 50μl of 1 x SDS sample buffer (with or without DTT for reducing or non-reducing respectively) and boiled prior to electrophoresis.

2.5.3 Gel electrophoresis: Proteins were separated by gel electrophoresis either using pre-cast NuPAGE 4-12% Bis-Tris gradient polyacrylamide mini gels (1.5mm thickness) (Novx, San Diego, CA) or laboratory prepared 8% acrylamide gels (1.5mm thickness). The pre-cast gels were composed of a 4% acrylamide stacking gel and a 4-12% gradient resolving gel. The laboratory made gels used were composed of a 5% stacking gel (125mM Tris-HCl pH6.8, 0.1% (w/v) SDS, and 5% (w/v) acrylamide) and 8% resolving gel (380mM Tris-HCl pH8.8, 0.1% (w/v) SDS and 8% (w/v) acrylamide). 50μl of prepared protein sample (described above) was loaded onto the wells of the stacking gel. Pre-stained molecular weight markers (All blue standards, Biored, Hercules, CA) were run alongside the samples

to allow size determination of proteins. The gels were then run at 120V (laboratory made gels) or 140V (Pre-cast gels) for approximately 1.5hr in Tris glycine running buffer (25mM Trizma-base, 192Mm glycine, 0.1% (w/v) SDS) or NuPAGE MOPS-SDS running buffer (Invitrogen, UK) respectively in the XCell SureLock Mini gel electrophoresis apparatus (Novex, CA). After separating the proteins the gels were either further western blotted, silver or coomassie blue stained.

2.5.4 Western blotting: The proteins on the gel were transferred onto Polyvinyldifluoride (PVDF) membranes (Millipore, Bedford, MA). The PVDF membranes were first pre-hydrated in 100% methanol for 1min and then washed in transfer buffer (25mM Trizma base, 190mM glycine and 20% (v/v) methanol). Proteins were transferred in a sandwich between wet filter paper and sponges in a trans-blot chamber (Biored, Hercules, CA) containing transfer buffer at 100V for 80min. The transfer buffer was kept at 4º C by a water circulating cooling pump. After the transfer, the membranes were blocked in 5% (w/v) Marvel made up with 1 x TBS-tween buffer (1x Tris buffered saline and 0.1% tween-20) for 1hr at room temperature. The membranes were then incubated with primary antibody made up in 5% BSA/TBS-tween solution overnight at 4ºC. Membranes were then washed three times with TBS-tween (3 x10 min) before being incubated with 5% Marvel/TBS-tween containing secondary horseradish peroxidase conjugated antibody (1:2000). Finally the membranes were washed again for three times with TBS-tween, and the signal was developed by enhanced chemiluminescence (ECL) (Amersham, Little Chalfont, UK). The luminescence of protein bands was then exposed onto a blue sensitive film (Fuji film Super RX, Fuji Film, Bedford, UK) in a safe light room.

2.5.5 Stripping and re-blotting: In some instances, membranes were required for re-blotting with a different antibody. Antibodies were first stripped from membrane by incubating in Stripping buffer (Millipore, Billerica, USA) at room temperature for 20min. Extensive

washes in TBS-tween were required before blocking and then re-blotting with another primary antibody.

2.5.6 Silver staining: Silver staining was performed in order to determine the purity of the purified His-tagged recombinant CTGF protein. After proteins were separated by gel electrophoresis, the gel was first fixed in 50% (v/v) methanol/ 5% (v/v) acetic acid for 20min. Following fixation, the gel was washed in 50% (v/v) methanol for 20min and then in distilled water for 2hr. The gel was incubated in sensitiser solution (0.02% (w/v) sodium thiosulphate) for 1min followed by 2 x 1min washing with distilled water and then incubated at 4ºC for 20min with silver reagent (0.1% (w/v) pre-cooled silver nitrate solution). The gel was washed 2 x 1min with distilled water and then incubated with the developing solution (2% (w/v) sodium carbonate and 0.04% (v/v) 35% formaldehyde). The developing reaction was stopped with 5% (v/v) acetic acid when protein bands were clearly visible. Finally, the gel was washed and stored in distilled water at 4ºC.

2.5.7 Coomassie Brilliant Blue G-250 staining: SDS-PAGE gels were placed in the staining solution containing 0.1% (w/v) CBB, 50% (v/v) methanol and 20% (v/v) acetic acid for 45min and destained with 30% (v/v) methanol and 1% (v/v) formic acid. The destaining solution was changed every 10min for 1hr and the gels were further destained overnight at room temperature. The gels were either dried or the protein bands of interest were cut from the gel in distilled water in an eppendorf tube and sent for mass spectrometry analysis.

2.5.8 Protein extraction from unstained SDS-PAGE gel: For protein extraction, samples were loaded on the SDS-PAGE gel in duplicates. After gel electrophoresis, the gel was cut into two. One half was quickly stained with coomassie blue to determine the position of the desired protein. On the other half unstained gel, gel pieces were cut out by referring to the position on the coomassie stained gel. In eppendorf tubes gel pieces were minced and incubated with an elution buffer containing 50Mm Tris-HCl, 150mM NaCl, 0.1mM EDTA, 1%

SDS and 10mM DTT pH7.5 at 30ºC overnight with constant agitation. The gel pieces were then centrifuged to the bottom of the tubes and the supernatant was collected and mixed with sample buffer before gel electrophoresis.

2.5.9 Protein extraction of chondrons for mass spectrometry

The protein extraction method from chondrons was established by previous PhD student Celia McLean. Human chondrons were processed and the proteins were electrophoresed by Dr. Thanos Didangelos.

Chondron pellets were obtained as described in 2.2.4. The pellets were extracted for 15min at room temperature in 1ml RIPA lysis buffer containing protease inhibitors. Following centrifugation at 16200x g at room temperature for 5min, the supernatant was retained and the pellets were further extracted using 4M guanidine hydrochloride (GnHCl) with constant agitation at 4°C for 1h and the supernatant was analysed by gel electrophoresis. The gel containing the extracted proteins were coomassie blue stained and the bands were cut in clean eppendorf tubes for mass spectrometry (Cambridge Centre for Proteomics, Cambridge, UK).

2.6 Purification of CTGF from HEK293 conditioned medium

The method of CTGF purification from the cultured medium of stably transfected HEK293 cells was also established by Celia McLean. The protocol is as follows:

2.6.1 Stable transfection of HEK293-EBNA cells: Stably transfected human embryonic kidney 293 (HEK293) cells expressing His-tagged human CTGF protein were already available in our group (Celia McLean PhD thesis, 2009). HEK293-EBNA cells were originally obtained from Invitrogen (Groningen, Netherlands). These cells have Epstein-Barr virus nuclear antigen 1 (EBNA-1) gene inserted into the chromosome. As a result, these cells have the ability to induce high-level expression of an inserted plasmid containing an Epstein-

Barr virus origin (oriP) and maintain the plasmid as an extrachromosomal episome within transfected cells. These cells contain a neomycin resistance gene and therefore cells were maintained in the presence of the G418 (PAA laboratories Ltd, UK) to enable selection of EBNA-1 positive cells. The ctgf construct was subcloned from a pcDNA3.1-ctgf vector (provided by Prof. Roger Mason) into a pCEP4 expression vector. pCEP4 is an episomal mammalian expression vector carries an oriP and a hygromycin resistance gene to permit extrachromosomal replication and be selected by an antibiotic hygromycin B (Invitrogen, Paisley, UK). Stably transfected HEK293 cells were stored in liquid nitrogen until use. Frozen cells were quickly thawed in a 37°C water bath, mixed with 20ml of warm DMEM in a falcon and centrifuged at 235x g at room temperature for 5min. The pellet was then resuspended and cultured in 15cm dishes with 10% FCS DMEM containing 250µg/ml of the antibiotic G418 and 100µg/ml hygromycin B. After reaching confluence, cells were washed and cultured in fresh serum free medium containing 250µg/ml G418 for 24hr. The conditioned medium (containing secreted CTGF) was removed and stored at -20ºC until purification.

2.6.2 Nickel affinity chromatography (ÄKTA system, GE Healthcare, Little Chalfond, UK): 150ml of the conditioned medium from the HEK293 cell culture (described above) was supplemented with 20mM imidazole before being filtered and loaded onto a 5ml nickel affinity column (GE Healthcare Ltd, Buckinghamshire, UK) which has been equilibrated in the binding buffer containing 20mM sodium pyrophosphate, 0.5M sodium chloride and 20mM imidazole, pH7.4. The flow-through was collected and the column was washed with binding buffer until the absorbance returned to the background level. The proteins were then eluted in 1ml fractions by a 10ml linear gradient ranging from 20mM to 500mM imidazole. Fractions were western blotted for the presence of CTGF and silver stained to assess purity.

2.6.3 Dialysis and ultrafiltration: Positive fractions identified by western blot were pooled together and dialysed using a 10kDa molecular weight cut off SpectraPor Float-A-Lyzers

(Fisher scientific, Hampton, NH) in 1 x PBS for 8-12hr at 4ºC prior to be used in cell culture. Dialysed protein solution was then centrifuged in a 10kDa cut off ultrafiltration spin column (Vivaspin, Sartorius, Goettingen, Germany) to concentrate the protein by 10 times. The retained fractions were resolved by electrophoresis and then coomassie blue stained. The concentration of CTGF was determined by NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, USA) and confirmed by comparing to a range of known concentrations of BSA on a coomassie blue stained gel. The fraction was then aliquoted and stored at -20ºC until use. The yield of the recombinant CTGF production was 12µg from 150ml of HEK293 conditioned medium.

2.7 Partial protein purification of TGFβ/CTGF complex from cartilage injury conditioned medium (CM)

2.7.1 Ion exchange chromatography (ÄKTA system): 1ml of cartilage explantation CM was mixed with 4ml binding buffer containing 20mM Tris-HCl pH 7.4 and passed through a 1ml HiTrap SP HP column (GE Healthcare, Little Chalfond, UK). The flow-through was collected and the column was washed with binding buffer until the absorbance returned to the background level. The proteins were then eluted in 1ml fractions by a 10ml linear gradient ranging from 0 to 1M NaCl. Fractions were electrophoresed and western blotted for CTGF, TGFβ and LAP1.

2.7.2 Gel filtration chromatography (SMART system, Artisan Technology Group, Champaign, USA): Either the positive ion exchange fractions or crude cartilage recutting CM was processed in gel filtration chromatography. Sample proteins were concentrated to 0.5mg/ml so that 50µl of sample contains 25µg protein. First, 15µl of mixed standard proteins were injected into a Superose 12 PC3.2/30 column (GE Healthcare, Little Chalfond, UK) and the column was continuously loaded with 1 x PBS until the UV absorbance reached the basal level (at least 2 column volume has passed through (4.8ml)). Then 50µl of the sample proteins was injected into the system and the flow-through was collected in 200µl

fractions until the absorbance reached basal level (approximately 24 fractions). By comparing to the standard protein profile, the fractions within the range of correct sizes were analysed by gel electrophoresis and western blotted for CTGF, TGFβ and LAP.

2.8 Molecular biology

2.8.1 RNA extraction from cells: After stimulations of NHDFs or HACs, medium was removed and the cell layer was washed twice with 1 x PBS. The cell layer was lysed and RNA was extracted using Qiagen RNeasy kit (Qiagen, Clawley, UK) according to manufacturer's instruction. RNA was eluted in 40µl of RNase free water and its concentration and purity was determined by measuring the absorbance at 260nm, 280nm and 230nm using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA).

2.8.2 Reverse transcription: Complementary DNA (cDNA) was generated from the purified RNA using the ABI high capacity cDNA Reverse Transcription Kits (Applied Biosystems Inc.). According to manufacturer's instructions, 200ng of RNA was reversely transcribed into cDNA in a total volume of 20µl mixture containing 10µl of RNA, 2µl of 10 x reverse transcription buffer, 2µl of random primer, 0.8µl dNTP, 1µl RNase inhibitor, 1µl reverse transcriptase and 3.2µl RNase free water, using a following programme: 25ºC for 10min, then 37ºC for 120min and finally 85ºC for 5min.

2.8.3 Quantitative reverse transcription polymerase chain reaction (RT PCR): cDNA was processed using TaqMan® RT PCR system (Applied Biosystems Inc.). 5µl of 5 times diluted cDNA was mixed with 7µl of RT-PCR master mix containing 6µl TaqMan® 2 x universal PCR mix (Applied Biosystems Inc.), 0.6µl TaqMan® primer/probe (Applied Biosystems Inc.) and 0.4µl RNase free water. All samples were in triplicates. The thermocycling condition were as follows: hold at 50ºC for 2min for optimal activity of UNGerase enzyme, then 40 cycles of denaturation at 95ºC for 2min, followed by an

annealing step at 60ºC for 30s. The thermocycling reaction was carried out in a Rotor-Gene 6000 real time rotary analyser (Corbett Life Science, Corbett Research UK Ltd) and all data analysis was carried out using Rotor Gene 6000 software version 1.7 (Corbett Life Science, Corbett Research UK Ltd). The expression levels of the genes of interest were normalised to the gene expression of a house keeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Table 3 summarises the TaqMan® primers used for RT PCR.

Gene	ABI catalogue number	Amplicon size (bp)	Genbank number
GAPDH	Hs99999905_ml	122	NM_002046.3
CTGF/CCN2	Hs01026927_gl	70	NM_001901.2
PMEPA1	Hs00375306_m1	77	NM_199170.1
BMPR2	Hs00176148_m1	69	NM_001204.6
LTBP2	Hs00166367_m1	78	NM_000428.2
Perlecan	Hs00194179_m1	70	NM_005529.5
Col1A1	Hs00164004_m1	66	NM_000088.3
FN1	Hs00365052_m1	81	NM_002026.2

Table 3 TaqMan® Gene Expression Assay primer/probe mixes (Applied Biosystems) used in TaqMan® RT-PCR.

2.8.4 Microarray study: Purified RNA from experiments was sent to Cambridge genomic centre for microarray study. Quality of the RNA was first determined using Agilent 2100 Bio-analyser (Agilent Technologies, Inc. CA) following the manufacturer's instruction to ensure RNA integrity (RIN), purity (260/280 for protein contamination, 260/230 for carbohydrates or phenol contamination) and concentration. RNA quality should satisfy the criteria of microarray analysis: RIN >8, 260/280 = ~2.0 and 260/230 >2. The microarray study was

carried out using Illumina platform on 2 HumanHT-12 v4 chips which covers more than 47,000 human genes. Briefly, prepared RNA was transcribed into cDNA which incorporates biotin-labelled nucleotides. Following the steps of array hybridization, washing, blocking and streptavidin-Cy3 staining, the chips were then scanned by BeadArray reader (Illumina Inc.) and the scanned images were quantified to determine the signal intensity of each spot. The acquired microarray raw data were first filtered and normalized to remove noise. An unsupervised clustering analysis was carried out by using the GenomeStudio Data Analysis software (Illumina, San Diego, CA).

2.8.5 CTGF silencing RNA transfection: 3 x 10^5 NHDFs or HACs were plated in a 6 well plate as described above and transfected with CTGF siRNA oligos (Applied Biosystem Inc.) in Opti-Mem reduced serum medium (Gibco, Life Technology, NK, USA) at a final volume of 2.5ml. siRNA oligo was first mixed with Lipofectamin 2000 transfection reagent (Invitrogen, NY,USA) at 1:20 ratio (v/v) in 0.5ml of Opti-Mem medium for 20min, which was then added to the each well of the culture plate containing 2ml of Opti-Mem drop by drop with gentle shaking. siRNA oligo will reach a final concentration of 15nM. After 4hr transfection at 37°C, cell culture was changed with fresh serum containing DMEM and cultured for further 24hr before stimulation.

2.8.6 Genomic DNA extraction from mice ear biopsy for genotyping: 2mm wide tissue was biopsied from a mouse ear at age around 4 weeks and kept frozen. Upon extraction, the ear tissue was digested in 100µl of a lysis buffer containing 50mM Tris-HCl, pH8.0, 0.1M NaCl, 1% SDS, 20mM EDTA and 8u/ml proteinase K (Promega, Southampton, UK) at 55ºC for 18hr. The digests were mixed with 100µl of 3.75M NH_4OAc (Sigma, Dorset, UK) and 100µl of chloroform (VWR International, Leicestershire, UK) and then centrifuged at 15600x g at room temperature for 5min to turn the digest into three layers. Liquid from the top layer was carefully pipetted into a clean eppendorf tube containing 500µl of 100% ethanol, which was inverted to mix well. Precipitated DNA was then centrifuged at 15600x g for 10min. After

discarding the supernatant，the pellet was dried using a vacuum dryer and resuspended in 50µl TE buffer containing 10mM Tris-HCl and 1mM EDTA pH8.0.

2.8.7 The polymerase chain reaction (PCR) for genotyping: PCR master ready beads (GE healthcare, Buckinghamshire, UK) were used for PCR reaction. Each ready mix bead was dissolved in 25µl of distilled water and then split into two 0.5ml tubes. Each reaction (12.5µl) was mixed with 1µl of each primer (10µM) (both sense and anti-sense) (Eurofins MWG Operon, Ebensburg, Germany) and 1µl of DNA in TE buffer (50mg/ml). The reaction tubes were then incubated in a thermocycler to allow PCR reaction to take place. Table 4 below shows the primers sequence and the PCR program used for genotyping.

Primers	**Sequences**
CTGF sense	5'-AAT ACC AAT GCA CTT GCC TGG ATG G-3'
CTGF antisense	5'-GAA ACA GCA ATT ACT ACA ACG GGA GGG G-3'
Ub Cre sense	5'-GCA TTA CCG GTC GAT GCA ACG AGT GAT GAG-3'
Ub Cre antisense	5'-GAG TGA ACG AAC CTG GTC GAA ATC AGT GCG-3'

PCR programmes	
CTGF	**Ub Cre**
Step 1: 94ºC 3min	**Step 1:** 93ºC 3min
Step 2: 94 ºC 30sec	**Step 2:** 93ºC 30sec
Step 3: 65 ºC 30sec	**Step 3:** 68 ºC 30sec
Step 4: 72 ºC 1min	Go to step 2 for 29 more cycles
Go to Step 2 for 34 more cycles	**Step 4:** 4ºC ∞
Step 5: 72 ºC 10min	
Step 6: 4ºC ∞	

Table 4 Primer sequences and PCR programmes for CTGF and Ub Cre genotyping.

2.8.8 DNA electrophoresis: The PCR products are analysed on 1.5% DNA Agarose gel (Invitrogen, Parsley, UK) in 0.5 x TBE buffer containing 45mM Tris-borate and 1mM EDTA at 100V for 1.5hr. PCR samples were mixed with 6x blue/orange dye (Thermo Scientific, Wilmington, USA) before loaded onto the gel. After the run, bands on the gel were visualised in a Gel Doc machine (Bio-Rad, Hemel Hempstead, UK). The sizes of the DNA fragments were determined by comparing to the size of the DNA ladder (100bp) (Invitrogen, Parsley, UK). The ctgf primers can produce two DNA fragments during PCR reaction. A larger DNA fragment (1000bp) is produced when ctgf exon is franked by two Lox P sites i.e. floxed ctgf, whereas a smaller fragment (800bp) is produced from a wild type ctgf allele. Therefore, the genotype can be determined by the size of the resulted PCR bands, i.e. wild type (a single band at 800bp), homozygous floxed ctgf (a single band at 1000bp) or heterozygous floxed ctgf (two bands at 1000bp and 800bp). The presence of the Cre allele was determined by the observation of a 400bp band.

2.8.9 Statistical analysis: All statistical analysis was performed using Prism 4 programme (Graphpad Software Inc., USA). Significance was calculated using t- test. Not significant (ns) when p value > 0.05, significant (*) when p value ≤ 0.05, very significant (**) when p ≤ 0.01 and highly significant (***) when p ≤ 0.001.

Chapter 3:

Results

Identification and binding of CTGF in the PCM of human articular cartilage

3.1 Introduction

My project started as an extension of the work performed by Celia McLean, a previous PhD student. In her studies she determined that CTGF was an abundant PCM protein of porcine cartilage and characterised a successful method for the expression and purification of the recombinant protein. These results, taken together with the known *in vivo* role of CTGF in the musculoskeletal development (Ivkovic *et al*. 2003) as well as the increased expression of CTGF in OA cartilage (Hermansson *et al*. 2004), have justified the further study of this interesting protein.

In this chapter I determine that CTGF is also present in the PCM of human cartilage and investigate to what is it bound in the PCM.

3.2 Results

3.2.1 Proteomic analysis of the PCM of human articular cartilage

3.2.1.1 Isolation of human cartilage chondrons

In early studies of the molecular composition of the PCM, Celia McLean developed a method of isolating chondrons from porcine cartilage and successfully extracting PCM proteins for mass spectrometry. Her optimised method involved limited enzymatic digestion of cartilage with a combination of dispase and collagenase for 4hr. I repeated a similar analysis only this time in human tissue. Human chondrons were isolated from normal human articular cartilage using Celia's methods described in 2.2.4. Chondron preparations were fixed onto chamber slides using methanol and then immunostained with antibodies against the known PCM protein perlecan to confirm the integrity of the PCM. Figure 3.1 shows the immunofluorescent staining of isolated human chondrons. Staining (purple) reveals a perlecan-rich coat around individual chondrocytes (red nuclei staining) confirming the presence of an intact PCM.

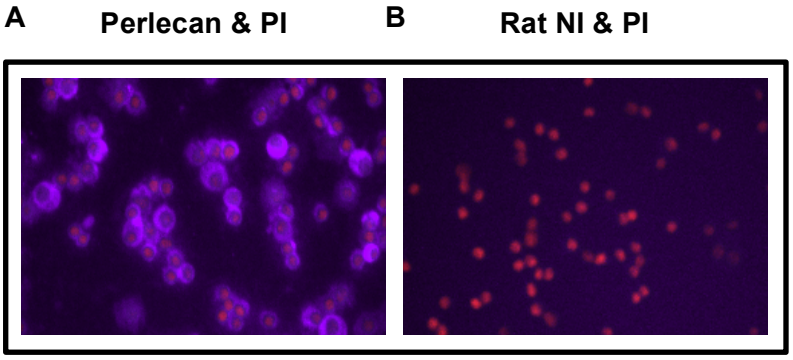


Figure 3.1 Immunofluorescent staining of perlecan in enzymatically isolated chondrons. Chondrons were isolated from human articular cartilage with a combination of dispase (0.2%) and collagenase (0.3%) for 4hr at 37ºC. Following isolation, chondrons were suspended in PBS and a small volume was allowed to adhere to Lab Tek II chamber slides. The chondrons were fixed in methanol, treated for 1.5hr with chondroitinase (10mu/ml) and then incubated with primary antibodies to perlecan (1:1000) (A) (purple) or rat non-immune control antibody (NI) (B). The chondrons were washed and incubated with secondary FITC labelled antibodies and propidium iodide (PI) (red). Fluorescence signals were detected using Ultraview confocal microscopy.

3.2.1.2 Purification of the PCM from isolated chondrons.

Chondrons contain both the chondrocyte and their surrounding PCM. In order to investigate the PCM by mass spectrometry, removal of chondrocytes from the chondron preparation was necessary to reduce contamination by intracellular proteins. Chondrons were incubated with a dissociative lysis buffer for 15min in an attempt to lyse intracellular proteins and wash them from the remaining PCM. The remaining pellet, enriched for PCM proteins, was then solubilised by guanidine hydrochloride (8M) to allow analysis by SDS-PAGE and mass spectrometry.

3.2.1.3 Identification of PCM proteins using mass spectrometry

Solubilised chondron proteins were separated on a 4-12% Bis-Tris gradient gel and stained with Coomassie Blue in a clean glass tray. Small gel pieces containing protein bands were cut into eppendorf tubes. Mass spectrometry analysis was performed by Cambridge Centre for Proteomics at the University of Cambridge. Table 5 shows all growth factors and ECM

proteins identified in the proteomic analysis of the human chondrons. Most of these proteins have already been described previously in human PCM (Wilusz *et al*. 2014; Hedman *et al*. 1979; Vincent *et al*. 2007; Omoto *et al*. 2004; Guo *et al*. 2010). Some of these molecules were also identified in Celia McLean's proteomic analysis using porcine cartilage, which are indicated in the table. There were also a large number of presumed non-specific proteins identified, which are not shown here in the table. These include intracellular proteins (e.g. histones and glutamine synthetase) from incomplete cell lysis or wash of the PCM pellet; secreted or transmembrane proteins that are commonly found in other tissues (e.g. claudin17, clusterin and lactadherin); foreign proteins (e.g. keratin and trypsin) from contamination of sample handling and uncharacterised proteins.

Selective PCM proteins identified from human articular cartilage	**Previously identified in porcine PCM (by Celia McLean)**	**Previously described human PCM (Wilusz *et al*. 2014; Hedman *et al*. 1979; Vincent *et al*. 2007; Omoto *et al*. 2004; Guo *et al*. 2010)**
Fibronectin	Yes	Yes
Fibroblast growth factor-2 (FGF2)	No	Yes
Hyaluronan binding protein	Yes	Yes
Chondroadherin	Yes	Yes
Cartilage oligomeric matrix protein (COMP)	Yes	Yes
Thrombospondin-1	No	Yes
Hyaluronan & proteoglycan link protein -1	Yes	Yes
Decorin	Yes	Yes
Biglycan	Yes	Yes
Collagen II	Yes	ECM
Collagen VI	Yes	Yes
Collagen IX	No	Yes

Collagen XI	Yes	ECM
Collagen XII	No	ECM
A disintegrin and metallopeptidase domain-7 (ADAM7)	No	ECM
ADAM metallopeptidase with thrombospondin type 1 motif -12 (ADAMTS12)	No	Yes
Heparin sulfate proteoglycan -2 (HSPG2/perlecan) protein core	Yes	Yes
Aggrecan protein core	No	ECM
Hepatocyte growth factor (HGF)	No	Yes
Connective tissue growth factor (CTGF)	Yes	Yes

Table 5 All growth factors and ECM proteins identified in the human chondrons by mass spectrometry. Solubilised human chondron proteins were analysed on gradient gel and cut in small pieces. The mass spectrometry study was performed by Cambridge Centre for Proteomics at University of Cambridge.

3.2.2 Localisation of CTGF in human articular cartilage

Having identified CTGF from the proteomic analysis of the PCM of human articular cartilage, we sought to confirm that CTGF localised to the PCM using immunofluorescent staining. Celia McLean developed a protocol for confocal microscopy of articular cartilage. Frozen tissue was processed as described in 2.3 and then immunostained for perlecan, type VI collagen, FGF2 or CTGF. Figure 3.2 shows the localisation of CTGF in adult human normal articular cartilage. Perlecan and type VI collagen were used as markers of the PCM as they are well-described pericellular proteins. The immunofluorescent staining of these molecules revealed a distinct pericellular pattern around each chondrocyte. The staining of CTGF co-localised with other PCM proteins such as perlecan and type VI collagen (Figure 3.2A and B). FGF2, which is known to bind to perlecan in the PCM, also demonstrated significant co-localisation with CTGF suggesting that they lie close to one another in the PCM (Figure 3.2C)

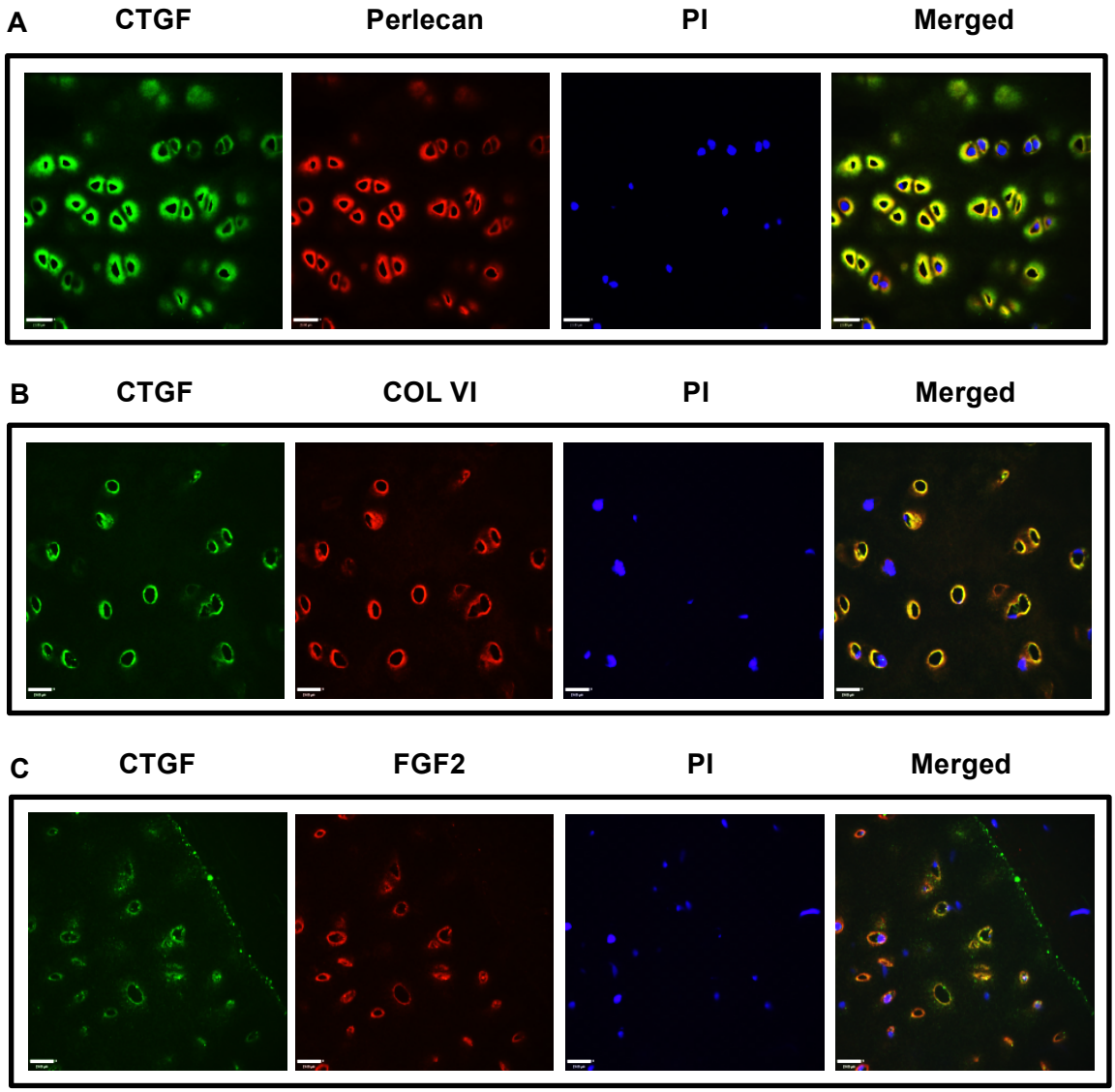


Figure 3.2 Immunolocalisation of CTGF and other known PCM proteins in human articular cartilage. Frozen sections (7µm) of human articular cartilage were pre-treated with chondroitinase ABC (10mU/ml for 120min). Sections were stained with primary antibodies to CTGF (1:500) (green), perlecan (1:1000) (red) (A), collagen VI (1:1000) (red) (B) and FGF2 (1:1000) (red) (C). Sections were washed and incubated with secondary FITC-labeled antibodies (1:1000) and propidium iodide (PI) (1:2000) (blue). Fluorescence signals were detected by UltraView confocal microscopy (n=3).

3.2.3 CTGF binds to perlecan *in vitro* via heparan sulphate chains

Immunohistochemistry confirmed that CTGF localised to the PCM of human chondrocytes, but did not indicate to what it was bound. CTGF has been described as binding to heparan sulphate (HS) in human chondrosarcoma-derived chondrocytic cell line HCS-2/8 *in vitro*

(Nishida *et al*. 2003), so I sought to determine whether this was the case in cartilage. To test this, I carried out an *in vitro* binding assay. Perlecan has different sulphation patterns according to the tissue from which it has been extracted. Commercial perlecan is from a variety of tissue sources that are known to have different sulphation patterns when compared with articular cartilage. These sulphation patterns determine the ability to bind to ligands. The perlecan used in my experiment was previously purified from adult (porcine) articular cartilage by Celia McLean. She used a method described by Hassell *et al,* involving extraction with guanidine hydrochloride (6M) followed by a series of caesium chloride gradient steps (Hassell *et al*. 1980). Perlecan is further purified by ion exchange chromatography and detected by dot blot (Celia Mclean's thesis, 2009). I then set up an ELISA-style assay in which perlecan (0.5 µg/ml) was plated onto the ELISA plate and following washing and blocking, incubated with different concentrations of recombinant CTGF (from 0 to 10 µg/ml). Bound CTGF was detected using an anti-CTGF antibody to which an enzyme-linked secondary antibody was attached. Figure 3.3A shows the detection sensitivity of this assay. CTGF binds to perlecan in a linear fashion with a larger slope from 0 to 1µg/ml, which then decreased from 1µg/ml to 10µg/ml. As mentioned previously, perlecan is substituted by both heparan sulphate (HS) and chondroitin sulphate (CS) chains. I determined whether CTGF bound to perlecan through either of these chains. The plates was coated with perlecan (0.5µg/ml) or 1% BSA as control. Heparitinase (H'ase) or chondroitinase ABC (C'ase) was used to remove the HS or CS chains respectively from the core protein of perlecan before CTGF (0.5µg/ml) was added to the assay plate and the amount of bound CTGF was measured. We found that in the presence of H'ase, the amount of bound CTGF was greatly reduced compared with the non-H'ase treated control, whereas C'ase did not change the binding of CTGF to perlecan (Figure 3.3B (CTGF)). Detection of perlecan (using an antibody against perlecan protein core) in the presence or absence of H'ase was carried out to ensure that the enzyme treatment did not reduce the amount of coated perlecan on the ELISA plates (Figure 3.3B (perlecan)).

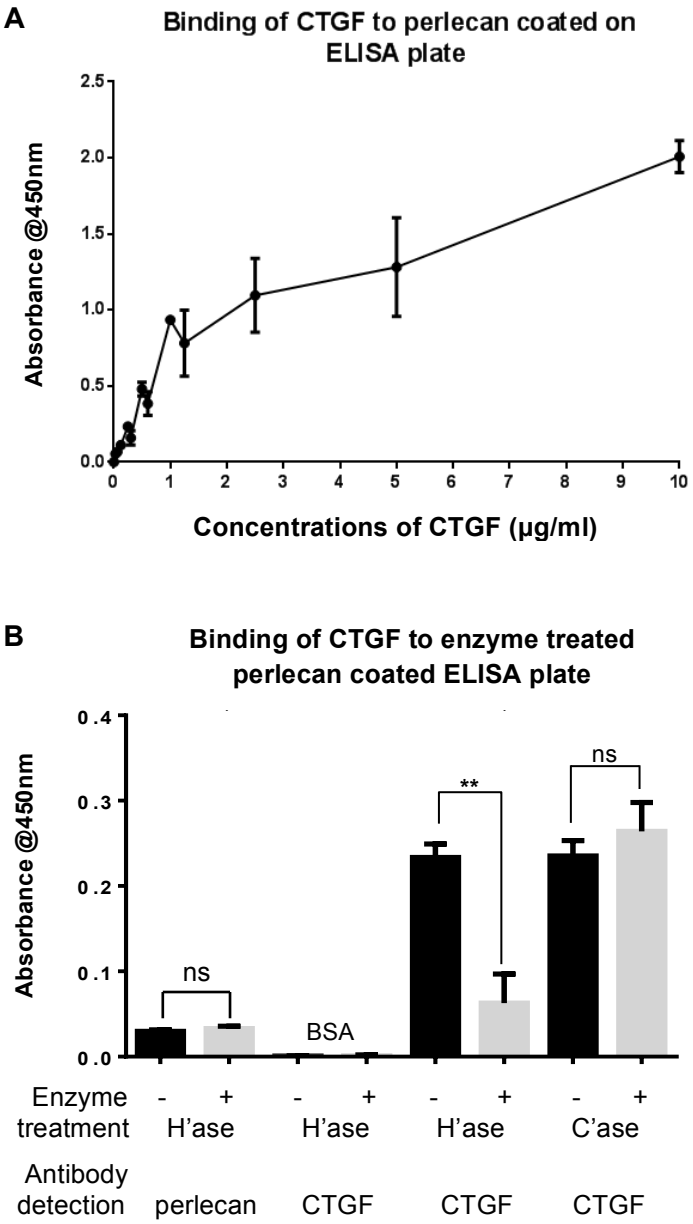


Figure 3.3 Dose dependent binding of CTGF to perlecan, which was abolished by heparitinase. An ELISA plate was coated with 0.5µg/ml of recombinant perlecan at 4°C for 18hr. After washing with PBS and blocking with 1% BSA in PBS, the plate was incubated with increasing concentrations of recombinant CTGF (0 to 10µg/ml) for 3hr in 1 x PBS at room temperature. After three washes in PBS-tween, the plate was incubated with primary and secondary antibodies to detect bound CTGF (A). For enzyme treatments, H'ase or C'ase (10mu/ml) was added to perlecan coated plates for 2hr at 37°C prior to addition of CTGF (0.5µg/ml). Perlecan or CTGF was detected using specific antibodies to the protein core followed by enzyme linked secondary antibodies (B). (A: n=3; B: n=3). **p<0.01 ns=not significant.

3.2.4 CTGF release following cartilage injury

FGF2 is also bound to the HS of perlecan, and it is from this bound pool that FGF2 released from the PCM in response to cartilage injury (Vincent *et al*. 2007). To test whether this is also the case for CTGF, I explored whether CTGF was also released upon cartilage injury. Porcine articular cartilage explants were dissected from pig trotter joints using a biopsy punch to ensure equal size. The dissected cartilage explants were immediately rested in serum free medium for 48hr to allow injury-induced responses to return back to basal level. The rested cartilage were then further cultured in fresh medium for another hour (this conditioned medium was used as control) before being recut in fresh medium. The conditioned medium (CM) was collected before (control CM) and after recutting at various time points (recut CM). Figure 3.4A shows the recut CM collected cumulatively (collect everything during the period) after 5min, 4hr and 24hr following recutting, alongside the control CM (1hr collection before recutting). The western blot showed strong CTGF detection in the recut CM of all the time points in contrast to the resting control. FGF2 release occurs very rapidly (within a few minutes of cutting) so I next determined whether this was also the case for CTGF release. Sequential recut CM (collect several times in sequence following recutting) was collected to determine the dynamic of CTGF release. Figure 3.4B shows the western blot of recut CM collected between 0 and 5min, then 5 and 30min following recutting. Strong CTGF was detected in the recut CM within the first 5min following recutting. The level of CTGF accumulating in medium beyond 5min was much lower.

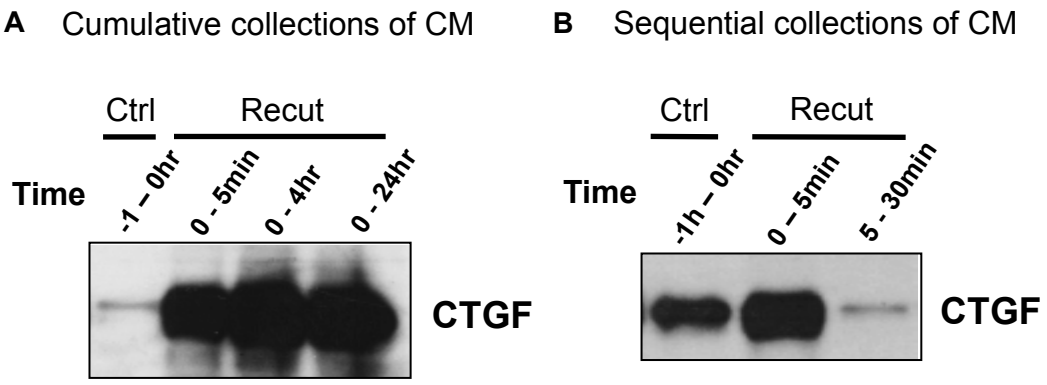


Figure 3.4 The western blots of recutting CM show accumulation of CTGF. Porcine cartilage explants were rested in serum free medium for 48hr, further cultured in fresh medium for another hour (Ctrl) and recut. The recutting CM (Recut) was collected cumulatively (A) or sequentially (B) after various time points following recutting and western blotted for CTGF.

Such rapid accumulation of CTGF in the recut CM (within 5min) following cartilage injury implies that CTGF is released from pre-made pool rather than from protein synthesis. To confirm this, I then carried out the recutting experiment in the presence of protein synthesis inhibitor cycloheximide (CHX). Porcine articular cartilage explants were again rested for 48hr and then further cultured for one extra hour, only this time some were in the presence of CHX (10μg/ml). Following recutting, medium was conditioned and collected for various times in the presence or absence of CHX (10μg/ml). Figure 3.5 shows the western blot of a sequential collection of recut CMs, which captures the protein generated from 0 to 1hr, 1 to 2hr, 3 to 4hr and 7 to 8hr following recutting. The results show that large amounts of CTGF protein accumulated in the CM within the first hour upon recutting irrespective of whether CHX was present. In the absence of CHX, the levels of CTGF detected in the following time points were significantly reduced, while in the presence of CHX there was no CTGF detected in the subsequent time points (Figure 3.5), suggesting that the protein produced in these later time points were newly synthesised.

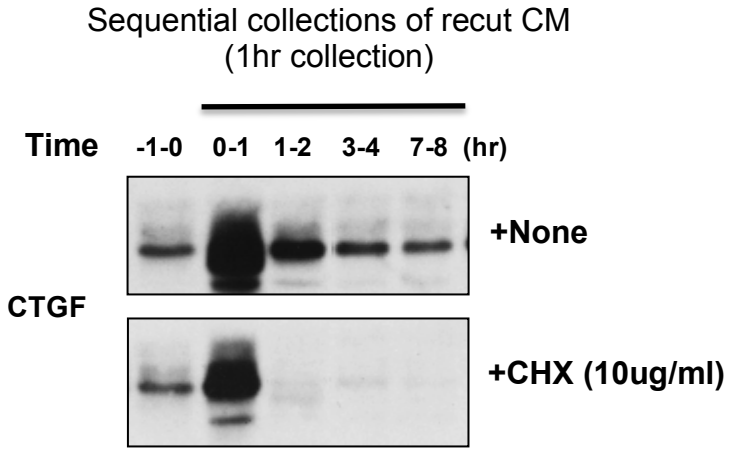


Figure 3.5 Porcine cartilage recutting caused release of CTGF from matrix either with or without CHX (10µg/ml). Rested porcine cartilage explants were recut and the conditioned medium was collected sequentially from 0 to1hr, 1 to 2hr, 3 to 4hr and 7 to 8hr. Fresh medium was replaced after each time point. The CM was analysed for CTGF by western blot. One representative result from 3 experiments (n=3).

Medium conditioned following cartilage explantation (without 48hr resting) was also analysed. This is a medium that captured proteins released from the injured cartilage that directly dissected from a joint. Dissected porcine cartilage explants were immediately cultured in serum free medium and these explantation conditioned media (explantation CM) were collected at various time points and analysed for CTGF release. Figure 3.6 shows the western blot of explantation CM collected sequentially from 0 to 1hr, 1 to 2hr, 3 to 4hr and 7 to 8hr following explantation in the presence or absence of CHX (10µg/ml). Again CTGF accumulation was strongly detected within the first hour of explantation with or without CHX. The amount started to decrease beyond the second hour in both cases. By the eighth hour following explantation the amount in the CHX treated group was significantly reduced. Taken together these results suggest that there is a rapid burst of CTGF protein release upon cartilage injury and this protein is passively released from a pre-made, most likely, the pericellular matrix pool. The protein accumulating at later time points is likely to be newly synthesised as this can be abrogated in the presence of CHX.

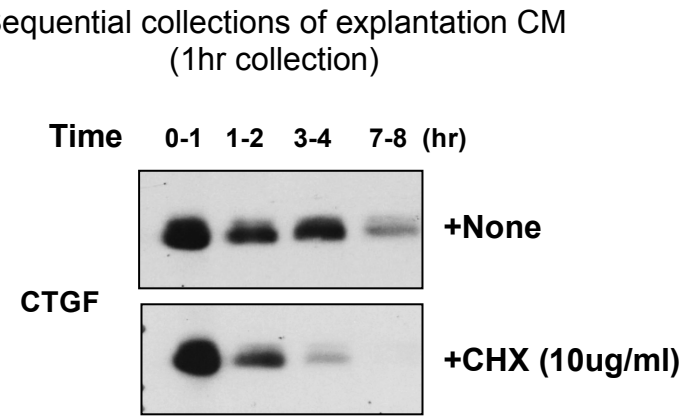


Figure 3.6 Porcine cartilage explantation caused release of CTGF from matrix either with or without CHX. Porcine cartilage explants were dissected from pig trotter joint and cultured in serum free medium for sequential time points 0-1hr, 1-2hr, 3-4hr and 7-8hr. Fresh medium was replaced after each time point. The CM was analysed for CTGF by western blot. One representative result from 3 experiments (n=3).

Secreted CTGF is rapidly internalised by cells through the low-density lipoprotein receptor-related protein 1 (LRP1) in a heparan sulphate dependent manner (Gao and Brigstock 2003). Therefore it was possible that the CTGF accumulation we observed upon injury was due to failure of LRP1 mediated re-uptake. To test this, we blocked LRP1 using its antagonist, receptor associated protein (RAP), or by adding an excess of soluble heparin. The same recutting experiment was carried out but this time the cartilage was pre-cultured and recut in the presence of these LRP1 blockers. The CM was collected after 15min at the beginning of each one hour period following recutting. As expected, RAP and heparin increased the resting levels of CTGF prior to recutting. They also increased the amounts released after recutting. They did not appear to alter appreciably the amount of CTGF released immediateltly upon injury. This may be because that LRP1 had been maximally saturated by CTGF or the action of recutting somehow disables the action of LRP1.

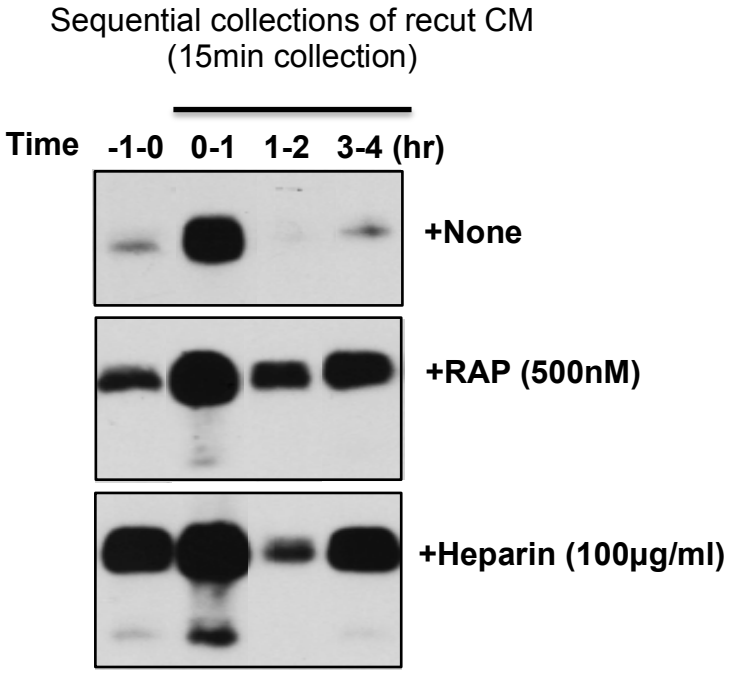


Figure 3.7 RAP and heparin increased CTGF protein accumulation in the CM. Explanted porcine cartilage were rested for 48hr, pre-incubated in the presence or absence of RAP (500nM) or heparin (100µg/ml) for 1hr and then recut. Recut CM was collected after 15min at the beginning of each hour (-1-0hr, 0-1hr, 1-2hr and 3-4hr) in the presence or absence of RAP (500uM) or Heparin (100µg/ml). Fresh medium was replaced after the 15min collection and at beginning of each hour. The CM was analysed for CTGF by western blot (n=1).

3.3 Discussion

From the proteomic analysis of human cartilage chondrons we identified a number of proteins that have been previously identified in the PCM of cartilage, including FGF2 and CTGF. Most of the identified proteins are pericellular matrix proteins, for example, perlecan, decorin, biglycan and type VI collagen. From previous confocal and electron microscopic studies it appeared that the PCM was rich in type VI collagen but not in type II collagen (Söder *et al.* 2002). The identification of type II collagen as well as aggrecan and type XI collagen may indicate incomplete removal of the further removed matrix upon enzymatic digestion or protein that is transiting through the PCM. The assemblies of type VI collagen and other small proteoglycan such as decorin and biglycan, which are specific to the PCM,

have been reported to participate in the assembly of aggrecan prior to their release into the ECM (Sandy *et al.* 1989; Larson *et al.* 2002). Some intracellular proteins, such as pyruvate kinase, were also identified in the study, which may represent contamination of intracellular proteins due incomplete cell lysis or washing. Most of the identified PCM proteins had also been identified previously in porcine chondrocytes by Celia McLean. By repeating the PCM proteomic analysis, alongside confocal studies, I have been able to confirm the pericellular localisation of CTGF in human articular cartilage. This is consistent with other studies in mice (Oka *et al*. 2007; Itoh *et al*. 2013). I also identified other interesting proteins which were not identified in porcine cartilage before, for examples, ADAMST12 and HGF. Their roles in human cartilage could be interesting to investigate.

Confocal microscopy showed co-localisation of CTGF with other known PCM molecules, perlecan, type VI collagen and FGF2. Binding assays were carried out to establish whether CTGF binds to perlecan. Administration of heparitinase significantly reduced the amount of bound CTGF but not chondroitinase, suggesting that CTGF binds to the heparan sulfate chains of perlecan as predicted. This finding is consistent with the observation described in HCS2/8 cell system (Nishida *et al*. 2003).

Molecules that are sequestered in the PCM can be made available to the cells very rapidly given the correct physiological stimuli (mechanical load in the case of FGF2). Sequestration may also act as a negative control of growth factor activity by holding ligands back from the cell surface until they are required. This was proved by the cartilage injury experiment, in which CTGF became rapidly available in the cultured medium upon cartilage recutting. This is consistent with the observation of FGF2 release, which in turn activates ERK MAPK and drive several cellular responses including induction of chondroprotective proteins such as activin A and TIMP1 (Vincent *et al.* 2007; Chong *et al*. 2013; Alexander *et al*. 2007). Increased protein releases in response to cartilage injury reported by others are mainly from newly protein synthesis. Anabolic proteins such as activin A was found elevated in response

to *in vitro* cartilage injury and OA (Stevens *et al*. 2009; Hermansson *et al*. 2004), which may play a role in tissue repair or a role in early inflammation and acute tissue injury response (Jones *et al*. 2004). Bone morphogenetic protein BMP13 was also found elevated in response to cartilage injury with a decrease in BMP antagonists including gremlin and chordin (Stevens *et al*. 2009). Elevated CTGF protein level was also found by others, but this was thought be actively mediated by other factors such as BMP2, TGFβ or macrophage colony-stimulating factor (M-CSF) (Nakao *et al*. 2005; Nakanishi *et al*. 1997b). There are also studies that show release of pre-existing proteins in response to cartilage injury, but these are mainly structural matrix proteins such as COMP and aggrecan (Lohmander *et al.* 1994; Stevens *et al.* 2009), which is thought to be the result of cartilage degradation mediated by MMPs. Injurious compression on bovine cartilage caused increased release of pre-existing aggrecan protein as well as increased synthesis of MMP1, 3, 9 and 13 in the same injury conditioned medium (Stevens *et al*. 2009). There is a substantial literature that is emerging from the Marfan syndrome field where TGFβ, which is normally sequestered on fibrillin in the matrix, is inappropriately active when fibrillin is misfolded (Neptune *et al*. 2003). The presence of regulatory factors in the PCM suggests that they are having important cellular functions with tightly controlled bioavailability. My finding of rapid CTGF release from pre-existing PCM matrix pool upon cartilage injury implies that CTGF is important in an early response following cartilage injury. The mechanism by which this is changing cellular behaviour will be addressed in later chapters.

Chapter 4:

Results

Effects of recombinant CTGF on articular chondrocytes

Part I: Investigating the role of CTGF in human articular chondrocytes

4.1 Introduction

CTGF is described as a matricellular protein. Matricellular proteins are non-structural proteins that are dynamically expressed in the ECM, serving regulatory roles. They are thought to function by modulating cellular responses through their ability to interact with multiple cell surface receptors, cytokines, growth factors, proteases, and structural proteins (Bornstein *et al.* 2002). CTGF, as described in chapter 1, contains 4 conserved modular domains that can bind to a large number of molecules. These interactions enable CTGF to participate in many essential biological functions including cell communication, proliferation, differentiation, adhesion and migration, regulation of skeletal growth and development, wound healing and angiogenesis (Oemar and Lüscher 1997).

4.1.1 *In vitro* studies of CTGF

The majority of published data on the role of CTGF has been from *in vitro* studies. CTGF has been described to exhibit numerous effects in different cell types including fibroblasts, chondrocytes, osteoblasts and vascular endothelial cells. Depending on the type of the cell with which it interacts, CTGF promotes cell proliferation, adhesion, chemotaxis, migration and extracellular matrix formation. CTGF has been shown to promote cell proliferation and migration of mesangial cells (Crean *et al.* 2004) and to induce apoptosis and caspase-3 activity in human vascular smooth muscle cells (Hishikawa *et al*. 2000). Takigawa's group has shown that CTGF promotes the proliferation of human chondrosacoma derived chondrocytic cell line (HCS2/8) and primary articular chondrocytes from rabbit cartilage, with increased gene expression of aggrecan, type II and type X collagen in these cells (Nakanishi *et al*. 2000). CTGF has also been reported to activate a number of signalling pathways for example ERK1/2 and Junk signalling in mesangial cells (Wahab *et al.* 2007) and to inhibit for example BMP and Wnt signalling in mesenchymal stem cells (Luo *et al.* 2004).

4.1.2 *In vivo* studies of CTGF

A wide variety of methods have been reported for investigating the role of CTGF. With regards to *in vivo* studies, the generation of CTGF null mice has suggested a role for CTGF in growth plate angiogenesis, skeletal development and chondrogenesis (Ivkovic *et al*. 2003). *In vivo* delivery of recombinant CTGF into the femoral marrow cavity of rat induced osteogenesis with increased angiogenesis (Safadi *et al*. 2003). Overexpression of CTGF in Xenopus embryos modulated Wnt signaling, suggesting a role in embryonic development (Mercurio *et al.* 2004). Another study using transgenic mice that overexpressed CTGF under the control of the *Col2α1* promoter showed increased cartilage and bone growth, with a 4% increase in the length of the tibial diaphysis in postnatal day 1 transgenic mice and increased proteoglycan and type II collagen accumulation (Tomita *et al.* 2013).

A number of studies have investigated the role of CTGF using animal models of disease. Increased expression level of CTGF has been found associated with chronic obstructive pulmonary fibrosis in rats (Tannheimer *et al.* 2012), human thyroid carcinoma (Wang *et al.* 2013), human renal allograft fibrosis (Metalidis *et al*. 2013) and human osteoarthritis (Hermansson *et al*. 2004), suggesting the involvement of CTGF in these diseases. In a mouse skin fibrosis model, injection of recombinant CTGF into the subcutaneous tissue of newborn mice caused the development of persistent fibrosis (Mori *et al.* 1999). Transgenic mice overexpressing CTGF after 20 weeks showed 100% protection from aging induced OA whereas 50% of wild-type mice developed an OA-like phenotype (Itoh *et al*. 2013). Another study using the monoiodoacetic acid induced OA in which recombinant CTGF was injected into rat knee joints, showed repair to cartilage defects implicating the role of CTGF in the regeneration of articular cartilage (Nishida *et al*. 2004).

These findings suggest that CTGF may have a beneficial role in articular cartilage. However, other reports investigating the effects of overexpression of CTGF in mouse knee joints showed increased gene expression of catabolic molecules including MMP3, MMP13,

ADAMTS4 and ADAMST5, suggesting its involvement in cartilage destruction (Blaney Davidson *et al*. 2006). Treatment with anti-CTGF neutralising antibody significantly ameliorated collagen induced arthritis in mice (Nozawa *et al*. 2013). These reports are contradictory and early attempts in our group were unable to confirm (see later also) the induction of any of these genes in human articular chondrocytes. Therefore we designed a microarray study to investigate the role of CTGF in human articular chondrocytes.

Prior to investigating cellular responses of CTGF, it was essential for us to obtain high quality recombinant protein. Overexpression of CTGF either by transient or adenoviral transfection methods have been used in a number of cell types (Hishikawa *et al.* 2000; Hishikawa *et al.* 1999; Liu *et al.* 2011; Luo *et al.* 2004; Wahab *et al.* 2001; Safadi *et al.* 2003). Many other groups used the recombinant protein from FibroGen, a company involved in the development of therapeutics for fibrotic disease, but they later scaled back their operation and the recombinant protein was no longer available. Other commercial sources were regarded as unreliable by others in the field (personal communications). The generation of our own recombinant CTGF became a prerequisite for our planned studies. Celia McLean established a method by which recombinant histidine tagged (His-tagged) CTGF can be generated from stably transfected HEK293 cells. I adopted this technique to generate the recombinant CTGF used in all the following experiments.

4.2 Results

4.2.1 Purification of His-tagged CTGF using nickel affinity chromatography

There are a number of published methods for the generation of recombinant human CTGF including: the use of transformed HeLa cells followed by purification with a combination of heparin and antibody affinity chromatography (Nakanishi *et al*. 2000); expression in a bacculoviral system followed by purification using heparin affinity chromatography (Frazier *et al*. 1996); expression in competent M15 [pREP4] *E.coli* followed by nickel affinity chromatography (Tong and Brigstock 2006); expression in transformed human mesengial

cells, followed by purification with either metal or heparin affinity chromatography (Wahab *et al*. 2001). Celia McLean attempted to purify CTGF protein using the different reported methods. None of the reported methods produced high yield of homogenous protein. After a series of optimisations, she established a highly successful method which involved stably transfecting human embryonic kidney 293 (HEK293) cells with His-tagged CTGF pCEP4 plasmid DNA (acquired from Prof. Roger Mason, Imperial College London) followed by nickel affinity chromatography. The polyhistidine (His) –tag is an amino acid motif consisting of six histidine residues. Metal ion affinity chromatography utilises the ability of histidine residues to interact with positively charged metal ions such as nickel.

Prior to investigating the function of CTGF, I first purified His-tagged human CTGF recombinant protein using Celia's method (Celia McLean PhD thesis, 2009). 150ml conditioned medium from stably transfected HEK293 cell culture was collected, filtered, supplemented with 20mM imidazole and passed through a 5 ml column containing charged nickel (Ni^{2+}) beads. After a series of washes, protein fractions were eluted by a linear gradient of imidazole (20-500mM). From the chromatographic trace (Figure 4.1A) there appeared to be a single absorbance peak corresponding to fraction 5 and 6. The increase in absorbance appeared after the peak was likely due to absorbance of imidazole at wavelength 280nm. Following elution, fractions were assayed using an anti-CTGF antibody (Figure 4.1B). In order to estimate losses following purification, eluted fractions, flow through (FT) and starting material (SM) were loaded in equivalent amounts (taking into consideration the difference in volumes). CTGF (37-39kDa) was detected within the SM and also the FT. This may indicate that the capacity of the column had been exceeded. However, detection of CTGF in fraction 5 and 6 showed bands with similar intensity as the band in the SM, suggesting good recovery. The source of the immunoreactive materials in the FT could also be endogenously expressed CTGF (not His-tagged) from the cultured HEK293 cells. In the silver stained gel, 20ul of each fraction (1ml) and 40µl of the SM (not equivalent) were loaded to determine protein purity. The amount of SM loaded was 20 times less than the

amount loaded for fractions (4 to10). A clear doublet band resolving at 38KDa was apparent in both fraction 5 and 6 with some weak protein staining seen at higher molecular weight in fraction 5 (Figure 4.1C).

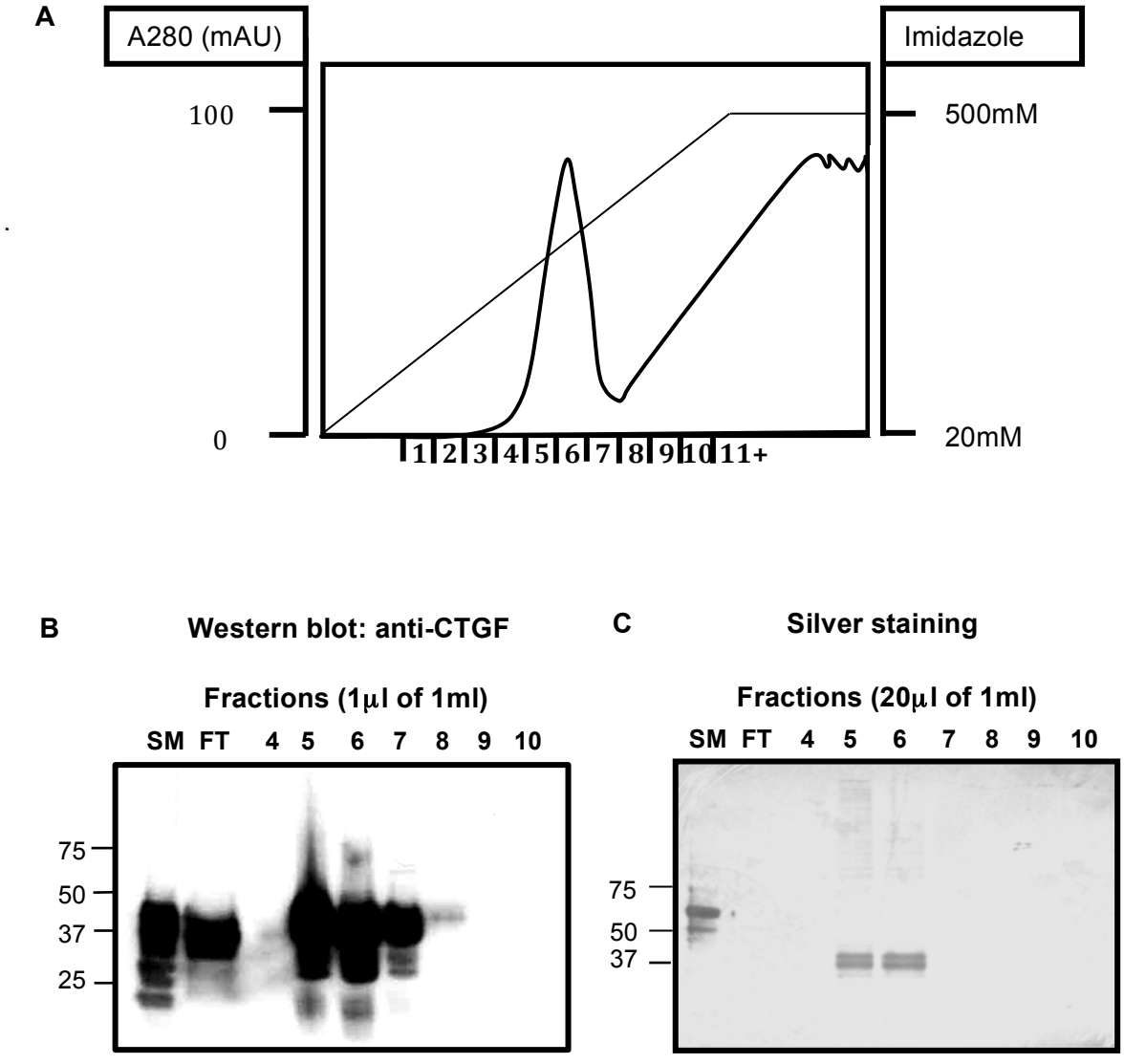


Figure 4.1 Purification of CTGF from conditioned medium of stably transfected HEK293 cells using a 5ml Ni^{2+} affinity column. Conditioned medium (150ml) from stably transfected HEK293 cells was applied to a 5ml nickel affinity column. Protein was eluted using a linear gradient ranging from 20-500mM imidazole. Figure A shows the chromatographic trace of purification for His-tagged recombinant CTGF (A). Samples were collected in 1ml fractions. Fractions (1μl for western blot or 20ul for silver staining of 1 ml) were run on 4-12% Bis-Tris gels and western blotted with an anti CTGF antibody (B) or silver stained (C).

4.2.2 Purified CTGF is retained upon dialysis and ultrafiltration

As indicated in Materials and Methods, purified CTGF fractions after elution contained approximately 150mM imidazole and 0.5M NaCl. Therefore, it was necessary to remove the contaminating salt before using it for experiments. The optimised de-salting method is to dialyse the protein solution in 1x PBS which is isotonic and non-toxic to cells. Fractions 5, 6 and 7 were pooled together and dialysed against 1x PBS across a 10kDa dialysing membrane. The resulting solution was concentrated using a 10kDa cut-off ultrafiltration spin column. The concentrated protein was loaded onto a gel with an equivalent amount of pre-dialysed material and protein was visualised by western blot (Figure 4.2). Eluted fractions from the nickel column were successfully retained upon dialysis and concentration. The activity of the purified CTGF was examined by looking at its effect on the gene expression of candidate matrix molecules in human chondrocytes. This will be addressed in section 4.2.4.

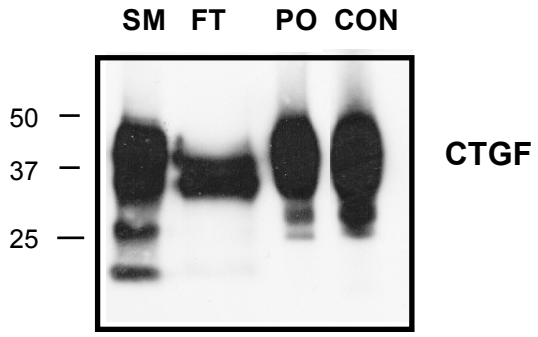


Figure 4.2 Purified CTGF is retained upon dialysis and spin concentration. Three fractions (3ml) positive for CTGF were pooled (PO) together, dialysed against 1x PBS across a 10kDa membrane for 18hr and concentrated 5 times using a 10kDa ultrafiltration column (CON). 1μl of pooled fraction, 0.2μl of concentrated solution and 50ul of starting material and flow through solution were loaded onto a gel and western blotted using an anti- CTGF antibody.

4.2.3 Quantification of purified CTGF following dialysis and ultrafiltration

The concentration of the purified protein was determined by comparing the intensities of CTGF bands to that of known concentrations of BSA (as standard protein) on a colloidal Coomassie Brilliant Blue G-250 stained gel. The concentration of protein in the preparation was first estimated by measuring the absorbance at 280nm using NanoDrop

spectrophotometer, where the measured concentration was 25µg/ml (25ng/µl). According to this, 200ng to 1000ng of BSA were loaded on a 4-12% gradient gel, along with 5µl, 10µl and 30µl of purified CTGF solution. The gel was stained with colloidal Brilliant Blue G-250 and the relative intensity (intensity per mm^2) of the bands was measured using Quantity One software (Figure 4.3A). Intensity and dose values of BSA bands were plotted to generate a standard curve, and the concentration of the purified CTGF was determined by coordinating to its intensity values (Figure 4.3B). As indicated, 30µl of purified material contained 600ng of CTGF, thus the concentration of CTGF was estimated at 20µg/ml. The final yield of protein obtained was 12µg from 150ml of conditioned medium.

A

	BSA					CTGF		
Dose/volume	200 (ng)	400 (ng)	600 (ng)	800 (ng)	1000 (ng)	5 (µl)	10 (µl)	30 (µl)
Intensity/mm^2 ($x10^6$)	2.31	3.67	4.91	5.95	6.46	1.73	2.35	4.64

B

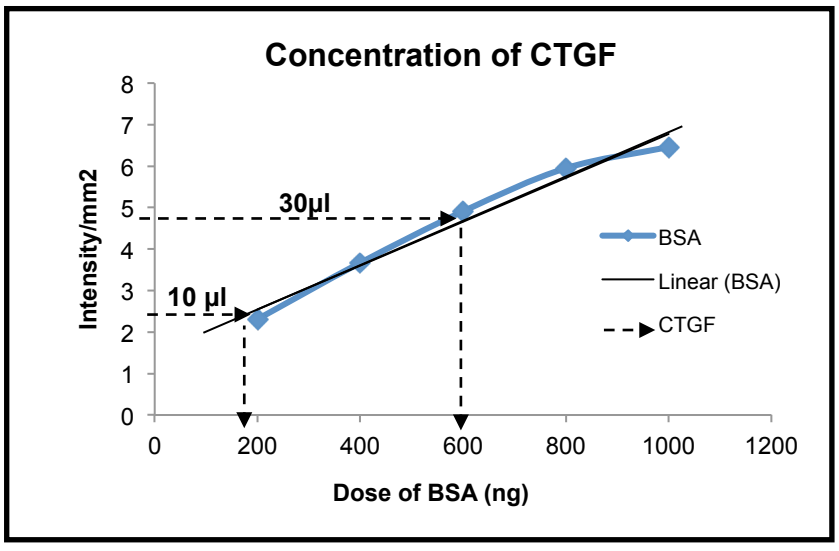


Figure 4.3 Quantification of purified CTGF. Purified material was dialysed and concentrated. Purified material (5,10 and 30µl) was loaded onto a 4-12% Bis-Tris gel alongside known concentrations of a standard protein (BSA). The gel was then coomassie stained and the intensity of the bands was measured using Quantity One (Bio-Rad ltd, USA) (A) and the values were plotted (B) to estimate the concentration of purified CTGF.

4.2.4 Purified CTGF did not affect the gene expression of candidate matrix genes in human chondrocytes

CTGF has been reported to regulate the expression of a number of matrix proteins including type I collagen, type II collagen, aggrecan and fibronectin, as well as molecules involved in matrix remodelling such as MMP1, MMP2, MMP3, TIMP1, ADAMTS4 and ADAMTS5 (Blaney Davidson *et al.* 2006; Chen *et al.* 2001; Fan *et al*. 2009; Frazier *et al*. 1996). Therefore it was decided to investigate whether stimulation of HACs with purified CTGF would reveal changes in the expression pattern of these molecules. HACs (passage1, P1) were stimulated with TGFβ1 (10ng/ml) as a positive control or purified recombinant CTGF at 100ng/ml for 24hr. This time point and concentrations were chosen on the basis of other publications (Tomita *et al*. 2013). RNA was then extracted and gene expression was measured relative to GAPDH using RT-PCR. TGFβ at 10ng/ml induced gene expression of type I collagen by 2 fold and perlecan by 2.5 fold while no significant response was seen with CTGF stimulation (Figure 4.4). Similar experiments looking at other matrix proteins such as type II collagen, fibronectin and aggrecan were also carried out in HACs (one experiment for each gene, results are not shown). Again no significant regulation was observed in response to CTGF. The failure to demonstrate the effect of CTGF in regulating candidate genes could have been due to a lack of biological activity in the expressed protein or due to high constitutive expression of CTGF in human chondrocytes (Celia McLean had shown previously that these cells make CTGF constitutively). Therefore it was decided to stimulate cells in which endogenous CTGF had been silenced using siRNA.

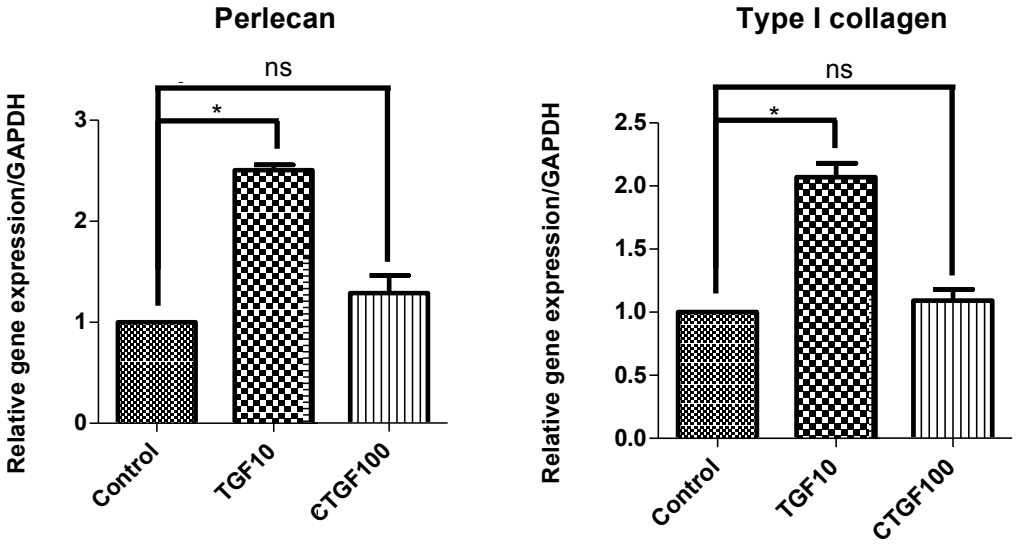


Figure 4.4 Purified CTGF did not affect gene expression of type I collagen and perlecan in HACs. HACs were starved for 18hr in serum free DMEM and stimulated with TGFβ1 (10ng/ml) or recombinant CTGF (100ng/ml) for 24hr. RNA was extracted and purified, relative gene expression of perlecan and type I collagen to GAPDH were measured using RT-PCR. Left panel shows the expression of perlecan and the right panel figure shows expression of type I collagen. Each bar represents the mean of three experiments with error bars representing standard error of the mean (n=3). *p<0.05, ns= not significant.

4.2.5 CTGF knockdown using siRNA

HACs were transfected with either of the two CTGF siRNA oligos (siCTGF A and B) or a scrambled siRNA (siSCR) as control. Following 48hr transfection, RNA was extracted from the cells and gene expression for CTGF was measured relative to GAPDH. Both CTGF siRNA oligos effectively reduced the relative RNA level of CTGF, compared to the siSCR transfected cells (90% for oligo A, 80% for oligo B). siSCR had a small non-specific effect on the gene expression of CTGF, compared to untransfected cells (Figure 4.5A).

Protein level of CTGF upon knockdown using siCTGF oligo A and B was also determined. First, the assay was optimised to detect secreted CTGF. TGFβ1 is known to induce CTGF in chondrocytes and fibroblasts (Grotendorst *et al.* 1996; Nakanishi *et al.* 1997b). Adding heparin into the medium blocks LRP-mediated uptake of CTGF allowing it to accumulate in the medium (Celia McLean PhD thesis, 2009; Troeberg *et al.* 2008). Therefore, cells were treated with TGFβ1 in the presence or absence of heparin, following siRNA knockdown of

CTGF. After 24hr stimulation, both cultured medium and cell lysates were acetone precipitated and run on a 4-12% SDS-PAGE to detect CTGF (Figure 4.5B). In the medium, CTGF was only detected when cells were stimulated with TGFβ1 in the presence of heparin, while in the lysates CTGF protein was expressed constitutively and its level increased slightly upon TGFβ stimulation (Figure 4.5B). To examine the efficiency of CTGF knockdown at protein level, stimulation with TGFβ1 in the presence or absence of heparin was carried out in siRNA transfected cells. These cells showed significant reduction at the protein level of CTGF for both siCTGF oligo A and B, compared to siSCR oligo transfected cells (Figure 4.5C).

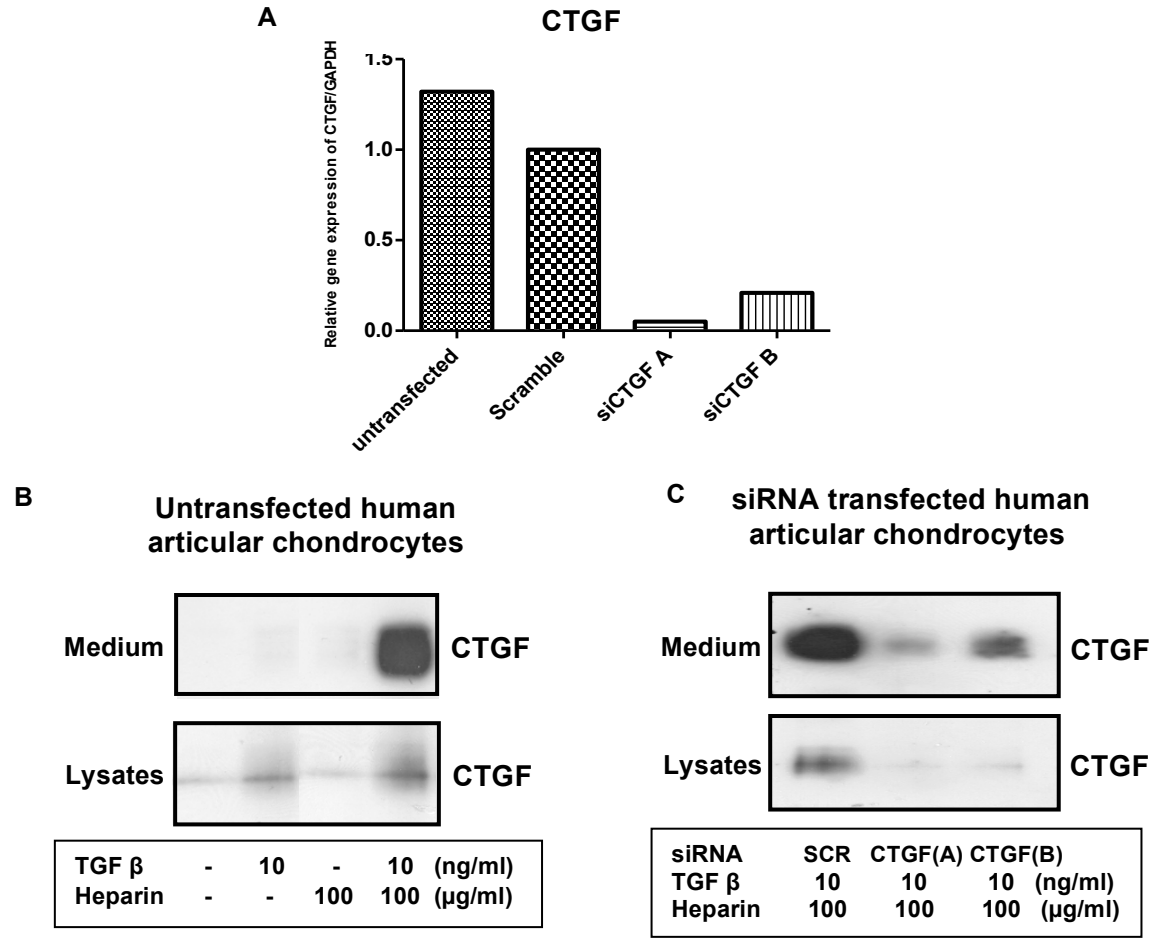


Figure 4.5 CTGF was effectively silenced by siRNA oligos A and B at both gene and protein levels. 3 x 10^5 HAC was plated for 18hr and transfected with 15nM of siCTGF oligos (A or B) or siSCR (SCR) with lipofectamine for 48hr. RNA was extracted from the cells and gene expression of CTGF was measured relative to GAPDH (A). For protein detection, untransfected (B) or transfected (C) HACs were stimulated with TGFβ1 (10ng/ml) with or without heparin (100ug/ml) for further 24hr. The conditioned medium and cell lysates were collected for acetone precipitation and run on 4-12% Bis-Tris gels. CTGF was detected on western blot using an anti-CTGF antibody.

4.2.6 CTGF knockdown did not affect the gene expression pattern of ECM molecules in human chondrocytes

Having validated CTGF knockdown by siRNA, it was now possible to test the effect of purified recombinant CTGF on the expression of candidate ECM molecules in human chondrocytes in the absence of constitutive levels of CTGF. It was also possible to examine whether the effects of TGFβ on chondrocytes were occurring, as some have proposed, through induction of CTGF. HACs transfected with either scrambled or CTGF siRNA were stimulated with TGFβ1 (10ng/ml) or CTGF (100ng/ml) for 24hr. Gene expression (CTGF, perlecan, type I collagen and fibronectin) was measured relative to GAPDH (Figure 4.6). CTGF RNA was reduced by 82% (average of four experiments) by siCTGF oligo A, compared to siSCR, confirming good knockdown. Despite this, the expression of perlecan, type I collagen and fibronectin remained unchanged between the two groups (siSCR and siCTGF), indicating firstly that constitutively expressed CTGF is not significantly contributing to expression of these genes and secondly that the induction of the genes by TGFβ was not dependent upon CTGF mRNA. In these experiments (at 24hr stimulation) CTGF does not stimulate the synthesis of perlecan, fibronectin nor type I collagen.

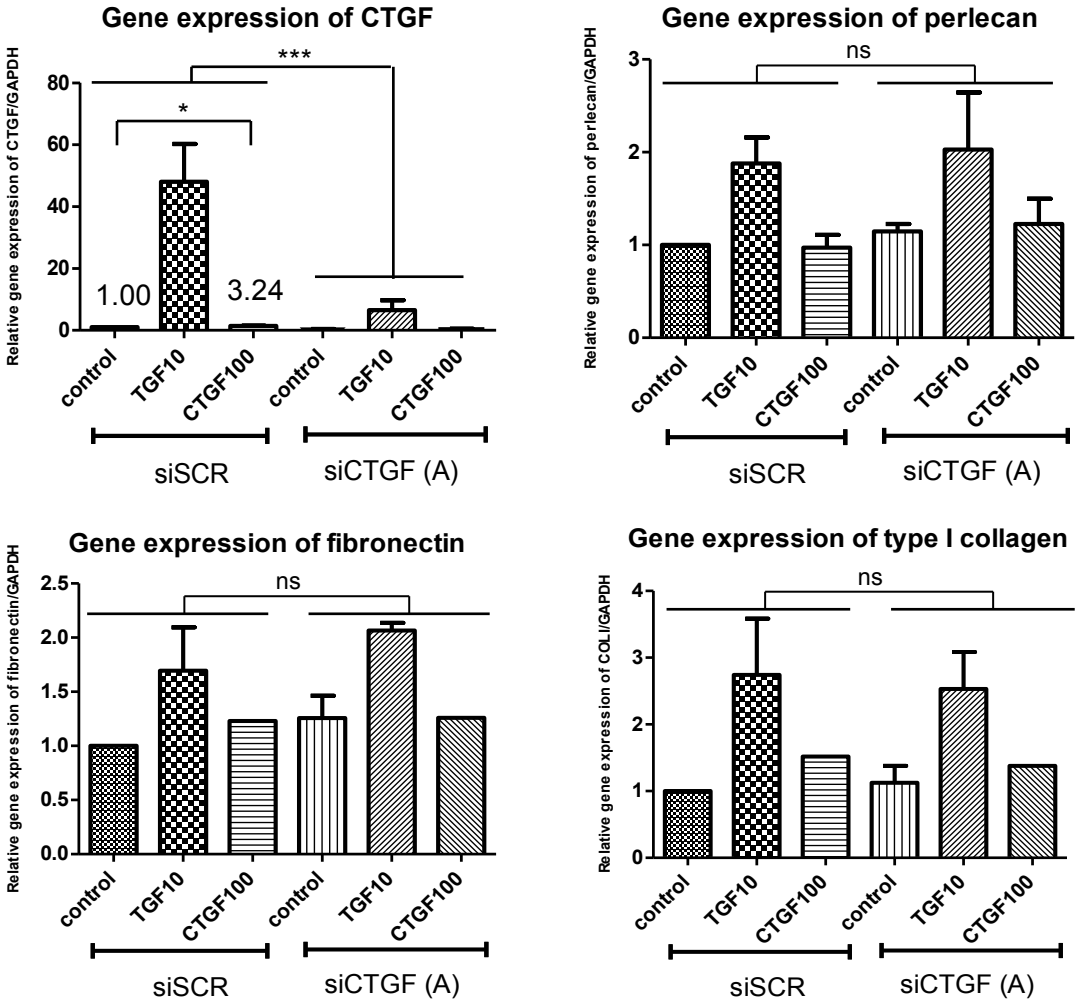


Figure 4.6 CTGF knockdown did not affect the gene expression of candidate ECM molecules in human chondrocytes. 3 x 10^5 HACs were plated for 18hr and transfected with 15nM of siRNA oligos (A for siCTGF and siSCR as control) with lipofectamine for 48hr. Transfected cells were stimulated with either TGFβ1 (10ng/ml) or CTGF (100ng/ml) for 24hr. RNA was extracted from the cells and gene expression of CTGF, perlecan, type I collagen and fibronectin was measured relative to GAPDH. Bars represent the mean of three separate experiments, with error bars representing the standard error of the mean. Statistical test suggested no significant difference between the two groups (siSCR and siCTGF) (ns: not significant; *** $p<0.001$,* $p<0.05$).

4.2.7 CTGF is induced strongly by activin A and recombinant CTGF in both HACs and NHDFs

As TGFβ induces CTGF in human chondrocytes, we examined whether activin A, another TGF β family member, also induced CTGF. Activin A was especially of interest to us because it was one of the proteins found overexpressed from human OA cartilage (Alexander *et al*. 2007) and it is a highly regulated early gene following induction of OA in mouse (Burleigh *et al.* 2012). Moreover, it is induced upon cartilage injury *in vitro* and *in vivo* in an FGF2-dependent manner (FGF2 is stored and released from the PCM upon cartilage injury) (Chong *et al*. 2013). Activin A and CTGF have been both found overexpressed in osteoarthritis (Hermanson *et al*. 2002) while Activin A's role in articular cartilage is unclear.

An activin A dose response was first carried out in order to determine the optimal concentration of activin A induced gene expression of CTGF. Normal human dermal fibroblasts (NHDFs) were used during optimisation as they are easily obtained and maintained in culture and it is known that TGFβ induces CTGF in this cell type. NHDFs were stimulated with either TGFβ1 (10ng/ml) as a positive control or increasing concentrations of activin A (2ng/ml to 200ng/ml) for 24hr. RNA was then extracted and gene expression of CTGF was measured relative to GAPDH using RT-PCR. As the concentration of activin A increased, the relative gene expression of CTGF also increased, peaking at 100ng/ml (Figure 4.7A). At this dose the fold induction was similar to that seen with TGFβ. Protein levels of CTGF were also determined in response to activin A stimulation in the presence of heparin. Following stimulation, conditioned medium was collected and loaded onto 4-12% gradient gels to detect CTGF by western blotting. Strong induction of CTGF by TGFβ1 and activin A can be observed in both cultured HACs and NHDFs in the presence of heparin (Figure 4.7B). No obvious accumulation of CTGF was seen with heparin alone, suggesting that addition of heparin alone did not cause the induction of CTGF in these cells. In a separate experiment, when cells were stimulated with CTGF (100ng/ml) we observed that

RNA of CTGF was also induced about 5 fold in HACs and 4 fold in NHDFs (Figure 4.7C and D), suggesting that CTGF induces itself.

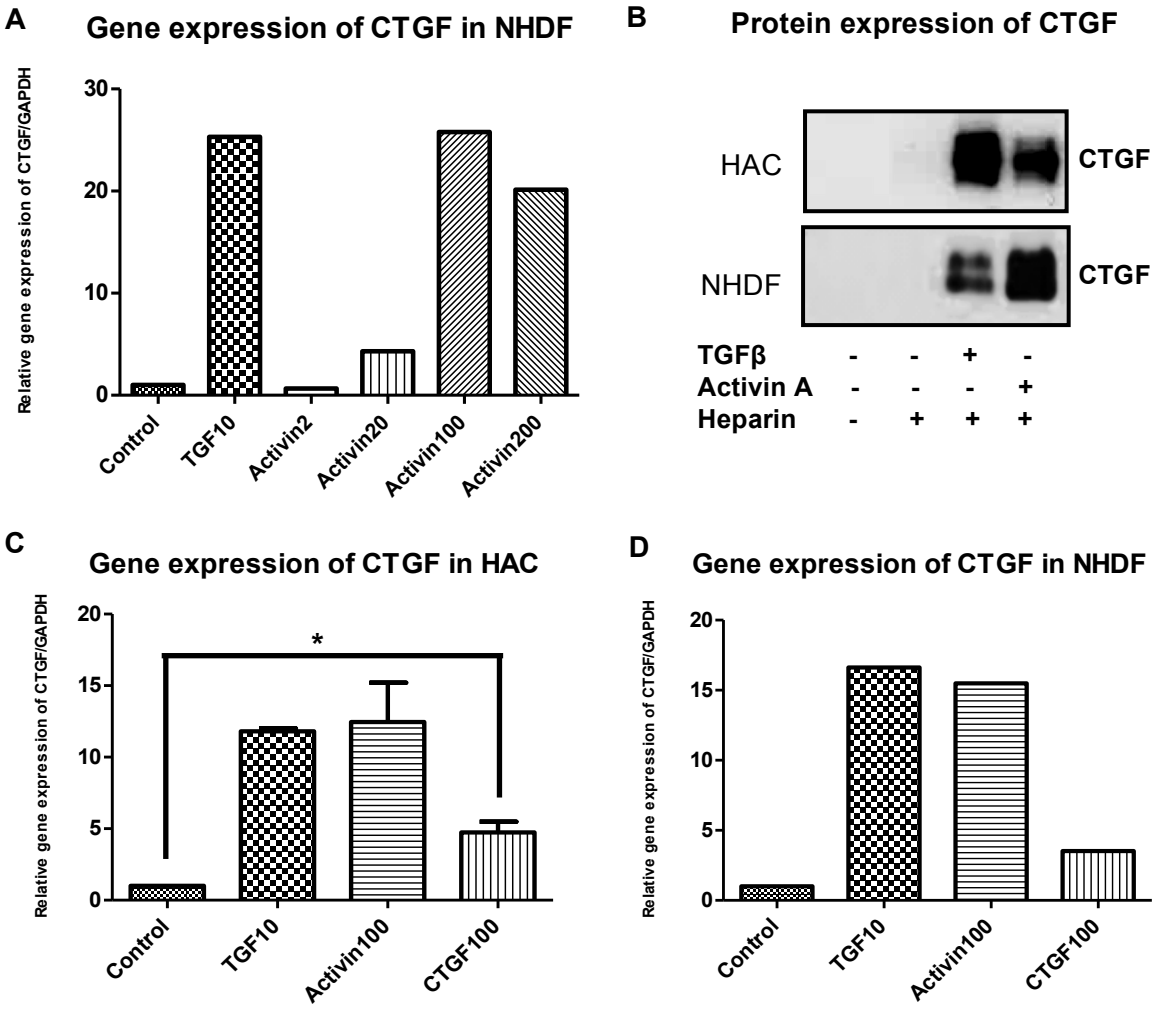


Figure 4.7 Induction of CTGF in P1 HACs and NHDFs. NHDF were stimulated with TGF β (10ng/ml) or activin A (2 to 200ng/ml) for 24hr and the relative gene expression of CTGF was measured (A). For protein, conditioned media were collected after treating the cells with TGFβ (10ng/ml) or activin A (100ng/ml) in the presence or absence of heparin (10mg/ml) for 24hr and western blotted to detect CTGF (B). HACs (C) or NHDFs (D) were stimulated with TGFβ1 (10ng/ml), activin or CTGF (100ng/ml) for 24hr and gene expression of CTGF was measured (A: n=1, B: one representative results of three experiments, n=3, C: n=3 *p<0.05 and D: n=1).

These experiments were important as they demonstrated a biological activity for our recombinant CTGF (induction of CTGF) and provided a possible biological link between CTGF and activin A (activin A strongly induced CTGF).

4.2.8 Optimisation for microarray study

So far CTGF was the only target gene shown to be induced by CTGF and activin A in human chondrocytes. Therefore it was decided to carry out a microarray study to identify novel target genes prior to studying their functions. Prior to microarray, the optimal concentration and time course for stimulation was determined. HACs were stimulated with increasing concentrations of recombinant CTGF (40ng/ml to 200ng/ml) or activin A (10ng/ml and 100ng/ml) for 24hr and gene expression of CTGF was measured relative to GAPDH using RT-PCR (Figure 4.8A). A dose response curve was demonstrated for each ligand and we decided to use 100ng/ml for both ligands. NHDFs were used for the time course experiment. NHDFs were stimulated with CTGF and activin A at 100ng/ml for different time periods ranging from 4 to 48hr. Relative gene expression of CTGF was measured (Figure 4.8B). CTGF and activin A stimulations gave different temporal responses: the gene response to CTGF stimulation was early (peaking at 8hr), while activin A stimulation was late (optimal at 48hr). These results explain the low fold induction of CTGF with recombinant CTGF stimulation in experiment 4.2.7 (at 24hr).

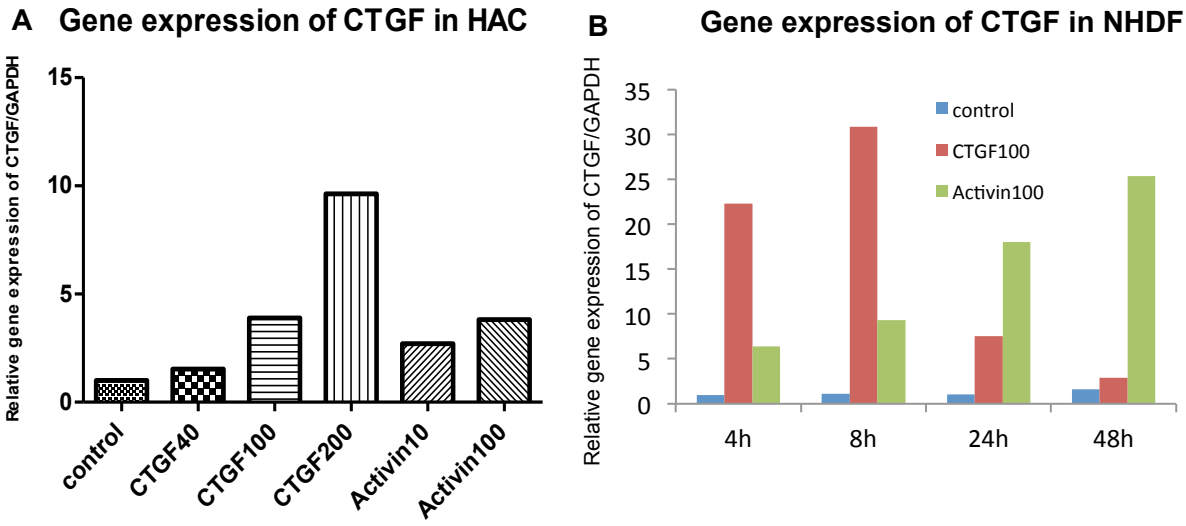
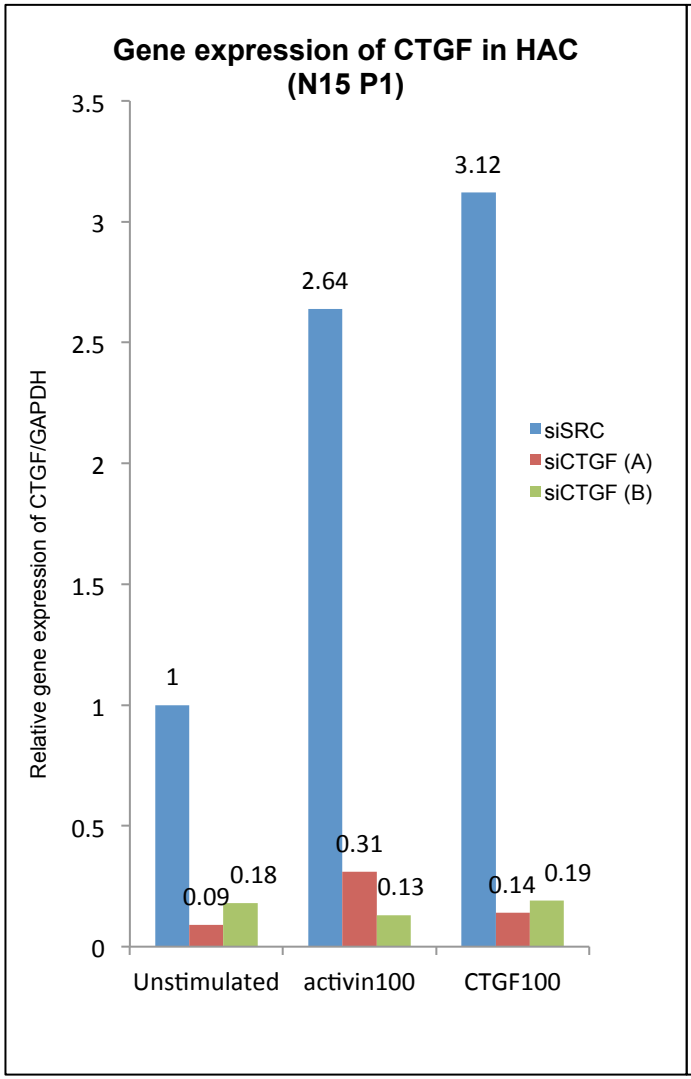


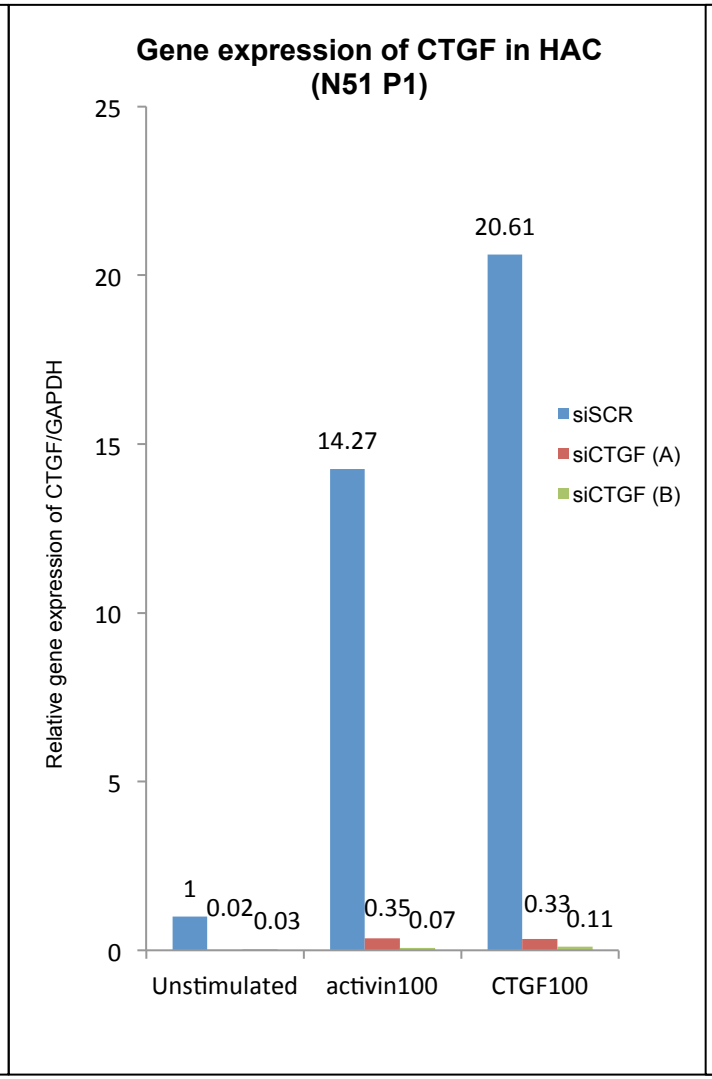
Figure 4.8 Optimisation of CTGF gene expression in response to CTGF and activin A stimulations. HACs were stimulated with increasing concentrations of purified CTGF (40ng/ml to 200ng/ml) or activin A (10ng/ml and 100ng/ml) for 24 hours and gene expression of CTGF was measured relative to GAPDH using RT-PCR (A). NHDFs were stimulated with CTGF and activin A at 100ng/ml for increasing time periods ranging from 4hr to 48hr. Relative gene expression of CTGF was measured (B) (n=1).

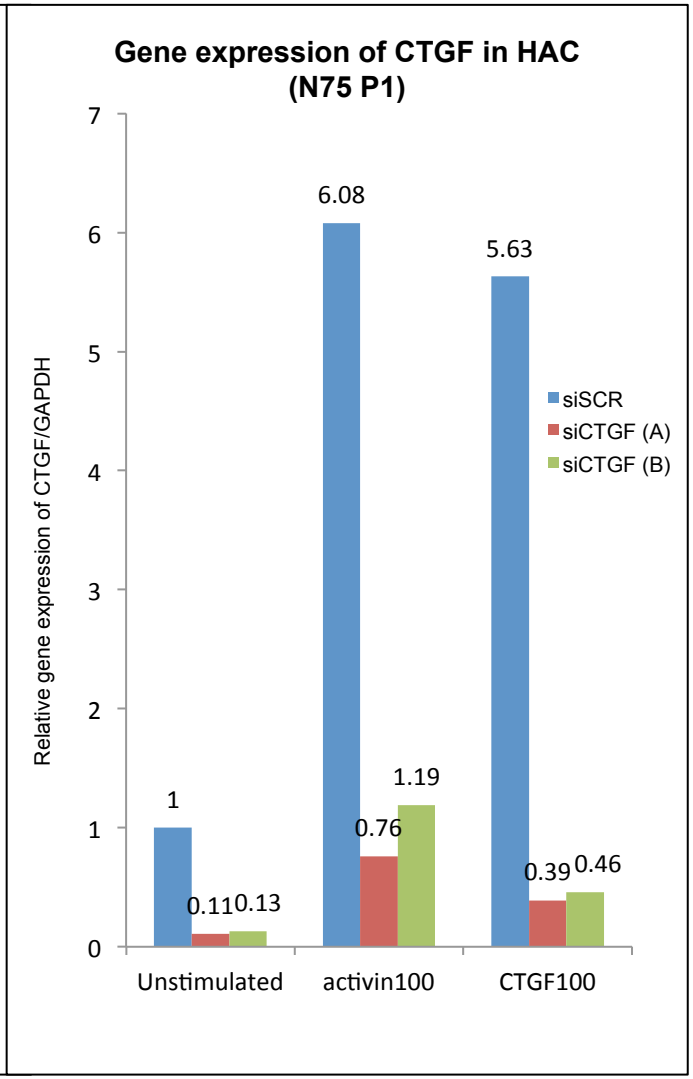
4.2.9 Design and preparation for microarray

In order to accommodate biological variation, HACs from three donors (N15, N51 and N75, all P1) were used. HACs were either transfected with siSCR or siCTGF oligo A or B and stimulated with the recombinant CTGF or activin A at 100ng/ml for 8hr. CTGF and activin A induced gene expression of CTGF peaked at 8hr and 48hr respectively (Figure 4.8B). For the microarray study, the earlier time point (8hr) was chosen because the regulation of immediate responsible genes would likely be captured after short time stimulation. RNA prepared from these experiments was first measured to determine the efficiency of the knockdown by RT-PCR. All three experiments showed high knockdown efficiency: 95% for oligo A and 82% for oligo B in N15 HAC, 98% for A and 97% for B in N51 HAC and 89% for A and 87% for B in N75 HAC (Figure 4.9A). Stimulation with the recombinant CTGF or activin A induced CTGF RNA in all three experiments but by different folds, which appear to be due to different basal levels of CTGF and activin A in these cells. Indeed the measurement of CTGF RNA in the unstimulated cells confirmed different basal levels of CTGF RNA in the HACs of different donors (Figure 4.9B). The result showed that the RNA level of CTGF in the unstimulated N75 HAC was about 4 times that of N51 HAC while N15 was about 4.7 times that of N51. This probably explains why we observed less induction in N75 (5 and 6 fold) and N15 HAC (2 and 3 fold) compared with N51 (14 and 20 fold) (Figure 4.9A).

A


Gene expression of CTGF in HAC (N15 P1)
Relative gene expression of CTGF/GAPDH
3.5
3
2.5
2
1.5
1
0.5
0
1
0.09
0.18
2.64
0.31
0.13
3.12
0.14
0.19
Unstimulated
activin100
CTGF100
siSRC
siCTGF (A)
siCTGF (B)


Gene expression of CTGF in HAC (N51 P1)
Relative gene expression of CTGF/GAPDH
25
20
15
10
5
0
1
0.02
0.03
14.27
0.35
0.07
20.61
0.33
0.11
Unstimulated
activin100
CTGF100
siSCR
siCTGF (A)
siCTGF (B)


Gene expression of CTGF in HAC (N75 P1)
Relative gene expression of CTGF/GAPDH
7
6
5
4
3
2
1
0
1
0.11
0.13
6.08
0.76
1.19
5.63
0.39
0.46
Unstimulated
activin100
CTGF100
siSCR
siCTGF (A)
siCTGF (B)

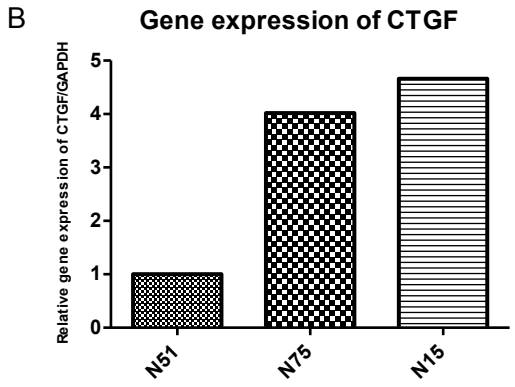


Figure 4.9 HACs from three donors showed high knockdown efficiency and different CTGF induction. HACs from three donors (N15, 51 and 75) were transfected with siSCR, siCTGF oligo A or oligo B, and stimulated with or without purified CTGF and activin A (100ng/ml). RNA was extracted and measured for the gene expression of CTGF relative to GAPDH (A). Basal level of CTGF (normalised to each patient specific GAPDH) was measured in the HACs of the three donors (B) (n=1).

4.2.10 Microarray analysis

Prior to sending the RNA samples for microarray analysis, the quality and quantity of the total RNA was examined. All RNA samples used (Table 6) satisfied the selection criteria detailed in 2.8.4. The microarray study was carried out by the Cambridge genomic centre at University of Cambridge. The hybridised chips were scanned with the BeadArray Reader (Illumina, San Diego, CA) to detect Cy3 fluorescence emission. The Genome Studio Data Analysis software (Illumina, San Diego, CA) was used to extract the fluorescence intensity measurements for each probe and to measure the quality of the hybridisations. Illumina BeadChips have internal control features to measure data quality control. The extracted features were processed in the R environment using the bioconductor package software lumi (Gentleman *et al*. 2004; Du *et al.* 2008). Selection of detected probes is performed using the detection p-value ($p < 0.01$) provided by Illumina. It represents the confidence that a given transcript is expressed above the background defined by the negative control probe. Probes need to be successfully detected in at least one sample to pass selection. 21886 probes were successfully detected. Data were log transformed and variance stabilised prior to

normalisation by quantile normalisation. Quality control was also performed using the arrayQualityMetrics R package in lumi. No samples failed or are outliers to their replicates. We analysed the data using the Multiexperiment Viewer (MEV v.4.6) software (Saeed *et al.* 2003; Saeed *et al.* 2006). The non-parametric method significance analysis of microarrays (SAM) was used to identify genes that were differentially regulated among the treatment variables (Tusher *et al.* 2001; Chu *et al.* 2002). SAM computes a statistic for each probe in the dataset that measures the strength of the relationship between probe intensity and the response variable. It uses repeated permutations of the data to determine if the expression of any probe is significantly related to the response. The cut-off for significance is determined by tuning a parameter delta, based on a 0 false positive rate. 50 genes were found to be differentially regulated among the groups. These 50 genes were hierarchically clustered (Eisen *et al.,* 1998) into a tree whose branch lengths reflect the degree of similarity in the expression change amongst the genes measured based on Pearson correlation (Figure 4.10). By adjusting the gene tree lengths we got 5 clusters. Cluster 1 contained BMPR2, which was up-regulated by CTGF and activin A compared to unstimulated samples and down-regulated by both siCTGF oligo A and oligo B compared to siSCR. Cluster 2 contained 31 genes that were down-regulated by the siCTGF oligo A only. Cluster 3 contained PMEPA1 and LTBP2 which were up-regulated by CTGF and activin A, but showed no change upon knockdown of CTGF. Cluster 4, similar to cluster 1, contained CTGF only, and was up-regulated by CTGF and activin A and down-regulated by both siCTGF oligo A and B. Cluster 5 contained 13 genes that were only down-regulated by siCTGF oligo B. BMPR2, PMEPA1, LTBP2 and CTGF were the only genes found to be differentially regulated by CTGF or activin A. The remaining genes were either down-regulated by siCTGF oligo A or siCTGF oligo B alone, which suggests non-specific off target effects of the two siCTGF oligos.

Donor's code N (donor's age)	Sample No.	Samples name	RIN NO.	siRNA	Stimulation
N51	1	N51_CTRL	9.7	SCR	none
	2	N51_CTGF	9.1	SCR	CTGF100
	3	N51_Activin	8.7	SCR	Activin100
	4	N51_CTRL_siA	8.8	CTGF (A)	none
	5	N51_CTGF_siA	8.9	CTGF (A)	CTGF100
	6	N51_Activin_siA	9	CTGF (A)	Activin100
	7	N51_CTRL_siB	9.6	CTGF (B)	none
	8	N51_CTGF_siB	9	CTGF (B)	CTGF100
N75	9	N75_CTRL	9.3	SCR	none
	10	N75_CTGF	9.7	SCR	CTGF100
	11	N75_Activin	9.2	SCR	Activin100
	12	N75_CTRL_siA	9.1	CTGF (A)	none
	13	N75_CTGF_siA	9	CTGF (A)	CTGF100
	14	N75_Activin_siA	9.6	CTGF (A)	Activin100
	15	N75_CTRL_siB	9.7	CTGF (B)	none
	16	N75_CTGF_siB	9.9	CTGF (B)	CTGF100
N15	17	N15_CTRL	10	SCR	none
	18	N15_CTGF	9.5	SCR	CTGF100
	19	N15_Activin	8.7	SCR	Activin100
	20	N15_CTRL_siA	8.3	CTGF (A)	none
	21	N15_CTGF_siA	8.7	CTGF (A)	CTGF100
	22	N15_Activin_siA	9.2	CTGF (A)	Activin100
	23	N15_CTRL_siB	8.6	CTGF (B)	none
	24	N15_CTGF_siB	9	CTGF (B)	CTGF100

Table 6 Summary of the RNA samples used for microarray study. RNA was extracted from HACs isolated from cartilage of three donors: N15, 51 and 75, aged 15, 51 and 75 respectively, either transfected with siSCR, siCTGF oligo A or B and stimulated with or without CTGF and activin A at 100ng/ml. RIN number of each RNA sample was measured prior to microarray analysis.

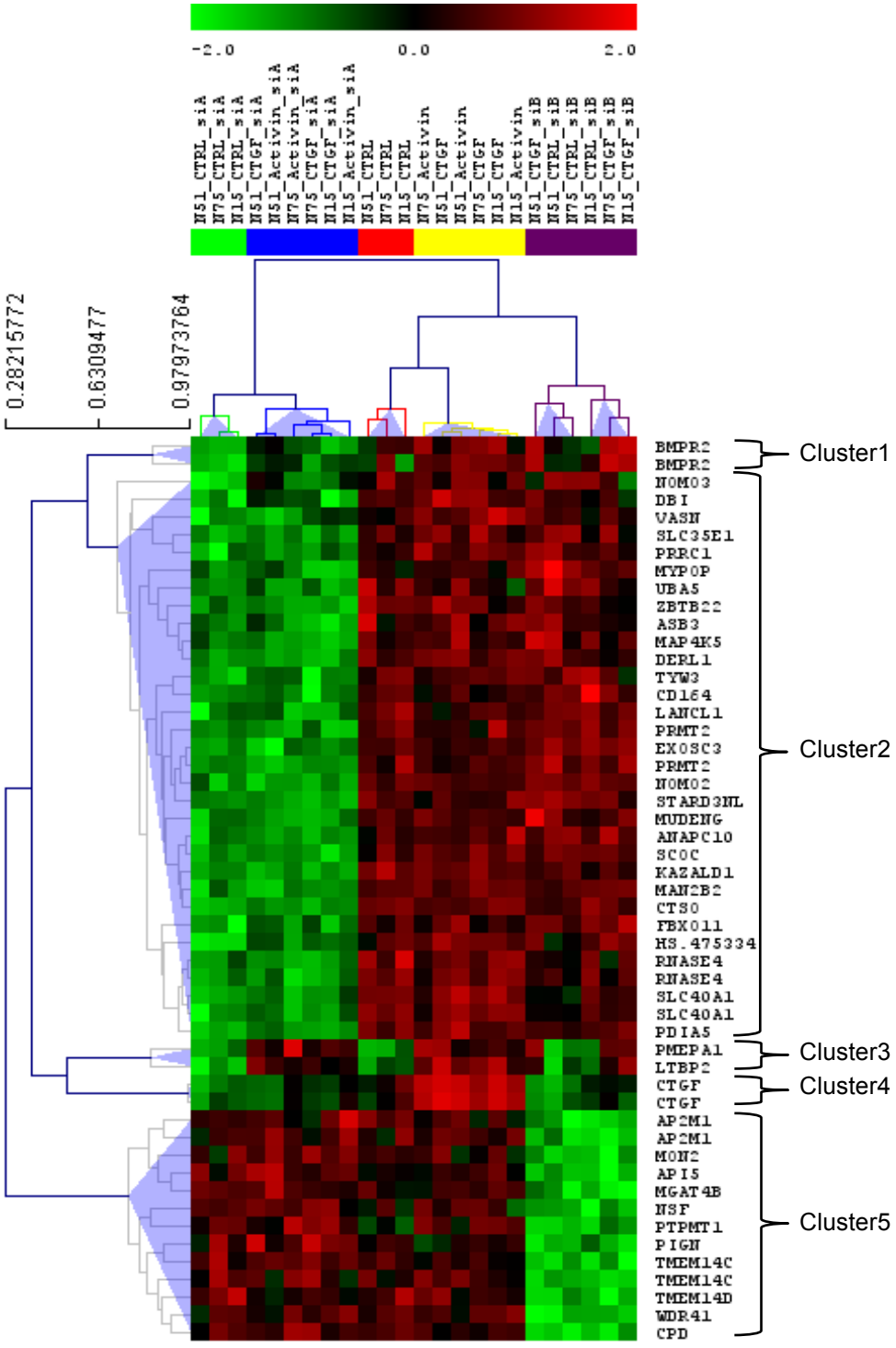


Figure 4.10 Heat map of the genes regulated from microarray analysis. 50 genes were found significantly regulated in the microarray study, whose signals were presented in heat map and orders were arranged in 5 clusters without supervision, according the degree of similarity between the gene responses. Samples were also arranged in 5 groups (colour boxes under samples' names) without supervision according the similarity between two treatments: green = unstimulated siA transfected group, blue= CTGF or activin A stimulated siA transfected group, red= unstimulated siSCR group, yellow= CTGF or activin A stimulated siSCR group and purple = unstimulated or CTGF stimulated siB transfected group. Gene expression levels are presented in heat map on a log scale (heat map bar on the top): red (up-regulation), green (down-regulation) black (no change).

4.2.11 Validation of microarray results using RT-PCR

The four genes (CTGF, PMEPA1, LTBP2 and BMPR2) were identified from the microarray study. Each was significantly regulated by CTGF and activin A. Before identifying their function, expression of these four genes was first validated in human chondrocytes and fibroblasts using RT-PCR. HACs or NHDFs were treated with CTGF or activin A at 100ng/ml for 8hr and the expression of the four genes were measured relative to GAPDH (Figure 4.11). In NHDFs, all four genes were regulated in response to activin A and CTGF whereas in HACs only PMEPA1 showed strong regulation. This may be due to the high basal level of CTGF and activin A in chondrocytes or the gene response to these stimulators may be optimal at different times. This will be addressed in the next section.

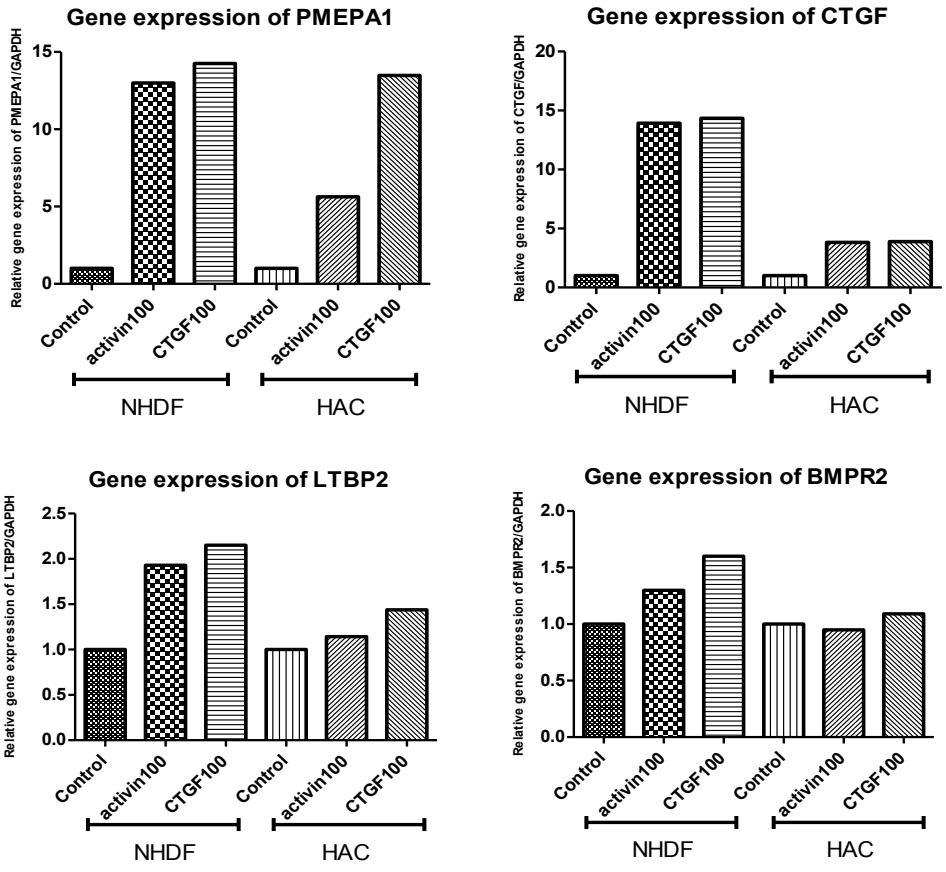
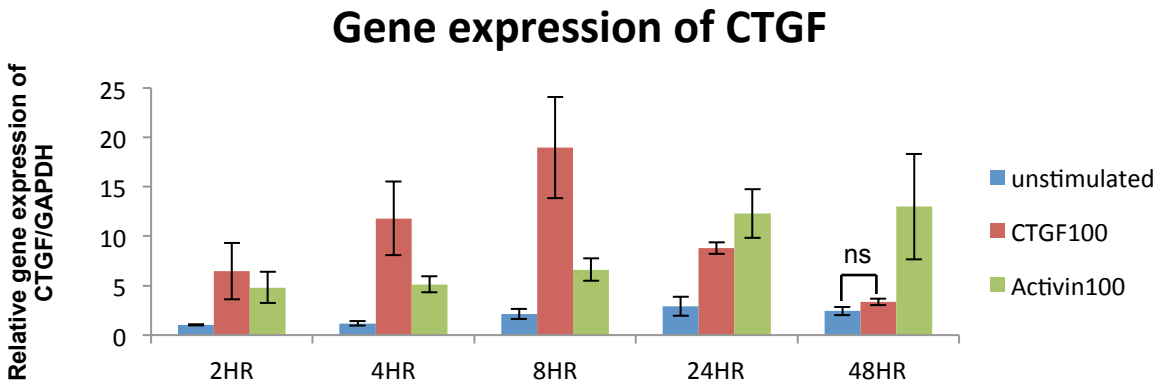


Figure 4.11 Validation of the four identified genes in HACs and NHDFs using RT-PCR. HNDFs or HACs were stimulated with CTGF or activin A at 100ng/ml for 8hr. Gene expression of CTGF, BMPR2, LTBP2 and PMEPA1 was measured relative to GAPDH. Bars represent fold change in gene expression (n=1).

4.2.12 Time dependent induction of the four identified genes by CTGF and activin A

NHDFs were stimulated with either CTGF or activin A (100ng/ml) for increasing time points ranging from 2hr to 48hr. Gene expression for CTGF, PMEPA1, LTBP2 and BMPR2 were measured relative to GAPDH. All four genes responded to CTGF stimulation earlier than to activin A (Figure 4.12). CTGF induced CTGF expression peaked at 8hr and declines afterwards while activin A induced CTGF expression became plateaued at 24hr. PMEPA1 again showed strongest induction in response to stimulation. It was induced by CTGF by 12 fold after 2hr stimulation, peaked at 23 fold after 4hr and declined beyond 8hr. Upon activin A stimulation, the PMEPA1 induction started at 2hr (9 fold), peaked at 4hr (17 fold) and declined from 8hr (8 fold). At 24hr, the induction of PMEPA1 by CTGF has returned to basal level, whereas for activin A, the expression was maintained up to 48hr. The induction of LTBP2 increased as the time of stimulation increased; it peaked at 24hr for both stimuli but had declined by 48hr for CTGF, while persisting for activin A. BMPR2 responded the least to the stimuli, was induced to the maximal 2.3 fold at 8hr by CTGF and 1.7 fold at 24hr by activin A.



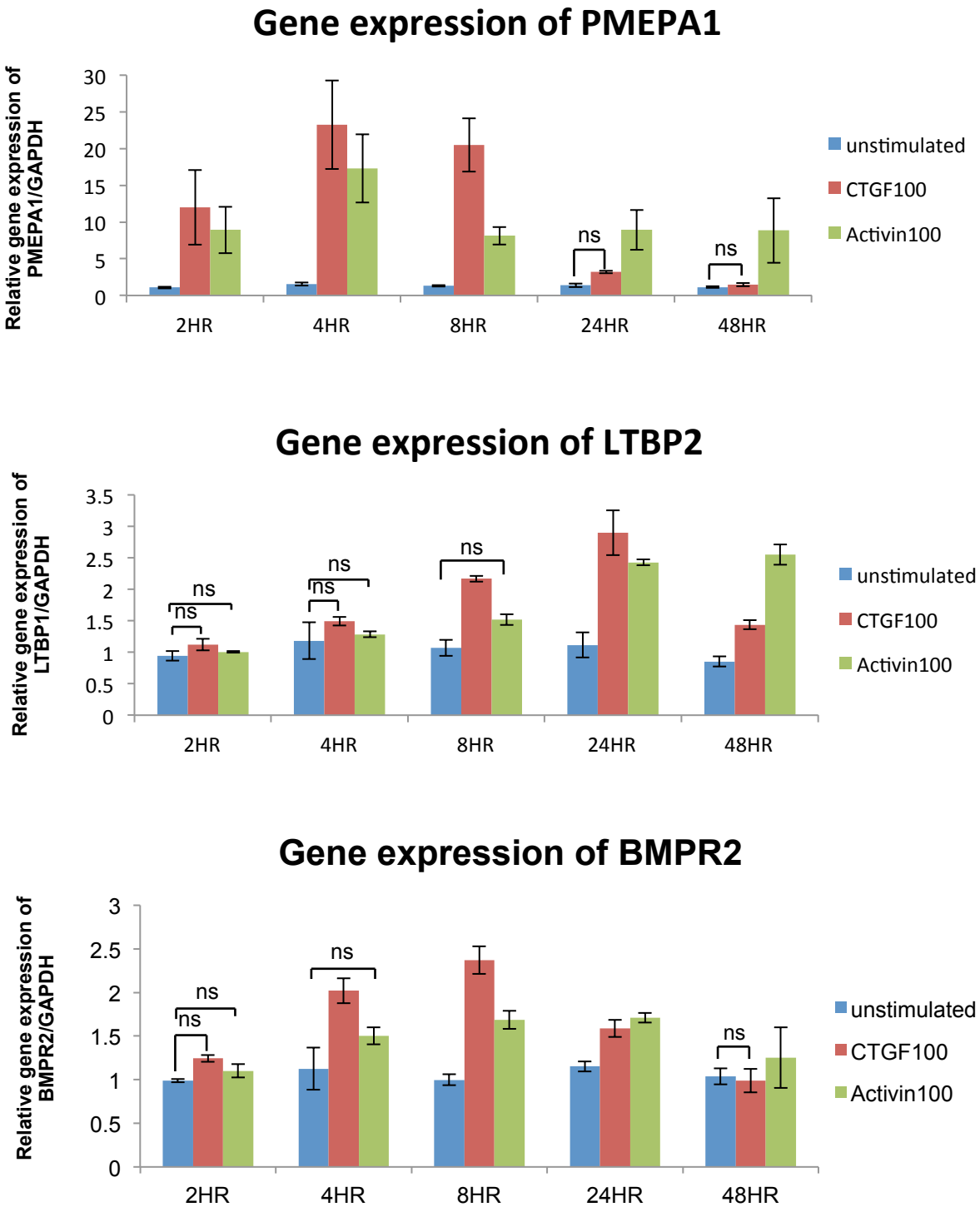


Figure 4.12 Validation of the four identified genes in time course experiments. NHDFs were stimulated with CTGF (100ng/ml) or activin A (100ng/ml) for increasing times from 2hr to 48hr. Gene expression of BMPR2, LTBP2, PMEPA1 and CTGF were determined using RT-PCR. Bars represent mean gene expression levels with error bars representing the standard error of the mean (n=4). All differences are significant compared with time-matched controls apart from those indicated (ns).

4.2.13 Activin A induced gene expression can be inhibited by cycloheximide

The late and persistent responses by activin A indicate that induction of gene responses could be mediated by an intermediate molecule. To test this, the protein synthesis inhibitor cycloheximide (CHX) was used. NHDFs were stimulated either with or without activin A in the presence or absence of CHX for 24hr. RNA was extracted for RT-PCR analysis. CHX significantly abolished the late gene expression (at 24hr) of CTGF, PMEPA1 and LTBP2 by activin A (Figure 4.13A). Activin A induced BMPR2 expression at 24hr in the absence of CHX was very modest and therefore non-interpretable. CTGF protein level was also measured in the presence or absence of CHX to examine the inhibition efficiency of CHX on protein synthesis. Under this condition CTGF protein induced by activin A was reduced by approximately 80% upon addition of CHX (Figure 4.13B). These results suggest that the late induction of genes by activin A may be indirect through a secondary mediator. Alternatively it could indicate that the CHX treatment used in the experiment was toxic to the cells. To test this, the CHX experiment was performed with TGFβ stimulation as a control. When CHX was added to TGFβ stimulated cells no reduction in gene expression of CTGF was demonstrated (at 24hr), indicating that the reduction in activin A induced gene expression after 24hr was not due to CHX toxicity to the cells (Figure 4.13C).

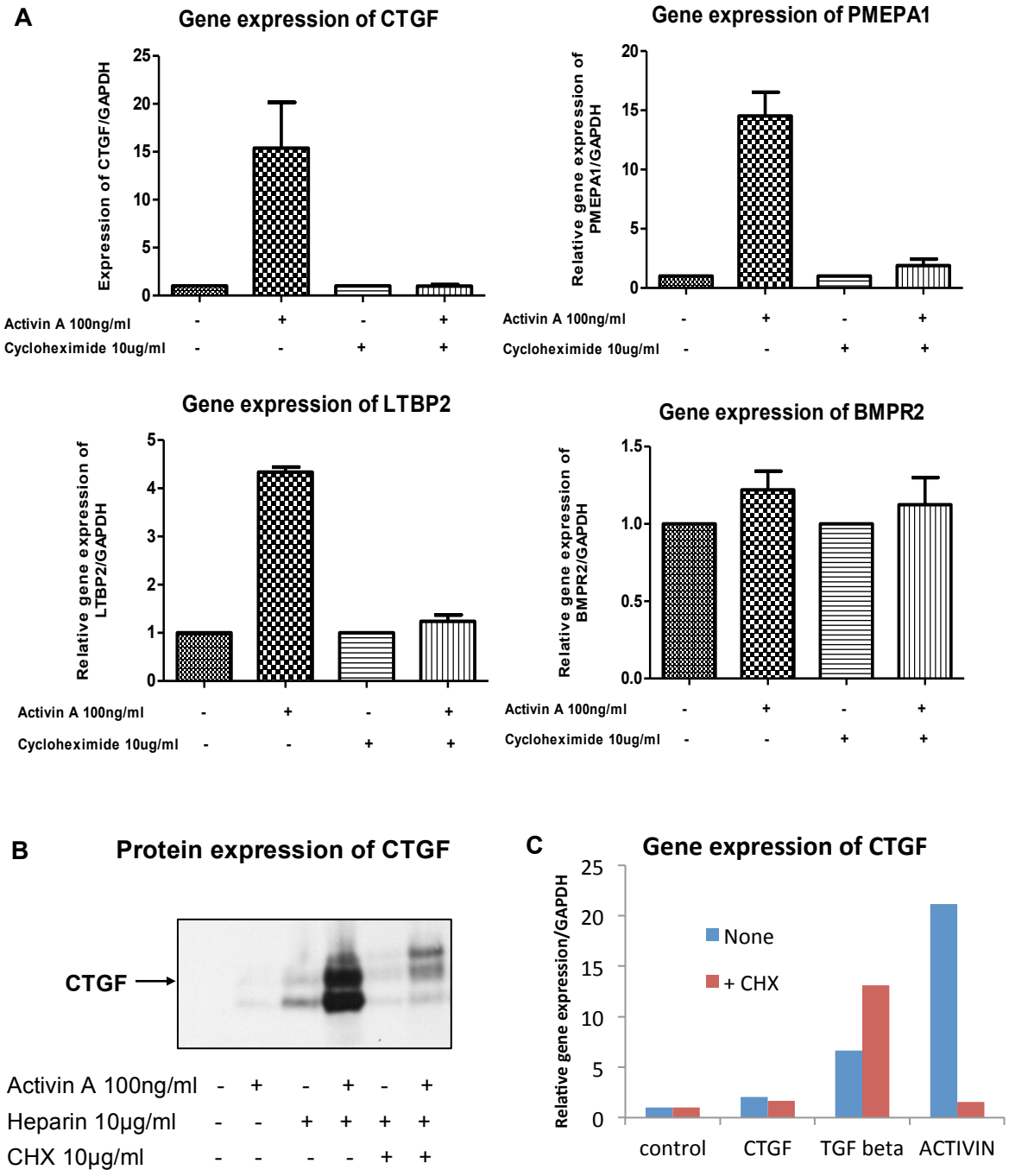


Figure 4.13 Induction of genes by activin A was inhibited by CHX at later stimulation time course. NHDFs were stimulated with or without activin A (100ng/ml) in the presence or absence of CHX (10µg/ml) for 24hr. RNA was extracted and analysed for the relative gene expression of CTGF, PMEPA1, LTBP2 and BMPR2 to GAPDH (A). Bars represent the mean of three experiments with error bars representing the standard error of the mean (n=3). For protein, cells were stimulated in the presence of heparin (10µg/ml) for 24hr. Conditioned medium was collected and acetone precipitated. CTGF protein was detected by western blotting using an anti-CTGF antibody (B). The effect of CHX on the gene expression of CTGF was also examined in CTGF and TGFβ stimulation, where no significant reduction was seen (C).

4.2.14 Activin A induced gene expression was not through expression of CTGF

The CHX experiment provided evidence that the late effect of activin A was through a secondary mediator. As activin A induced CTGF and CTGF could induce the three target genes, it was possible that the late gene effect of activin A was through induction of CTGF. To test this, activin A treatment was performed in NHDFs in which CTGF had been knocked down by siRNA. NHDFs were transfected with either siSCR oligo or siCTGF oligo A and then stimulated with or without activin A (100ng/ml) for 4hr or 24hr. Gene expression of CTGF, PMEPA1, LTBP2 and BMPR2 were compared between siSRC and siCTGF cells. 90% of CTGF RNA was silenced using siRNA oligo A, however, the gene expression of PMEPA1, LTBP2 and BMPR2 were unchanged (Figure 4.14). This suggests that activin A-induced gene expression was not dependent upon the induction of CTGF.

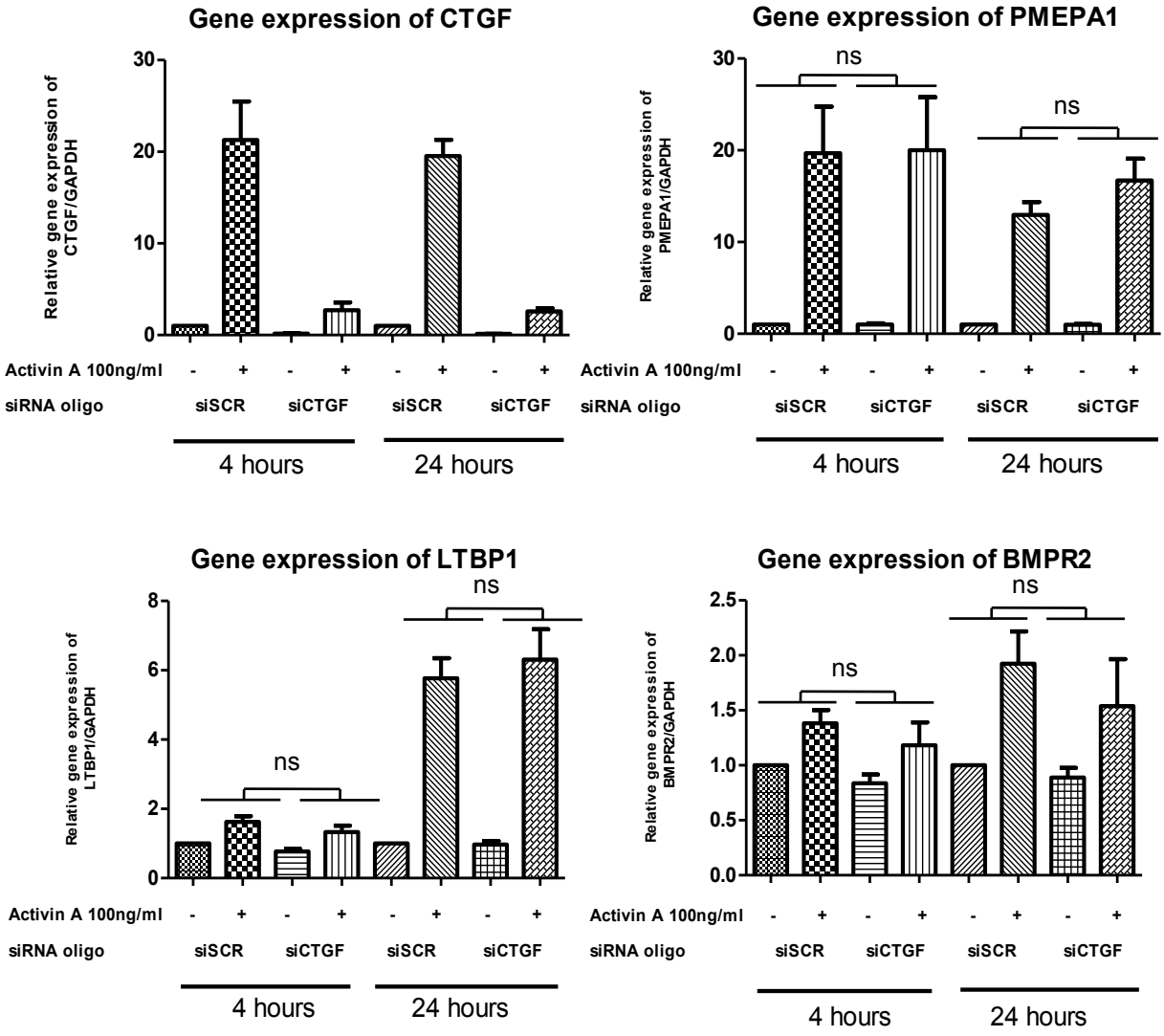


Figure 4.14 Activin A induced gene expression was not in part through CTGF. NHDFs were transfected with siSCR or siCTGF oligo for 48hr and stimulated with or without activin A at 100ng/ml for 4hr or 24hr. Gene expression of CTGF, PMEPA1, LTBP2 and BMPR2 was measured using RT-PCR. CTGF mRNA was reduced by 90% upon knockdown. Gene expression of the other three genes did not significantly change between siSCR and siCTGF treated cells. Bars represent mean of gene expression with error bars representing the standard error of the mean (n= 4).

4.3 Discussion

Central to the success of this project is the expression and purification of active recombinant CTGF; I used a previously established protocol using stably transfected HEK293 cells. The process of making of my own recombinant CTGF has been relatively easy and straightforward. The advantage of using HEK293 cells has been that they can be easily cultured and stably transfected. The fact that they are mammalian cells makes it a good source for expressing human recombinant protein and increases the likelihood that the expressed protein will be folded correctly. The purified recombinant CTGF was detected at a molecular weight of 37kDa by western blot and appeared as doublet on the silver stained gel. This is consistent with the published observations where recombinant CTGF appears as two bands at 36 and 38kDa. The higher molecular weight form is thought to be due to glycosylation of the protein (Grotendorst and Duncan 2005). From the silver stained fractions, the purity of the recombinant CTGF was high, estimated above 90%. Although the yield of my CTGF purification was not very high, about 16µg protein per 150ml of conditioned medium, the procedure is straightforward and new batches can be prepared easily.

The first challenge was to determine whether the purified CTGF was active or not. Although there are several published biological activities reported in the literature, many of the cellular responses e.g. phosphorylation of signalling molecules such as p38 and ERK1/2 (Nagai *et al*., 2008), inhibition of Wnt signalling (Luo *et al.* 2004), and gene expression of ADAMTS and MMPs (Blaney *et al*. 2006) have not been reproduced in our hands (Celia McLean PhD thesis, 2009). Some of these inconsistences may be due to the different recombinant CTGF preparations used in different labs and some may be cell type dependent. Early on we were concerned that negative results might also be due to high basal levels of endogenous CTGF protein in chondrocytes. We preformed CTGF RNA knockdown using two CTGF siRNA oligos. We achieved high knockdown efficiencies; 98% and 80% for oligo A and B

respectively. However, CTGF silencing did not affect putative CTGF inducible gene expression (type I collagen and perlecan etc). By chance during these experiments I found that the purified CTGF induced CTGF itself at gene and protein level as also shown in other's study (Parada *et al.* 2013). This response occurs early and is relatively transient (gone by 24hr) and is similar to that observed for TGFβ stimulation (see next chapter). These findings showed activity in our recombinant CTGF although it could not ruled out that there might be contamination with TGFβ (or similar) in the purified sample. As CTGF and TGFβ bind to one another (Abreu *et al.* 2002), it is possible that these proteins co-purified during chromatography. This is addressed in detail later in this chapter.

We were also interested in activin A as it was one of the elevated proteins identified in the conditioned medium of human OA articular cartilage (Hermansson *et al.* 2004). Activin A mRNA was up-regulated following induction of OA in mouse (Chong *et al*. 2013) and protein was induced upon cartilage injury *in vitro* and *in vivo* in a FGF2 dependent manner (Chong *et al.* 2013; Burleigh *et al*. 2012; Alexander *et al*. 2007). However the role of activin A in normal articular cartilage remains unclear. Activin A null mice show similar musculoskeletal phenotypes to CTGF null mice, therefore I suspected there might be some link between the two proteins. Activin A was able to induce CTGF at gene and protein level but with a relatively delayed time course. This finding is significant as our group had previously been unable to identify specific target genes of activin A in human chondrocytes (Alexander, PhD thesis, 2007; Alexander *et al.* 2007). I also intended to identify other target genes for activin A in the microarray study.

Having confirmed biological activity of the purified CTGF, I proceeded to a microarray analysis. The aim of the microarray study was to identify specific target genes of CTGF as well as activin A following the recombinant CTGF and activin A stimulation respectively, with or without removing basal CTGF by siRNA silencing in these cells. Human articular

chondrocytes used in this experiment were from three donors. This is to accommodate biological variations in the specimen and provide sample replication for statistics. These human chondrocytes were passaged once (P1) before used for the experiment. Freshly isolated (P0) chondrocytes are less suitable for siRNA transfection as P0 cells are likely to disassociate from the monolayer culture plate during transfection and have low transfection efficiency (personal communication within the lab). Cultured chondrocytes beyond three passages (P3) in monolayer system are likely to lose chondrocytic phenotype (reduced type II collagen and aggrecan expression and increased type I collagen expression) and become more fibroblastic (Benya and Shaffer 1982). Two validated siRNA oligomers were used to minimise off-target effects. Both recombinant CTGF and activin A stimulations were included in the experiment to identify their specific target genes. It would be interesting if TGFβ stimulation had been included in the study. However, considering the complexity of the study and many TGFβ inducible genes have already been identified in chondrocytes, I decided not to include TGFβ. From the microarray data, only four genes were found to be up-regulated by CTGF as well as activin A stimulation at 8hr. It is interesting that CTGF and activin A up-regulated the same four genes in chondrocytes, suggesting they may have related roles in cartilage. To understand it better I looked the four genes into details.

The four genes identified were: CTGF, bone morphogenetic protein receptor II (BMPR2), latent TGF binding protein 2 (LTBP2) and transmembrane prostate androgen induced protein (TMEPAI also known as PMEPA1). Aside of CTGF, the other three genes have activities related to TGFβ family protein signalling transduction. BMPR2 is the type II receptor of BMPs; upon binding to ligand, the type II receptor recruits BMP type I receptor and together phosphorylate SMAD molecules which then translocate to the nucleus to act as transcription factors. LTBPs are important molecules that control the bioavailability of TGFβ. Latent TGFβ molecules are shielded in an ECM sequestering complex consisting of TGFβ, LTBP and latency associated peptide (LAP) (more will be discussed in chapter 5). In human

there are 4 types of LTBPs, of which LTBP-1, -3 and -4 contain cysteine repeats that are responsible for the binding to TGFβ/LAP complex. LTBP2 does not bind any of the TGFβ/LAP complexes, but it does bind microfibrils where it may play a structural role or participate in formation of microfibril-associated protein complexes by competing with other LTBPs for binding to fibrillin-1 (Hirani *et al.* 2007). PMEPA1 has been found to negatively regulate growth of androgen responsive cells, and these functions may be mediated through the interaction of PMEPA1 with NEDD4 protein (neural precursor cell expressed developmentally down-regulated protein 4) involved in the ubiquitin-proteasome pathway (Li *et al.* 2008). Reduced PMEPA1 expression was found in prostate cancer but elevated expression in breast cancer (Xu *et al.* 2003). PMEPA1 has been found to be strongly induced by TGFβ (Brunschwig *et al.* 2003) and involved in a negative feedback loop in TGFβ signalling pathway by inhibiting the phosphorylation and translocation of SMAD3 (Watanabe *et al.* 2010).

The effects of CTGF and activin A stimulations on the four target genes were validated using single Taqman RT-PCR. The same results were reproducible in both human chondrocytes and dermal fibroblast, where CTGF and PMEPA1 gene expression showed the greatest change, followed by LTBP2 and BMPR2. Time course experiments showed that the response of BMPR2 and PMEPA1 gene expression to CTGF were early and transient whereas the response to activin A was late and persistent. The gene expression of LTBP2 following both CTGF and activin A stimulation was late, but declined after 24hr with CTGF stimulation while staying constant beyond 48hr with activin A stimulation. This result suggested the induction of a secondary mediator in activin A stimulated cells. Cycloheximide (CHX), which inhibits all newly synthesised protein, abolished the late gene response of activin A while not to that of TGFβ, confirming that there is a secondary protein being synthesised during activin A treatment. One candidate gene induced by activin A that could lead to late gene induction was CTGF. Knockdown of endogenous CTGF was unable

to reduce the late gene induction caused by activin A, indicating that CTGF was not the intermediate molecule involved in activin A's action. PMEPA1 and BMPR2 are also likely to be the early-induced mediator (expression peaked at 4hr and 8hr respectively) of activin A for its late gene induction. To test this, knockdown effect of these genes can be looked at and whether PMEPA1 and BMPR2 can induce other genes (e.g. CTGF and LTBP2) can also be examined.

The data obtained from the microarray study has been very interesting; I have identified four strongly CTGF-induced genes. These same four genes were also induced by activin A in human chondrocytes although the effects of activin A were not dependent upon CTGF expression. The reason why CTGF and activin A share similarities in gene regulation and share a relatively restricted gene expression profile remains unanswered. However it is notable that both molecules are closely linked to TGFβ; activin A is a TGFβ family member and signals through the SMAD signalling pathway; CTGF is known to bind to TGFβ itself to modulate signalling at the TGFβ receptor (Abreu *et al.* 2002); and finally all four genes that were identified on the microarray are known to be TGFβ-induced. The role of CTGF in TGFβ signalling and bioavailability was next investigated.

Part II Role of CTGF in TGFβ signalling and bioavailability

4.4 Introduction

CTGF has been proposed to act closely with TGFβ. During wound repair and fibrotic disorders, both CTGF and TGFβ mRNA and protein levels are found elevated at sites of damage (Igarashi *et al*. 1993; Kulozik *et al*. 1990) and increased connective tissue formation by TGFβ has been proposed to occur via an indirect mechanism involving CTGF (Grotendorst, 1997). The CTGF gene is strongly induced by TGFβ, but is not induced to any significant level by other growth factors including EGF, FGF, PDGF and insulin or IGFs (Igarashi *et al*. 1993). Many activities exhibited by CTGF are also similar to the actions of TGFβ. For example, CTGF stimulates cell proliferation in monolayer cultures of normal rat kidney (NRK) fibroblasts, which is a characteristic of TGFβ stimulation in the same assay (Frazier *et al.* 1996). CTGF is also reported to induce type I collagen, fibronectin and α5 integrin expression in NRK fibroblasts, which is another action shared with TGFβ (Frazier *et al*. 1996). A number of studies suggest that CTGF acts as a mediator or cofactor for TGFβ family members. Abreu *et al* (2002) showed that CTGF binds to both TGFβ1 and BMP4. This stabilises the binding of TGFβ to its receptor but inhibits the binding of BMP-4 to its receptor. The same authors also showed that CTGF was able to potentiate the effect of TGFβ on phosphorylating SMAD2 (Abreu *et al*. 2002). Song *et al* (2007) showed that silencing of CTGF expression using siRNA could inhibit mesenchymal cell condensation mediated by TGFβ, and Kothapalli *et al* (1997) showed antisense of CTGF was able to inhibit TGFβ induced anchorage independent growth of cells (Song *et al*. 2007; Kothapalli *et al*. 1997).

In human chondrocytes, we found CTGF was strongly up-regulated by TGFβ1 and activin A (Figure 4.13C). The three genes (PMEPA1, LTBP2 and BMPR2) that it induced were known to be TGFβ-induced and were related to TGFβ family signalling pathways. This suggested

that CTGF might act indirectly by modulating the effects of TGFβ family proteins. Therefore it was decided to investigate the role of CTGF in TGFβ-induced responses.

4.5 Results

4.5.1 TGFβ also induces the expression of the four identified CTGF inducible genes

If CTGF was acting indirectly through the action of TGFβ family proteins, it was first important to investigate whether TGFβ was also able to induce the expression of the four identified genes itself. A time course experiment was first carried out in NHDFs with TGFβ1 stimulation for 4hr, 8hr and 24hr and the RNA was extracted for the analysis of relative gene expression of CTGF, PMEPA2, LTBP2 and BMPR2 using RT-PCR. Unsurprisingly, TGFβ was able to induce these four genes strongly (Figure 4.15). Similar to that which we had observed for CTGF stimulation, the regulation of PMEPA1 and BMPR2 to TGFβ stimulation was also early and transient: peaked at 4hr and declined after 8hr. For LTBP2, the fold of induction increased at late time points, from 3.5 fold at 4hr and 8hr to 5.5 fold at 24hr.

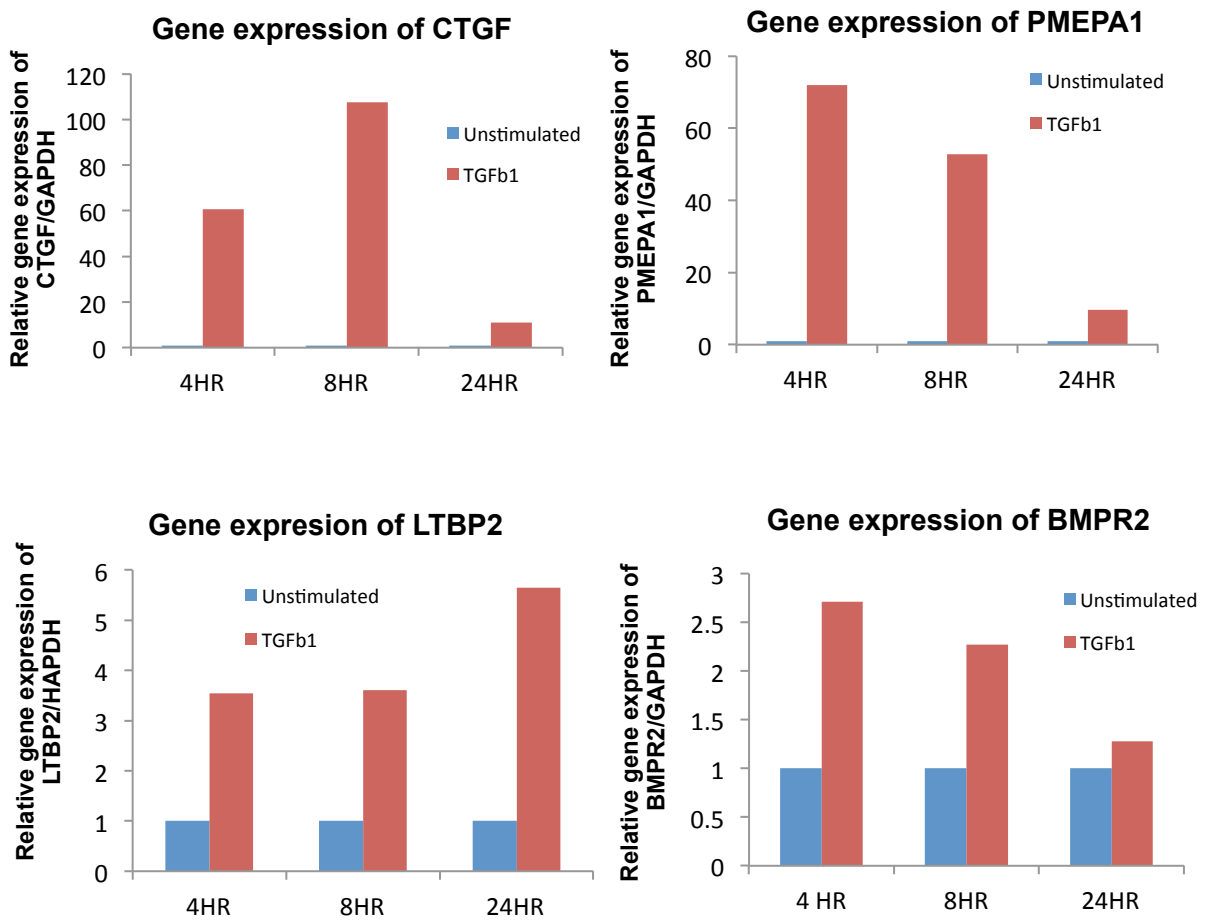


Figure 4.15 TGFβ induces the gene expression of CTGF, PMEPA1, LTBP2 and BMPR2 in NHDFs. NHDFs were stimulated with or without TGFβ1 at 10ng/ml for 4, 8, or 24hr. Gene expression of CTGF, PMEPA1, LTBP2 and BMPR2 were measured using RT-PCR. Bars represent one experiment (n=1).

4.5.2 CTGF knockdown did not affect TGFβ1 induced gene expression

Strong induction of CTGF by TGFβ1 can be seen at early time points (as early as 4hr). Therefore it was possible that TGFβ induced gene expression was through CTGF. To test this, CTGF RNA expression was silenced by siRNA oligo A (siCTGF) (siSCR was used as control) in cells prior to stimulation with or without TGFβ for 8hr. RNA was then extracted and analysed for gene expression (CTGF, PMEPA1 and LTBP2). 90% of CTGF RNA was silenced using siRNA oligo A (Figure 4.16). In cells transfected with siSCR, CTGF mRNA increased by 105.52 fold (mean of several experiments) upon TGFβ1 stimulation. siCTGF

transfected cells showed approximately 90% reduction in CTGF gene expression either with or without TGFβ1 stimulation. No change was observed in gene expression of PMEPA1 and LTBP2 upon TGFβ stimulation when CTGF had been silenced, indicating that the effects of TGFβ on these genes were not dependent upon induced or constitutive CTGF.

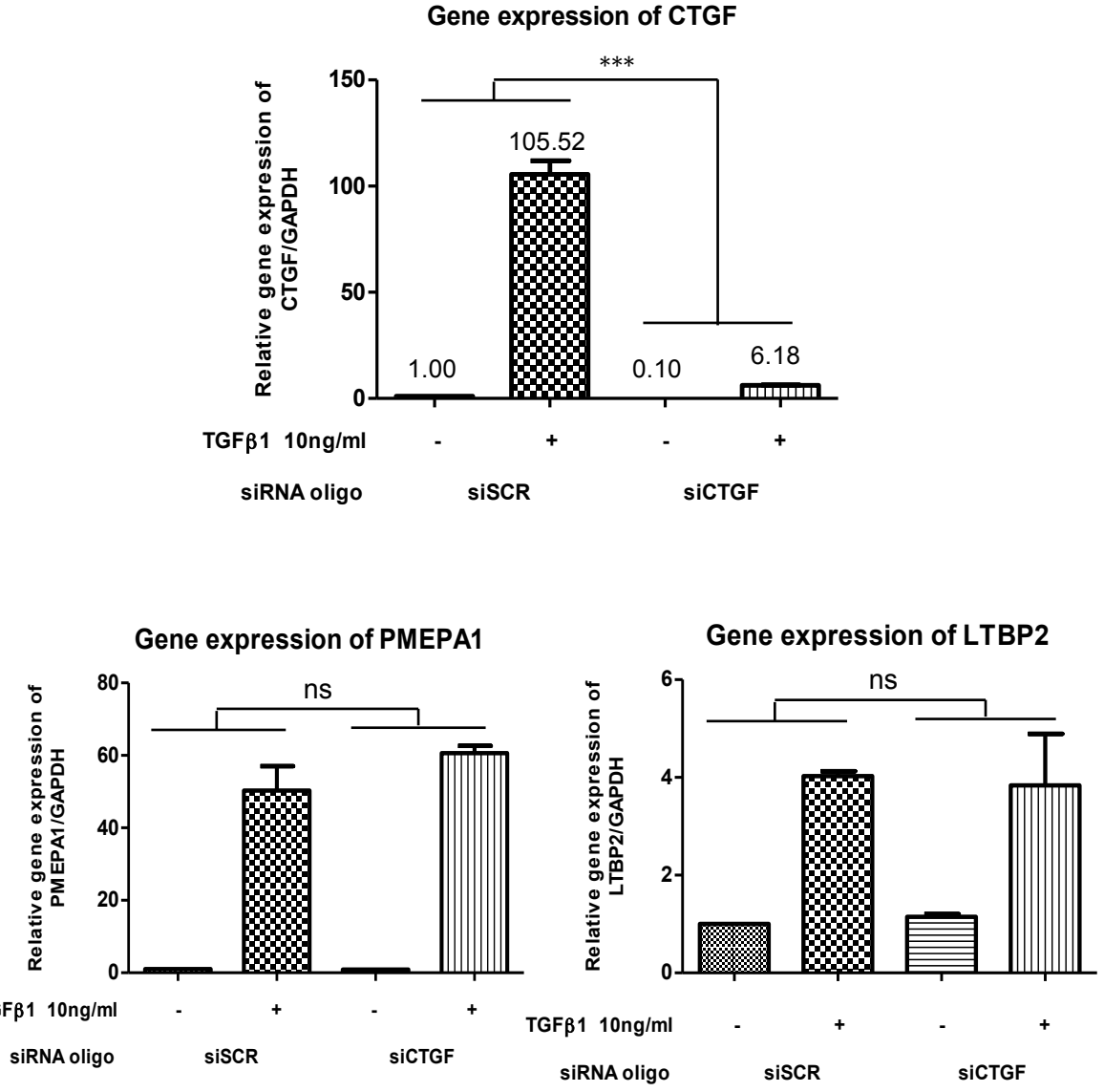


Figure 4.16 CTGF knockdown did not affect TGFβ induced gene expression. NHDFs were transfected with siSCR or siCTGF oligo A for 48hr and stimulated with or without TGFβ1 at 10ng/ml for 8hr. Gene expression of CTGF, PMEPA1 and LTBP2 was measured using RT-PCR. RNA of CTGF was reduced by 90% upon silencing. Gene expression of PMEPA1 and LTBP2 did not show significant change between siSCR and siCTGF treated cells. Bars represent fold change in gene expression with error bars representing the standard error of the mean (n=3, ns: not significant, *** $p<0.001$).

4.5.3 TGFβ family type 1 receptor inhibitor SB431542 attenuated CTGF induced gene expression and SMAD2 activation

So far, TGFβ, activin A and CTGF have all been showed to induce the PMEPA1, LTBP2 and BMPR2 but the pathway through which this occurs has not been examined. It is known that the canonical signalling pathway of TGFβ family members is via phosphorylation of SMAD proteins. Ligands first bind to their specific type II receptor which recruits the type I receptor. A small molecule inhibitor SB431542 selectively inhibits ALK4 (activin A type I receptor) and ALK5 (TGFβ type I receptor), thus suppresses activin A and TGFβ SMAD2/3 signalling but not BMP SMAD1/5/8 signalling. Therefore, by using a receptor inhibitor we can determine whether CTGF induces the three genes through the TGFβ/activin A receptor signalling pathway. NHDFs were treated with TGFβ1, activin A, CTGF or BMP2 in the presence or absence of SB431542 for 8hr and the gene expression of CTGF and PMEPA1 was measured (CTGF and PMEPA1 are most strongly induced by these ligands). SMAD activation was detected using anti-phospho-SMAD antibodies by western blotting. Stimulation of cells in the presence of SB431542 reduced TGFβ and activin A induced gene expression, confirming that the canonical TGFβ pathway was responsible for this TGFβ and activin A mediated change in gene expression (Figure 4.17). SB431542 also inhibited CTGF induced gene expression. This suggested that CTGF was acting through the ALK5 or ALK4 receptor (perhaps as a cofactor for TGFβ or activin A), or that the inhibitor had non-specific inhibitory effects or caused toxicity to the cells. This latter possibility was unlikely as similar quantities of good quality of RNA were extracted from all samples (with and without SB431542). Also, no inhibition of phosphorylation of BMP2 activated SMAD1/5/8 was seen after 45min, while phosphorylation of SMAD2 upon stimulations of TGFβ, activin A and CTGF was abrogated (Figure 4.18). This indicates that the effects of CTGF are acting through the TGFβ/activin A signalling pathway.

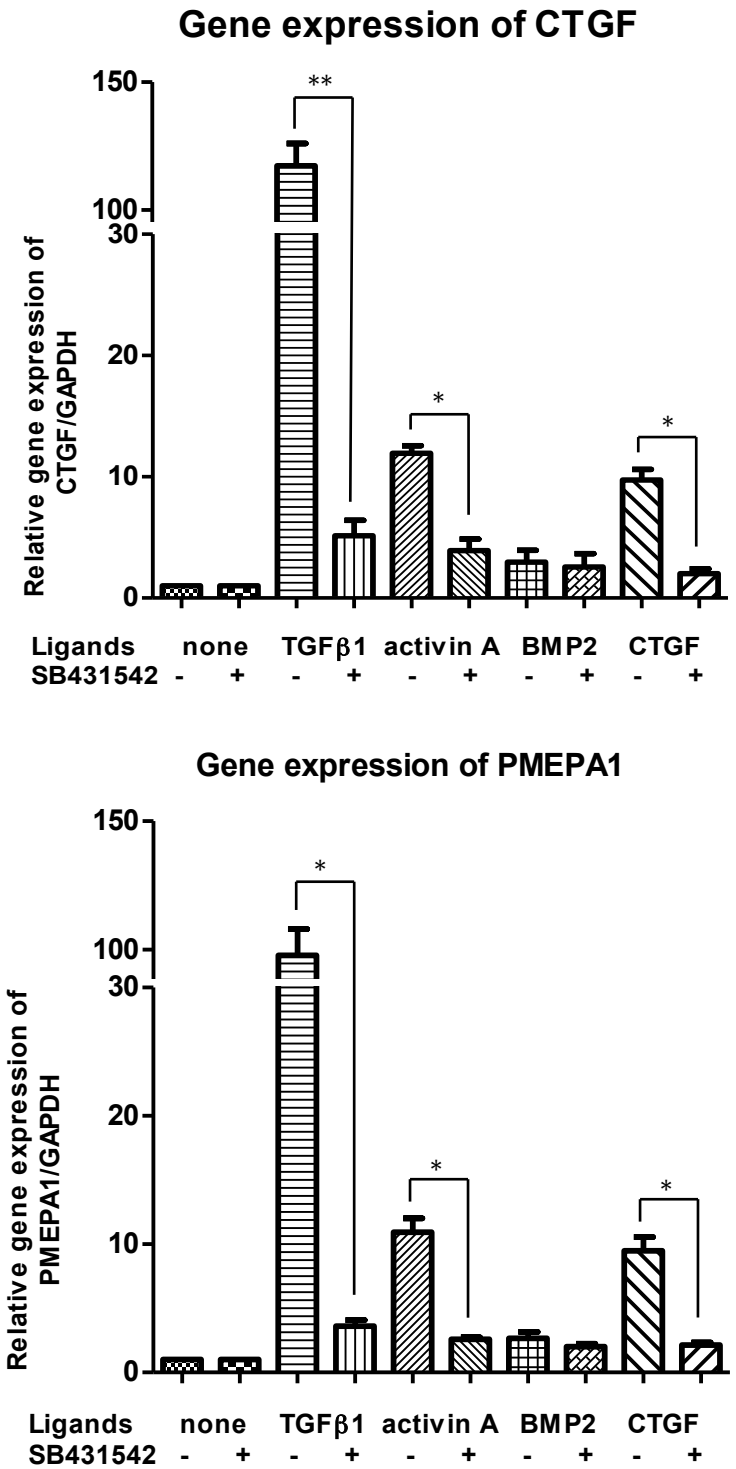


Figure 4.17 CTGF induced gene expression was inhibited by TGFβ/activin A type I receptor SB431542. NHDFs were treated with TGFβ1 at 10ng/ml, activin A at 100ng/ml, BMP2 at 100ng/ml or CTGF at 100ng/ml for 8hr in the presence or absence of SB431542 at 5μM. Gene expression of CTGF and PMEPA1 was measured using RT-PCR. Figure shows mean of gene expression with error bars represent the standard error of the mean (n=3 ** $p < 0.01$, *$p < 0.05$). Only two experiments were performed on BMP2 stimulation, therefore no statistical test was done for BMP2.

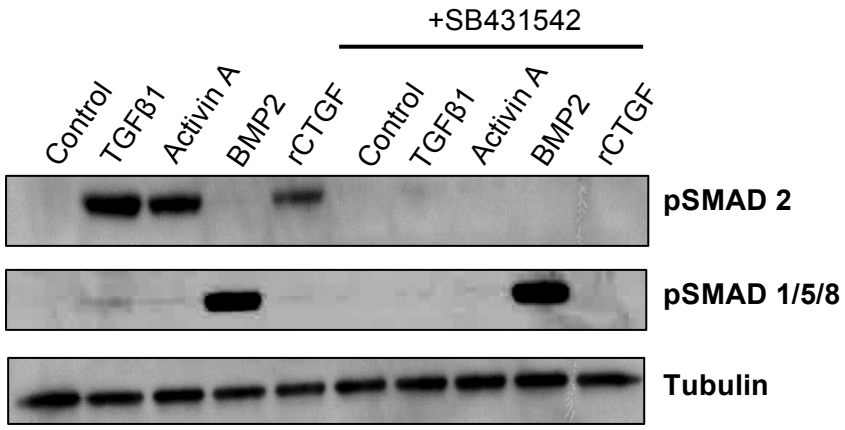


Figure 4.18 CTGF activated SMAD2 but not SMAD1/5/8, which was inhibited by TGFβ/activin A type I receptor SB431542. NHDFs were treated with TGFβ1 at 10ng/ml, activin A at 100ng/ml, BMP2 at 100ng/ml or CTGF at 100ng/ml for 45min in the presence or absence of SB431542 at 5μM. Lysates were western blotted for phospho-SMAD2 or phospho-SMAD1/5/8. Tubulin was used as a loading control. n=3

4.5.4 CTGF does not act synergistically with either TGFβ or activin A

In order to investigate whether CTGF acts independently through the TGFβ receptor or as a cofactor for TGFβ family ligands, we looked for a synergistic effect of CTGF and TGFβ proteins. First, a dose response experiment was carried out (Figure 4.19). NHDFs were treated with either CTGF (0-400ng/ml), activin A (0-400ng/ml) or TGFβ1 (0-10ng/ml) for 8hr and gene expression of CTGF, PMEPA1 and LTBP2 was measured. CTGF induced gene expression increased gradually with the increasing concentration of CTGF from 10 to 400ng/ml, with a strong induction in gene expression of PMEPA1 and CTGF (Figure 4.19A). Activin A induced CTGF and PMEPA1 expression showed an increasing pattern from 5 to 400ng/ml, while gene expression of LTBP2 plateaued at 20ng/ml (Figure 4.19B). TGFβ was able to induce the genes strongly at as low as 2ng/ml, suggesting that TGFβ is a potent ligand. TGFβ induced gene expression increased with the concentration of TGFβ from 2 to 10ng/ml (Figure 4.19C).

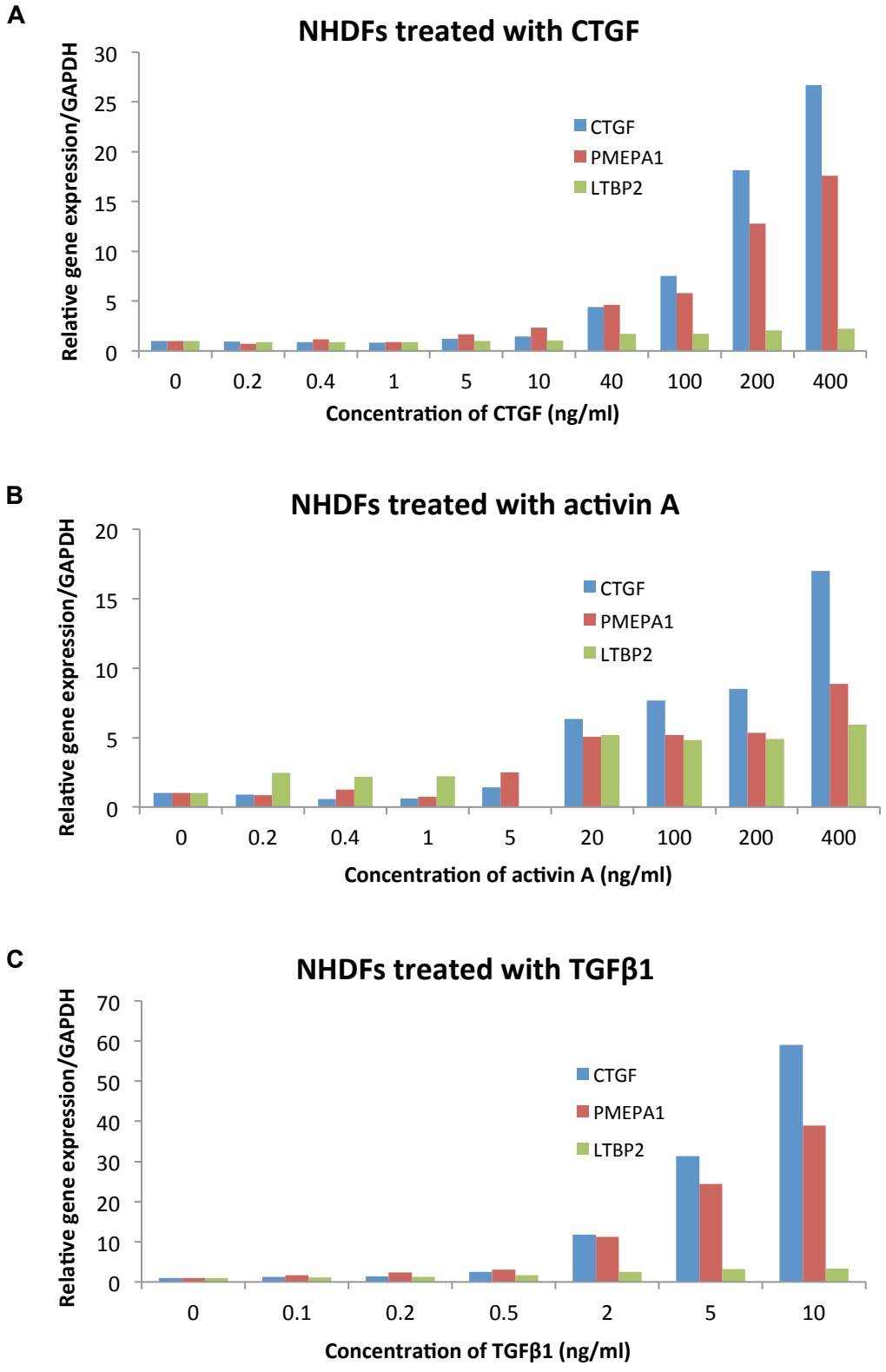


Figure 4.19 Genes responded to ligands in a dose dependent manner. NHDFs were treated with either CTGF (A), activin A (0.2 to 400ng/ml) (B) or TGFβ1 (0.1 to 10ng/ml) (C) for 8hr. Gene expression of CTGF, PMEPA1 and LTBP2 was measured relative to GAPDH. Bars represent relative gene expression from one experiment (n=1).

For synergistic experiments, NHDFs were treated with suboptimal dose of CTGF (40ng/ml), activin A (50ng/ml) and TGFβ1 (1ng/ml) alone or in combinations for 8hr. CTGF and PEMPA1 mRNA levels were measured and the fold changes were compared between the 'alone' and combined treatments. Optimal dose stimulations of activin A (400ng/ml) and TGFβ (10ng/ml) were also used to illustrate the maximal inductions for the two genes. For the synergy experiment of CTGF and activin A, the fold change in each 'alone' stimulation adds up to that of the combined stimulation, suggesting a possible additive effect of CTGF to activin A on the expression of the two genes (n=1, not statistically tested) (Figure 4.20A and B). For CTGF and TGFβ, addition of a suboptimal dose of CTGF to TGFβ did not show an additive effect. Indeed, there was no significant difference between TGFβ alone and in combination for either CTGF or PMEPA1 mRNA regulation (Figure 4.20C and D). I was able to confirm that at these suboptimal doses in all four experiments maximal gene induction had not been reached.

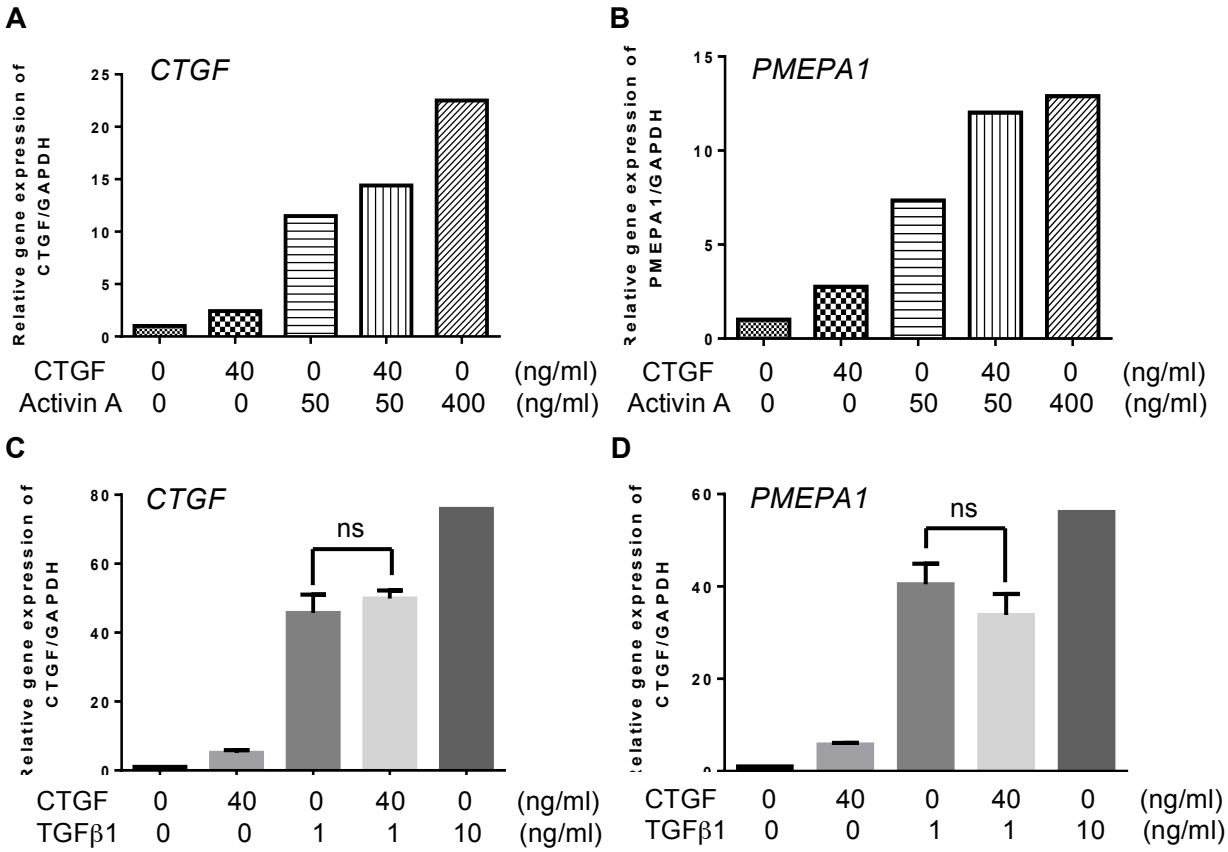


Figure 4.20 CTGF showed additive effect to activin A while no additional effect to TGFβ in the gene expression of CTGF and PMEPA1. NHDFs were treated with CTGF (40ng/ml) or activin A (50ng/ml) (A and B n=1) /TGFβ1 (1ng/ml) (C and D n=3) alone or in combination. Gene expression of CTGF and PMEPA1 was measured relative to GAPDH. Result shows additive effect of CTGF to activin A (not statistically tested) and no effect of CTGF on TGFβ1 induced gene expression. ns= not significant.

4.5.5 Anti-TGFβ neutralising antibody abolishes CTGF induced SMAD2 activation and gene regulation

It is known that TGFβ and activin A are constitutively expressed by articular chondrocytes (Vivien *et al*. 1995; Hermansson *et al*. 2004). Thus it was possible that CTGF was able to interfere, in some way, with the activity of these endogenous proteins. To test this, CTGF induced SMAD2 activation and gene expression were now analysed in the presence or absence of neutralising antibody specific to TGFβ or activin A. Porcine articular chondrocytes were treated with TGFβ1, activin A or CTGF in the presence of IgG (as an

isotype control), anti-TGFβ or anti-activin A. Figure 4.21A shows strong activation of SMAD2 by all the stimulators in the presence of the control immunoglobulin IgG. In the presence of the anti-TGFβ antibody, the signal of the pSMAD2 induced by TGFβ1 was largely inhibited and interestingly the activation by CTGF was also completely inhibited by the anti-TGFβ antibody, whereas anti-activin A antibody had only minor effect on the action of CTGF. CTGF induced gene expression of CTGF was also inhibited by anti-TGFβ neutralising antibody by about 70%, suggesting the action of CTGF in articular chondrocytes is likely to be TGFβ dependent (Figure 4.21B).

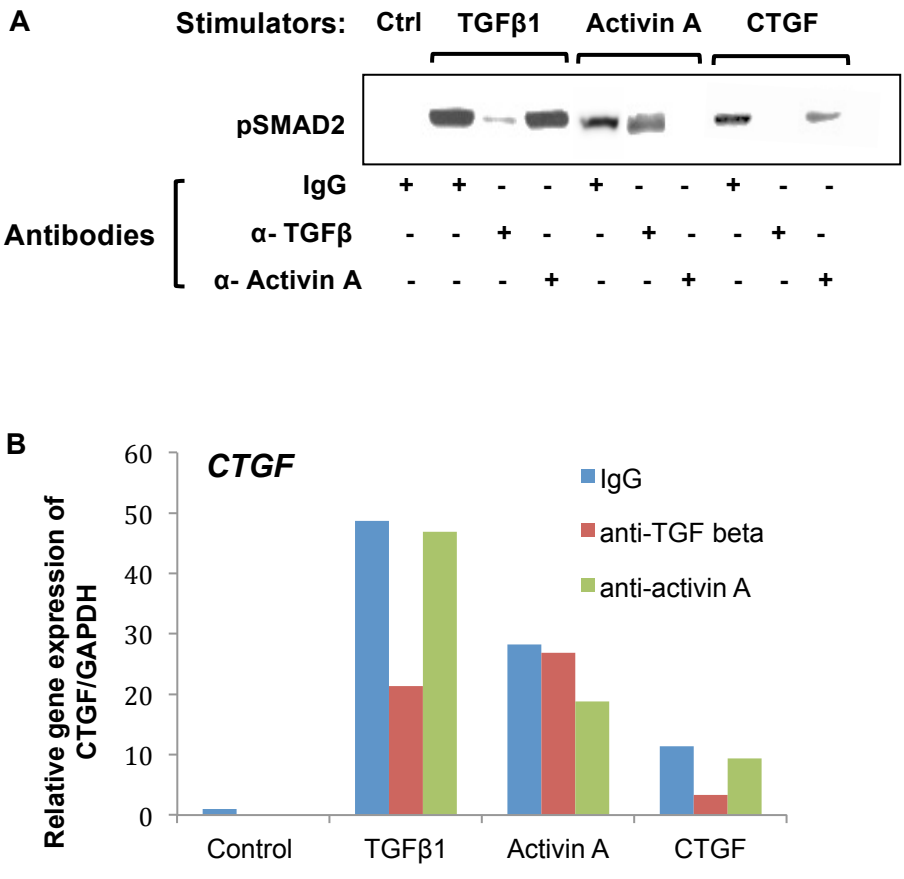


Figure 4.21 The activity of CTGF in SMAD2 activation and gene expression was inhibited by anti-TGFβ neutralising antibody. Porcine articular chondrocytes were treated with TGFβ1 (10ng/ml), activin A (100ng/ml) or CTGF (100ng/ml) in the presence of mouse IgG, anti-TGFβ, anti-activin A antibodies. The signal of pSMAD2 was measured by western blot (A). The gene expression of CTGF/GAPDH was measured by RT-PCR (B) n=2.

4.5.6 No detection of TGFβ protein contamination in the CTGF recombinant protein sample

The inhibitory effect of the TGFβ neutralising antibody on CTGF suggests that the action of CTGF requires the presence of TGFβ protein. Therefore it was important to determine the source of the protein. The first consideration was that whether our recombinant CTGF protein sample was contaminated with TGFβ protein. To test this, I immunoblotted our recombinant CTGF protein with an antibody against all the TGFβ isoforms, i.e. TGFβ1, 2 and 3. Commercially purchased recombinant TGFβ1 protein was used as a positive control. The presence of TGFβ1 monomer (12.5kDa) and dimer (25kDa) were detected from 0.1ng by western blot and were seen to increase with increasing doses of TGFβ1 (0.1 to 4ng). TGFβ1 propeptide (60kDa) also started to appear from 0.5ng. No TGFβ was detected in the CTGF sample at either 40 or 100ng despite strong CTGF immunostaining evident in these lanes. From the previous dose experiment of TGFβ (Figure 4.19), at least 0.2ng of TGFβ was needed to change gene expression (Figure 4.19C), thus ruling out significant TGFβ contamination as an explanation for the above observation.

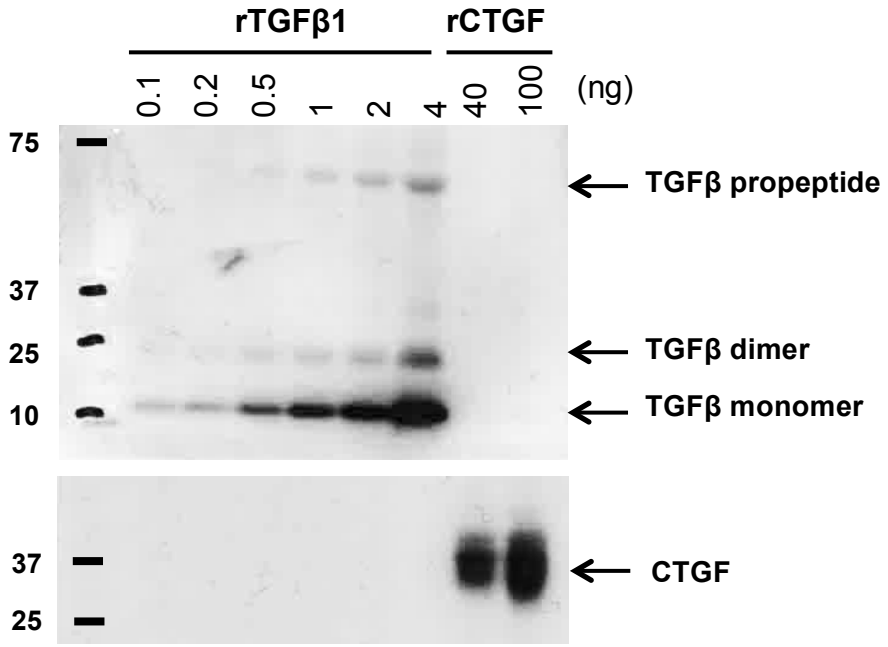
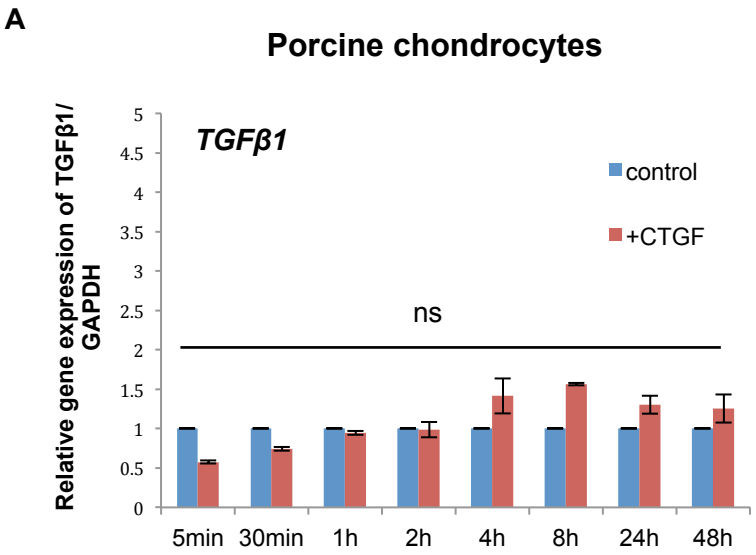


Figure 4.22 No detection of TGFβ1, 2, 3 in the recombinant CTGF samples. Recombinant TGFβ (rTGFβ1) and CTGF (rCTGF) were analysed by western blot at 0.1 to 4ng and 40 and 100ng respectively for TGFβ1, 2, 3 and CTGF. n=1

4.5.7 Recombinant CTGF increases TGFβ protein level

Knowing that the activity observed for CTGF was not due to TGFβ contamination, I decided to determine whether CTGF could control level of TGFβ protein. To test whether CTGF induces TGFβ protein synthesis, porcine chondrocytes were treated with recombinant CTGF at 100ng/ml for different time points and the relative gene expression level of TGFβ1 was measured against GAPDH. As shown in figure 4.23A, there was no significant gene induction was observed at any time point used in the experiment (Figure 4.23A). The protein level of TGFβ was also studied. Porcine chondrocytes were treated with TGFβ1 at 10ng/ml or CTGF at 100ng/ml for 45min. After the stimulation, the conditioned medium was collected and western blotted for TGFβ. The cell layer was then washed and lysed in RIPA buffer and the lysates were western blotted for pSMAD2, TGFβ and tubulin. Again CTGF and TGFβ1 strongly induced the phosphorylation of SMAD2. TGFβ protein was strongly detected in the

lysate of TGFβ1 treated cells, which possibly contained internalised recombinant TGFβ protein as not much TGFβ was detected in the conditioned medium after exogenous TGFβ was added. In the CTGF treated cells, a small amount of TGFβ was detected in the cell lysate, slightly stronger than the control; and no detection of TGFβ in the conditioned medium (Figure 4.23B). CTGF did not seem to increase the level of TGFβ protein in isolated chondrocytes. However, when I used porcine cartilage explants (with an intact ECM), I was able to detect TGFβ protein accumulation in the conditioned medium after 1hr CTGF stimulation (Figure 4.23C). These results suggest that CTGF can rapidly modulate TGFβ protein levels in tissues with an intact extracellular matrix. From the basis of these experiment I formulated a novel hypothesis that CTGF can control the bioavailability of matrix-bound TGFβ.



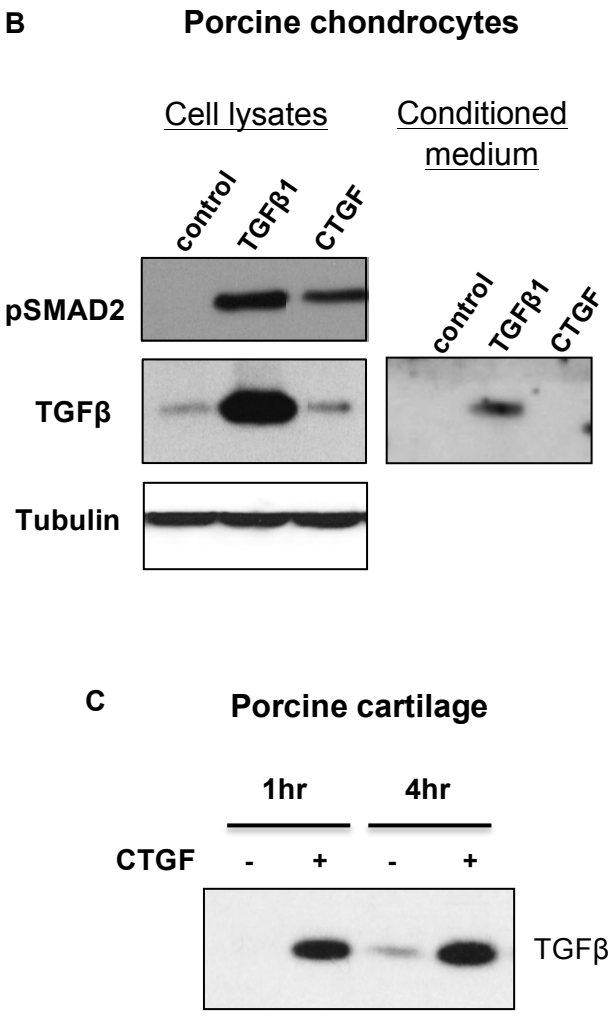


Figure 4.23 CTGF did not induce TGFβ protein synthesis but the release from the extracellular matrix. Porcine chondrocytes were treated with recombinant CTGF at 100ng/ml for 5min to 48hr and the relative gene expression of TGFβ was measured by RT-PCR n=3 (A). For protein porcine chondrocytes were treated with TGFβ (10ng/ml) or CTGF (100ng/ml) for 45min and the cell lysates and the conditioned medium were analysed for pSMAD2 and TGFβ (B). Porcine cartilage was treated with CTGF at 100ng/ml for 1 or 4hr and the conditioned medium was analysed for TGFβ (C).

4.6 Discussion

The bioactivities of the four genes identified from microarray suggested that CTGF may be involved in somehow TGFβ induced activities. We first tested whether TGFβ also induced the four genes. Unsurprisingly (from the literature), TGFβ strongly induced PMEPA1 (90 fold), LTBP2 (5 fold) and BMPR2 (2.5 fold) in addition to CTGF (100 fold). The pattern of expression of the genes induced by TGFβ1 in the time course experiments was similar to that which we observed following CTGF stimulation. The response of PMEPA1 and BMPR2 appeared to be early and transient whereas LTBP2 expression was persistent and increased at later time points. One possibility was that TGFβ regulated gene expression through CTGF. To test this, CTGF RNA was silenced using siRNA in these cells prior to stimulation with TGFβ. Upon CTGF knockdown, no change was seen in the gene expression levels of PEMPA1 and LTBP2 following TGFβ stimulation.

The type I receptor inhibitor SB431542 significantly reduced gene induction of CTGF, PMEPA1 and LTBP2 induced by CTGF, TGFβ and activin A stimulation. The specificity of the inhibitor was examined by showing that it did not inhibit phospho-SMAD1/5/8 following BMP2 stimulation. In the blot, we observed the pSMAD2 band for CTGF stimulation with relative lower intensity and this band disappeared when SB431542 was added. It is unlikely that CTGF directly acts through the TGFβ or activin A receptor to activate SMAD2/3 signalling pathway as there has not been any report showing CTGF directly binds to the receptors of TGFβ family proteins. It is more likely that CTGF indirectly elevates basal SMAD2/3 signals by binding to endogenous TGFβ or activin A, which stabilises their binding to the receptors thus potentiating SMAD2/3 signalling. This supports one published theory that CTGF acts as a cofactor to promote TGFβ signals (Abreu *et al*. 2002). A second possibility is that CTGF promotes SMAD2/3 signals by disturbing the negative feedback loop; there have been reports to suggest that CTGF suppresses the gene expression of the

negative regulator SMAD7 (Wahab *et al.* 2005). Both of these latter responses dependent upon some endogenous production of TGFβ or activin A, or require it to be present within cultures (it could be bound to matrix). Either way inhibiting TGFβ or activin A (by siRNA or neutralising antibody) should suppress the apparent biological effects of CTGF. Experiments looking for synergy between CTGF and TGFβ or activin A would provide further evidence for this. When suboptimal doses of CTGF and activin A or CTGF and TGFβ were used, no synergistic effect on regulation of CTGF or PMEPA1 was seen, indicating that CTGF does not potentiate the activity of activin A or TGFβ. When using neutralising antibodies to endogenous TGFβ (but not activin A), CTGF-induced SMAD2 activation and gene regulation was inhibited, suggesting that the effects of CTGF require the presence of TGFβ protein. I confirmed that this TGFβ protein was not from our recombinant CTGF preparation as a contaminant and was not newly synthesised upon CTGF stimulation.

TGFβ is known to be synthesised as a propeptide and secreted as a latent complex stored in the extracellular matrix through binding to the LTBP protein. The activation of TGFβ involves the release of latent complex from the matrix pool and dissociation of mature TGFβ dimer from the latent complex (Taipale *et al.* 1998). It is possible that the endogenous TGFβ involved in the action of CTGF has been released from its matrix pool. Indeed only stimulation of articular cartilage explants, which possess an extensive extracellular matrix, caused rapid release of TGFβ into the medium. This suggests that CTGF increases the bioavailability of matrix bound TGFβ. Appropriate control of TGFβ bioavailability is important for correct cell responses. Inappropriate TGFβ activation observed in the Marfan syndrome causes excessive production of matrix, resulting abnormalities in various tissues such as limbs, heart and lungs (Neptune *et al*. 2003). The question how CTGF may be involved in latent TGFβ activation in articular cartilage is addressed in the chapter 5.

Chapter 5:

Results

Effect of endogenous CTGF on mechanically injured articular cartilage

Part I Study of injury response using normal porcine cartilage

5.1 Introduction

5.1.1 Response of articular cartilage to mechanical injury

Mechanical injury on cartilage predisposes the joint to OA. Cuts or damage made on the surface of mature articular cartilage are thought to make an attempt at repair when defects extend to the subchondral bone. This leads to a fibro-cartilaginous tissue which fills the defect albeit with inferior mechanical integrity compared with hyaline cartilage. This was demonstrated by Ghadially's group who produced partial-thickness defects (not extending to subchondral bone) in the articular cartilage of rabbits and found no evidence for repair over a 2-year period. In these cases, a thin layer of matrix was formed over the injured surface early after injury, although they concluded that it was abraded away by joint movement (Ghadially *et al*. 1976). The lack of repair may be due to the inability of mature articular chondrocytes to divide or mount a sufficient repair reaction in magnitude to fill the defect (Bennett and Bauer 1932). Conversely, deeper defects that violate the underlying bone are rapidly filled with blood containing red and white blood cells and bone marrow elements (Campbell *et al*. 1969). These white and undifferentiated cells, which likely contain mesenchymal progenitor cells, form vascular fibroblastic repair tissue at the base of the wound (De Palma *et al*. 1966). Mitchell and Shepard demonstrated that such repair tissue was then converted to a hyaline-like chondroid tissue in the early course of deep defects in rabbits, with increased mitotic activity and histological staining showing increased proteoglycan. Despite this, after 12 months of the injury the repairing tissue became more fibrous rather than cartilaginous (Mitchell and Shepard 1976). Such tissue has reduced mechanical material functions, which may be acceptable if the defect is not too large. For larger defects, excessive mechanical load on newly constructed tissue is likely to result the development of OA (Radin *et al*. 1991).

5.1.2 Growth factors released upon mechanical stimuli to articular cartilage

These historical studies suggest that there are early innate cellular responses in place to respond to cartilage injury. Such cellular responses are likely to be mediated in part by the mechanically induced release of growth factors. Tonia Vincent's group discovered that mechanically injured or compressed articular cartilage led to the release of fibroblast growth factor 2 (FGF2) from a pericellular matrix-bound pool (Vincent *et al.* 2004). Released FGF2 activated the ERK MAPK pathway and induced the synthesis and secretion of tissue inhibitor of metalloproteinase 1 (TIMP-1) from injured cartilage explants (Vincent *et al*. 2004).

In my study (chapter 3) I found that explanting (cutting cartilage at the osteochondral junction) or recutting (cutting previously explanted and rested) articular cartilage resulted in the release of CTGF into the medium. This is perhaps not surprising as CTGF has similar physical properties to sequestered FGF2, and is also localised in the pericellular matrix and binds to perlecan through the heparan sulphate chains (chapter 3). In this chapter I will describe the consequences of CTGF release with specific reference to TGFβ activation.

5.1.3 TGFβ latency

5.1.3.1 Latent form of TGFβ

TGFβ is synthesised as a pre-pro-peptide containing an N-terminal secretion signal peptide, a latency associated peptide (LAP) or propeptide and a C-terminal TGFβ active peptide. Two TGFβ proprotein monomers associate to form homodimers linked by disulfide bonds (Gray and Mason 1990). During secretion, mature TGF-β is cleaved from its propeptide (LAP) by furin-like endoproteinase at a cleavage site (R-X-R/K-R) at the end of the propeptide (LAP) sequence (Leitlein *et al*. 2001). The LAP dimer remains associated with the TGF-β dimer by non-covalent interactions while both the mature TGF-β dimer and the LAP dimer are

disulfide bonded (Shi *et al.* 2011). The latent TGF-β complex consisting of LAP and TGF-β is referred as TGF-β small latent complex (SLC). The association between active TGF-β and its LAP propeptide is reversible and involves extensive structural changes in the LAP (Shi *et al*. 2011). The type of LAP is named after the TGFβ isomer that it associates with, for example LAP1 for TGFβ1. In cartilage, 40% of TGFβ produced is in TGFβ1 isoform with TGFβ2 and 3 at lesser abundance (Kanaan and Kanaan 2006). Therefore the antibody used to detect LAP in the injury-conditioned medium (CM) in the following experiments was only specific to LAP1 (see section 5.2.2).

When latent complexes of TGFβ were characterised, the complexes were found to contain another high molecular weight protein, which was cloned and named as latent TGFβ binding protein (LTBP) (Miyazono *et al*. 1991). LTBP is able to form a disulfide bond with the propeptide (LAP) of TGFβ to form large latent complexes (LLCs). It was subsequently found that TGFβs are mainly secreted as LLCs, consisting of TGFβ SLC covalently bound to LTBP (Taipale *et al*. 1996). *In vitro* studies have shown that the SLC is secreted very slowly and the majority of it is retained in the Golgi body (Miyazono *et al.* 1991). LTBP is secreted very rapidly, and enhances the secretion of TGFβ into LLCs (Taipale *et al*. 1996; Miyazono *et al*. 1991). LTBP also plays a role to target TGFβ to the extracellular matrix (ECM). This is achieved by interaction between LTBP and fibronectin or fibrillin. Fibrillins show structural homology with LTBP in the epithelial growth factor (EGF) like repeats and eight cysteine residue repeats. There are four LTBP isomers have been characterised, amongst which LTBP-1, -3 and -4 (LTBP-2, cannot bind TGF-β) are able to form LLCs (Munger and Sheppard 2011).

5.1.3.2 TGFβ activation

Most cells secrete TGFβ in a latent form. The activation of latent TGFβ involves the release of the SLC from the ECM pool followed by disruption of the non-covalent interaction between LAP and TGFβ or direct release of active TGFβ from matrix bound LLC, enabling TGFβ to bind its cell surface receptors. The existence of different TGFβ isoforms and latent forms, as well as the number of different LTBPs suggest that multiple pathways exist for the activation of TGFβs.

The *in vitro* activation methods of TGFβ include acidic or basic pH, heat treatment or different chaotropic agents (Lawrence *et al.* 1985). These *in vitro* conditions are used in many TGFβ purification procedures, thus the purified TGFβ is frequently obtained in its active state.

Proteolysis (Lyons *et al.* 1988; Lyons *et al.* 1990; Abe *et al.* 1998), acidic cellular microenvironments (Jullien *et al.* 1989), the matrix protein thrombospondin (TSP) (Schultz-Cherry and Murphy-Ullrich 1993) as well as integrin $\alpha_v\beta_6$ (Munger *et al.* 1999) have all been proposed to mediate TGFβ activation under physiological conditions. Proteolysis is involved in the release of the SLC from the ECM through cleavage of LTBP as well as the release of active TGFβ by degrading the LAP propeptide. TSPs are large, multimeric proteins that are able to activate both the LLC and recombinant SLC (Schultz-Cherry and Murphy-Ullrich 1993). The activation mechanism involves the interaction of N-terminal end of LAP and the type I repeats of TSP possibly by inducing a conformational change in LAP thus it is no longer able to trap the active TGFβ dimer (Schultz-Cherry and Murphy-Ullrich 1993). This activation can be achieved with small peptides having the KRFK amino acid sequence. Integrins such as $\alpha_v\beta_6$ are also able to activate TGFβ1 (Munger *et al*. 1999). The LAP propeptide contains an RGD-motif that is recognised by integrins $\alpha_v\beta_1$ and $\alpha_v\beta_6$ (Munger *et al.* 1999). Similarly, binding of integrin to LAP also induces a conformational change in LAP,

which as a result releases active TGFβ. The action of integrin can also be combined with proteolysis. The SLC presented by integrin on the cell surface becomes accessible by proteases such as MMP2 and 9 (Yu and Stamenkovic 2000). However, mice with mutations in MMP2 and MMP9 genes can still activate TGFβ and do not show any TGFβ deficiency phenotypes possibly suggesting redundancy among the activating enzymes (Annes *et al.* 2003).

5.1.3.3 TGFβ in cartilage

In cartilage, TGFβ is one of the most extensively studied regulators (Blaney Davidson *et al.* 2007) due to well-characterised ability to regulate the biosynthesis of a large number of cartilage extracellular matrix molecules (Chandrasekhar and Harvey 1988; O'Keefe *et al.* 1988; Recklies *et al.* 1998; Redini *et al.* 1988; Niikura and Reddi 2007; Schmidt *et al.* 2008). However, the extracellular regulation of TGFβ activity and the physiologic mechanisms through which chondrocytes access the growth factor continue to be unclear. In articular cartilage, TGFβ is also secreted in latent form and stored in the cartilage ECM as LLCs (Annes *et al.* 2003; Hyytiäinen *et al.* 2004). Although various mechanisms of TGFβ activation have been characterised in a number of tissues, studies have rarely addressed these mechanisms in articular cartilage. MMP3 (Maeda *et al.* 2001), MMP13 (D'Angelo *et al*. 2001) and transglutaminase (Le *et al.* 2001) have been shown *in vitro* to play a role in TGFβ activation in growth plate cartilage or aging articular cartilage. Mechanical forces may also activate TGFβ. This idea was originally demonstrated in myofibroblasts, where integrin-mediated cellular contractions were shown to pull on matrix bound latent TGFβ to induce activation (Wipff *et al.* 2007). This was then followed by a study by Ahamed *et al,* who demonstrated that mechanical shearing of blood serum was able to activate soluble TGFβ in the absence of integrins and cells (Ahamed *et al*. 2008; Ahamed *et al.* 2009). Using the same concept, Albro's group reported that shearing synovial fluid could also activate the

latent TGFβ present in it (Albro *et al.* 2012). It was postulated that hyaluronan macromolecules present in the synovial fluid, when sheared, become entangled with the latent TGFβ complex to break its weak non-covalent bond between LAP and TGFβ. When the same group carried out mechanical loading of bovine articular cartilage they were unable to demonstrate TGFβ activation (Albro *et al.* 2013). The studies I will now present show that TGFβ is activated by mechanically damaged articular cartilage and reveal a novel mechanism of TGFβ release and activation.

5.2 Results

5.2.1 Release of TGFβ protein in response to porcine cartilage injury

Having demonstrated that recombinant CTGF increased TGFβ release from rested porcine articular cartilage (section 4.5.7) and cartilage injury led to the release of endogenous CTGF in the conditioned medium (section 3.2.4), I next investigated whether the endogenous CTGF released from cartilage matrix in response to cartilage injury had effect on the bioavailability of TGFβ.

Porcine cartilage explants were rested in serum free medium for 48hr and recut conditioned medium (CM) was collected at various time points. Cumulative collection (collecting everything over the entire time period) of recut CM was made in the presence or absence of CHX. Samples were western blotted for CTGF and TGFβ. In these experiments, CTGF was detected as early as 15min and these levels were maintained over longer time points (1hr, 18hr and 24hr) (Figure 5.1A). In contrast, TGFβ was detected only after 1hr following recutting and increased in the medium at later time points (Figure 5.1A). In the presence of CHX, both CTGF and TGFβ could still be detected, with a slight reduction in the amount of CTGF at longer time points (Figure 5.1B). This suggests that both CTGF and TGFβ are

passively released rather than require protein synthesis. They also suggest that CTGF is released first followed by a delayed release of TGFβ.

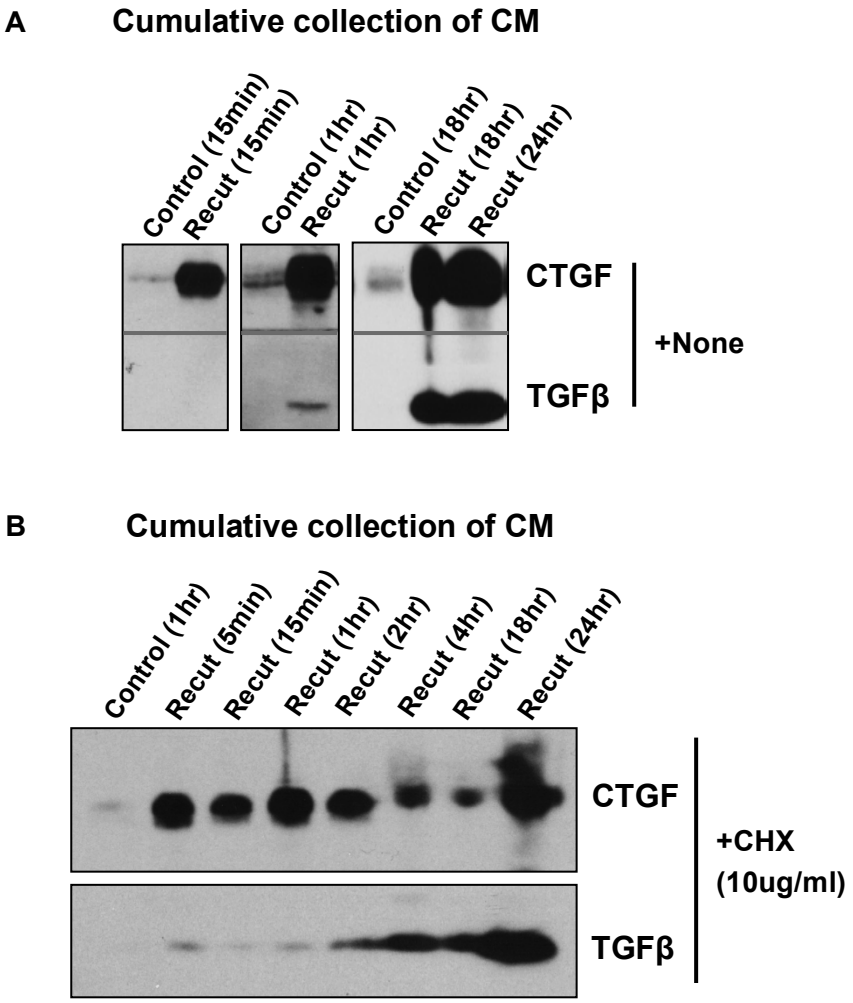


Figure 5.1 Cartilage injury causes early release of CTGF, followed by delayed release of TGFβ. Dissected porcine cartilage explants were rested in serum free medium for 48hr and recut in fresh medium in the absence (A) or presence (B) of CHX (10ug/ml). The recut CM was collected cumulatively at various time points (from 5min to 24hr) and western blotted for CTGF and TGFβ.

5.2.2 TGFβ protein released in the CM in a latent complex containing LAP

As described in the introduction, TGFβ is secreted in a large latent complex (LLC) and stored in the extracellular matrix through the interaction with LTBP. TGFβ activation involves liberation of the small latent complex (SLC) from the matrix and the release of the active TGFβ dimer at the cell surface. It was therefore important to determine whether the TGFβ protein detected in the cartilage injury CM was in a latent form. The CM had thus far been analysed in the presence of the reducing agent dithiothreitol (DTT) with boiling, which breaks disulfide bonds and non-covalent bonds respectively to linearise protein peptides. TGFβ protein detected under this reducing condition runs at 12.5kDa on gel electrophoresis, which represents the molecular weight of monomeric TGFβ. A small TGFβ latent complex consists of a disulfide bonded TGFβ dimer enclosed by a disulfide bonded LAP dimer via a non-covalent interaction. To determine whether TGFβ detected in the CM was in the latent form, I electrophoresed the injury CM (explantation 48hr) under non-reducing conditions (without DTT) in addition to the reducing (with DTT) condition and western blotted with antibodies against TGFβ and LAP1. I also ran alongside positive controls of recombinant latent TGFβ1 (latent TGFβ1) (10ng) and recombinant active TGFβ1 (TGFβ1) (1ng).

As shown in figure 5.2, under the non-reducing conditions (lane 1 to 3), no immunoreactive material could be detected by the anti-TGFβ antibody in the latent TGFβ1 sample (lane 1) nor the injury CM (lane 3). However, this antibody was able to detect the TGFβ dimer (25kDa) band as well as a higher molecular weight band which was likely a multimer of TGFβ present in the TGFβ lane (lane 2). This suggests that there is no free TGFβ molecule in the latent TGFβ1 sample nor in the injury CM. In contrast, under reducing conditions (lane 4 and 5), a protein band of 60kDa could be seen in the latent TGFβ1 sample (lane 4) and a strong 12.5kDa, 25kDa and a weak 37kDa bands were observed in the CM lane (lane 5). The 60kDa band observed in the latent TGFβ is likely to be the TGFβ pro-peptide, which has

been reported between 50 to 60kDa in other studies. The strong 12.5kDa and 25kDa bands seen in the CM are TGFβ monomer and dimer respectively. The strong detection of TGFβ in the CM under reducing condition but not non-reducing condition suggests that TGFβ released in the injury CM of porcine cartilage was in the latent form. Under non-reducing conditions, the TGFβ dimer is trapped in a 'shield' of LAP thus the antibody was unable to access its epitope probably explaining why no detection of TGFβ was seen under this condition. This phenomenon has also been reported by others (Kaur and Reinhardt 2012).

On the right hand panel where anti-LAP1 antibody was used, under non-reducing conditions no band could be seen in the latent TGFβ1 (lane 6), whereas several intense high molecular weight bands (80kDa, 120kDa, 150kDa and 250kDa) were detected in the CM (lane 7). Under reducing conditions, the same 60kDa band (TGFβ propeptide) could be seen in the latent TGFβ1 lane (lane 8) and several weak bands between 80kDa and 30kDa were detected by the antibody in the CM lane (lane 9) (Figure 5.2). These results suggest that the purchased recombinant latent TGFβ is largely intact TGFβ proprotein and does not contain much SLC. The immunoreactive material detected by LAP antibody in the CM under non-reducing condition had large molecular weight, implying the latent complex. It is not clear why there are so many immunoreactive LAP bands when the sample was reduced. The strong band at 75kDa is likely to be a LAP dimer.

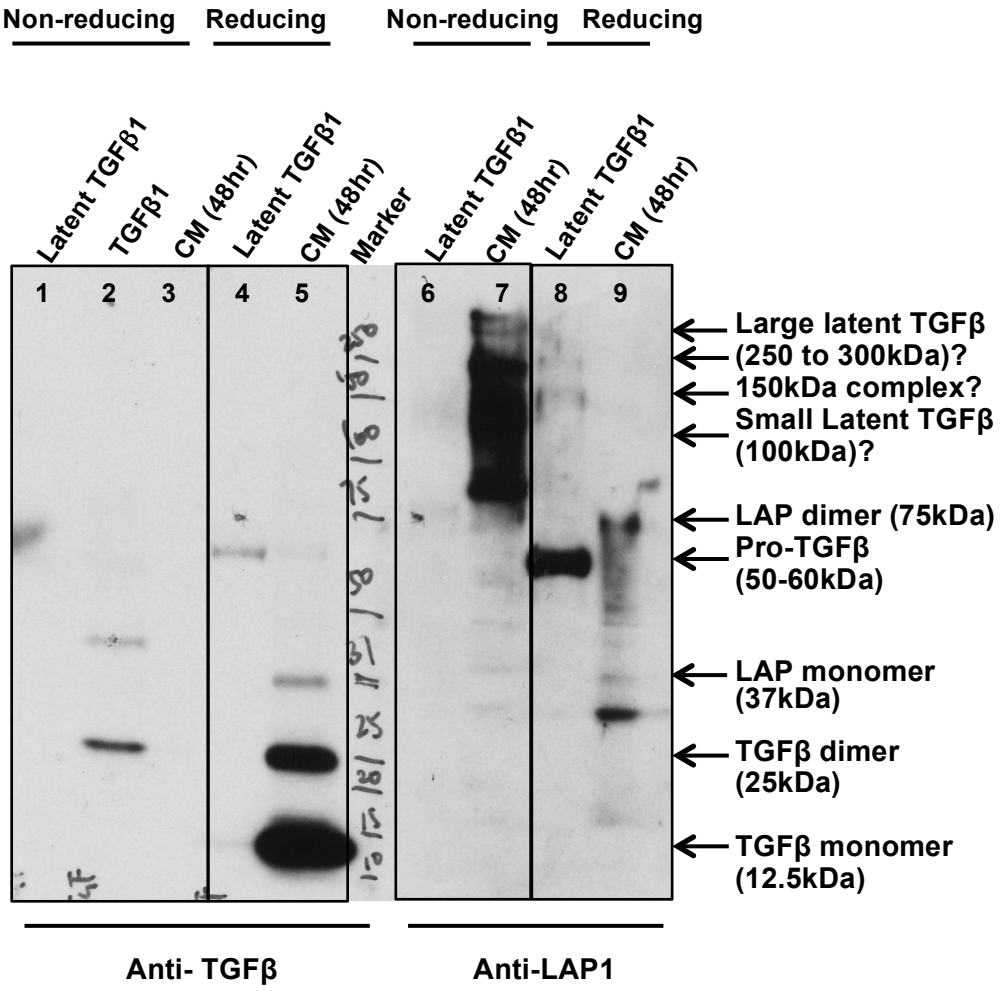


Figure 5.2 Non-reducing analysis of injury CM revealed released TGFβ molecules in latent form containing LAP. Explantation (48hr) CM was analysed under both non-reducing (no DTT) and reducing (containing DTT) conditions with heating and western blotted using antibodies against TGFβ (lane 1 to 5) and LAP1 (lane 6 to 9). Arrows indicate the speculated protein for each band in the lanes.

Although the TGFβ molecules released in the CM appeared to be in a latent form, I next examined whether this CM was capable of activating SMAD2 in porcine chondrocytes. Freshly isolated porcine chondrocytes were rested in serum free medium for 24hr and then treated with or without the explantation (48hr) CM in the presence or absence of anti-TGFβ neutralising antibody for 45min. The CM was also passed through a 50kDa cut-off ultracentrifuge spin column to separate protein molecules larger (>50kDa) or smaller (<50kDa) than 50kDa and then the two separated fractions were used to treat the cells at

equivalent concentration for 45min. Figure 5.3 shows the western blot of the cell lysates for phospho-SMAD2 (pSMAD2). The CM was able to activate SMAD2 similar to the positive control (TGFβ1). This activation was inhibited by TGFβ neutralising antibody, suggesting that activity is from TGFβ rather than another SMAD2 activator. This activity was present in the high molecular weight fraction (>50kDa) rather than the filtrate (<50kDa). This provides further support that TGFβ in the CM is present in a large complex.

The protein caused SMAD2 phosphorylation is larger than 50kDa

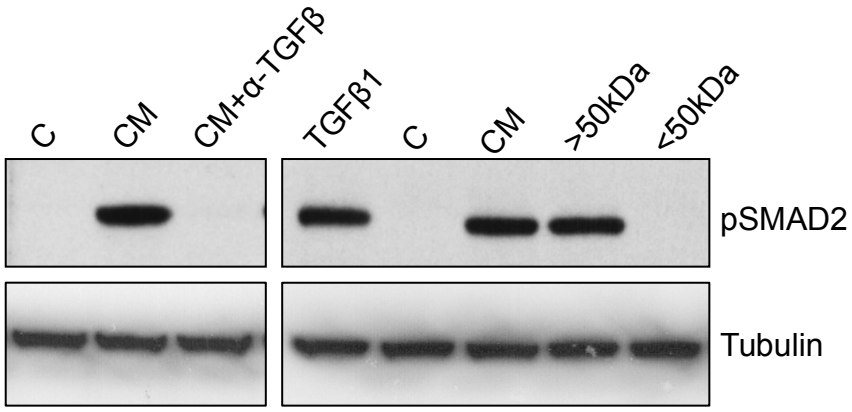


Figure 5.3 The explantation CM containing large TGFβ protein molecules was able to activate SMAD2 in isolated porcine chondrocytes. Rested (24hr) porcine chondrocytes were treated with or without explantation (48hr) CM in the presence or absence of TGFβ neutralising antibody (left panel). Molecules in the CM were separated by a 50kDa cut-off spin column and the resulted fractions were used to treat rested porcine chondrocytes for 45min (right panel). The lysates were analysed for pSMAD2 and tubulin for loading control.

5.2.3 TGFβ activation in cartilage injury CM is not through integrins or MMPs

There was no detection of free (active) TGFβ in the injury CM under non-reducing conditions suggesting that TGFβ activation took place at cell surface of the stimulated porcine chondrocytes. Reported mechanisms of TGFβ activation under physiological state involve mainly cell surface molecules including integrins and MMPs, which induce conformational change and cleavage of LAP respectively to release active TGFβ (Munger and Sheppard 2011). To test whether integrins or MMPs are involved in TGFβ activation in cartilage I treated porcine chondrocytes with cartilage explantation CM in the presence or absence of integrin and MMP antagonists: synthetic RGD peptide and GM6001 respectively. Rested porcine chondrocytes were pre-incubated with or without RGD (500μM) or GM6001 (100μM) for 1hr before being stimulated with the explantation (48hr) CM in the presence or absence of the inhibitors for 45min. The cell lysates were then analysed for pSMAD2. As shown in figure 5.4, there is no difference in the pSMAD2 signal with and without the inhibitors. This may suggest that TGFβ activation in cartilage in not solely mediated by integrins or MMPs.

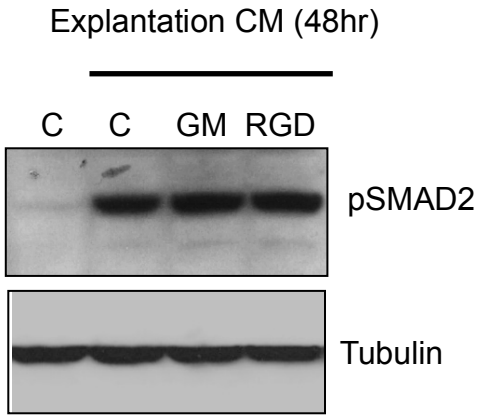


Figure 5.4 Inhibitors of integrin or MMPs did not inhibit TGFβ activation in chondrocytes. Rested porcine chondrocytes were pre-incubated with or without RGD (500μM) or GM6001 (100μM) for 1hr before being stimulated with the explantation (48hr) CM in the presence or absence of the inhibitors for 45min. The lysates were analysed for pSMAD2 and tubulin for loading control.

5.2.4 CTGF and LAP in the injury CM

Since there was both LAP and TGFβ in the injury CM and activity was exclusively associated with a high molecular weight complex, I explored the relationship between these molecules further. Rested porcine cartilage explants were treated with recombinant CTGF (100ng/ml) for 1hr or recut and medium was collected cumulatively at various time points. The resulting CMs were analysed under reducing condition for CTGF, LAP and TGFβ. As shown in figure 5.5, release of CTGF (37kDa) occurred within 5min of recutting and the protein level remained constant over time. The level of TGFβ protein (12.5kDa), as shown previously, was increased upon recombinant CTGF stimulation. TGFβ was not increased immediately after cutting, but accumulated in the CM over time as shown previously (Figure 5.5). Interestingly, LAP1 was also elevated by recombinant CTGF stimulation, and recutting caused LAP1 accumulation in the CM within the first 5min, decreasing at later time points (Figure 5.5). The predominant band picked up by the anti-LAP1 antibody in these experiments was at 75kDa, which coincides with the molecular weight of the LAP dimer. Repeated western blots under the same conditions also revealed LAP1 at 75kDa rather than its monomeric form of 37kDa, suggesting that reduction has been incomplete.

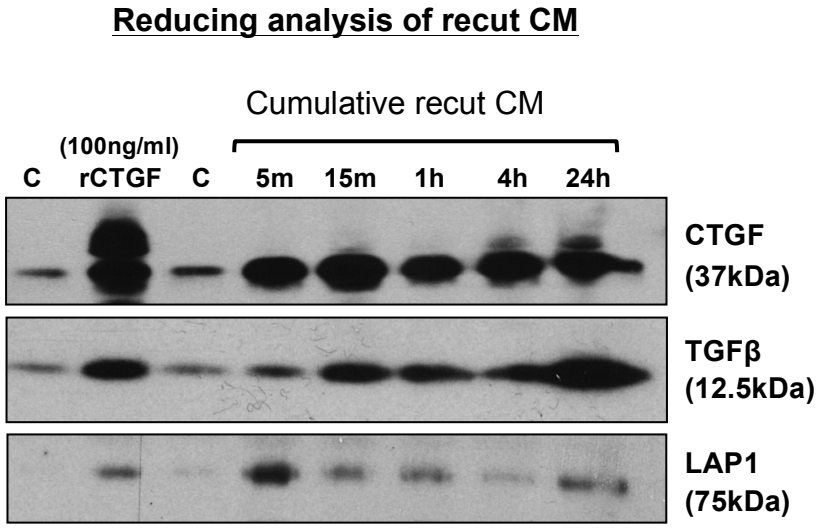


Figure 5.5 Reducing analysis of injury CM revealed release of LAP. Rested porcine cartilage explants were treated with recombinant CTGF (100ng/ml) for 1hr or recut and medium was collected for various times. The resulting CMs were analysed under reducing condition for CTGF, LAP and TGFβ.

Analysis of the injury CM under reducing conditions showed that the accumulation of LAP1 correlated better with levels of CTGF than TGFβ, so I examined the relationship between CTGF and LAP in the injury CM further. Explantation CM (5 to 120min and 4 to 48hr) was western blotted for CTGF and LAP1 under non-reducing conditions. Instead of running at 37kDa and 75kDa for CTGF and LAP respectively, both CTGF and LAP ran at a high molecular weight (around 150kDa) on non-reducing SDS-PAGE (Figure 5.6), suggesting that CTGF and LAP may associate in a large molecular weight complex. Samples in the first blot were overloaded; strong bands were detected at various high molecular weights. These may be CTGF oligomers or complex with other proteins. Only a small portion of monomeric CTGF was detected at 37kDa at 24 and 48hr. This monomeric CTGF may be newly synthesised protein or be disassociated from the large complex. The early detection (within 5min upon explantation) of both LAP and CTGF in the form of large complex may suggest

that SLC is released together with CTGF (in a pre-existing complex or independently and then rapidly bind to one another) in response to cartilage injury. Increased accumulation of high molecular weight CTGF and LAP at later time points may suggest continuous release of SLC as more CTGF being synthesised and complexes with the SLC to assist its release from the matrix pool.

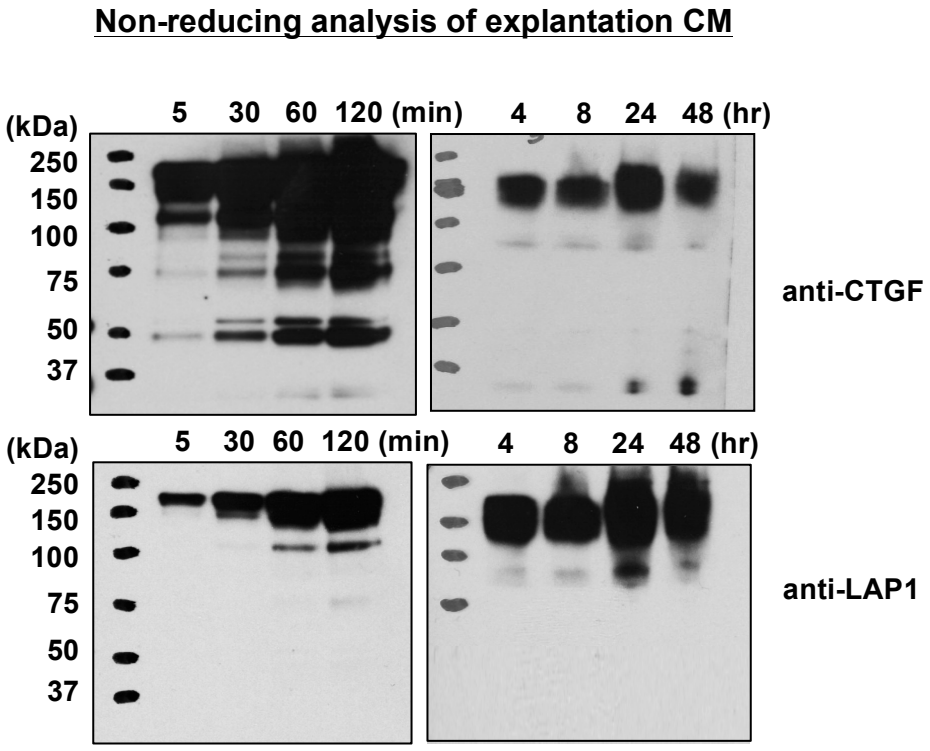


Figure 5.6 Non-reducing analysis of the explantation CM reveals that CTGF and LAP ran at high molecular weight. Porcine cartilage explants were dissected in serum free medium and the CM was collected after 5 to 120min or 4 to 48hr and then western blotted under non-reducing condition for CTGF and LAP.

The explantation CM (48hr) was also analysed for TGFβ and LTBP1 under both the reducing (R) and non-reducing (N) conditions. As shown before, no TGFβ could be detected in the CM under non-reducing conditions, whereas under reducing conditions 12.5kDa (monomeric TGFβ) and 100kDa bands (may be SLC) could be detected by the same anti-TGFβ antibody (Figure 5.7). LTBP1 antibody could detect bands under both reducing and non-reducing

conditions at around 150kDa, which coincided with the molecular weight of the monomeric LTBP1. Under the non-reducing conditions, a higher molecular weight band (around 250kDa) was also detected by the LTBP1 antibody, which may be the large latent complex (LLC). CTGF was usually detected in the CM under non-reducing conditions at around 150kDa, which is likely in a complex that only contains a SLC. Therefore I only investigated the relationship between CTGF and LAP in the following studies.

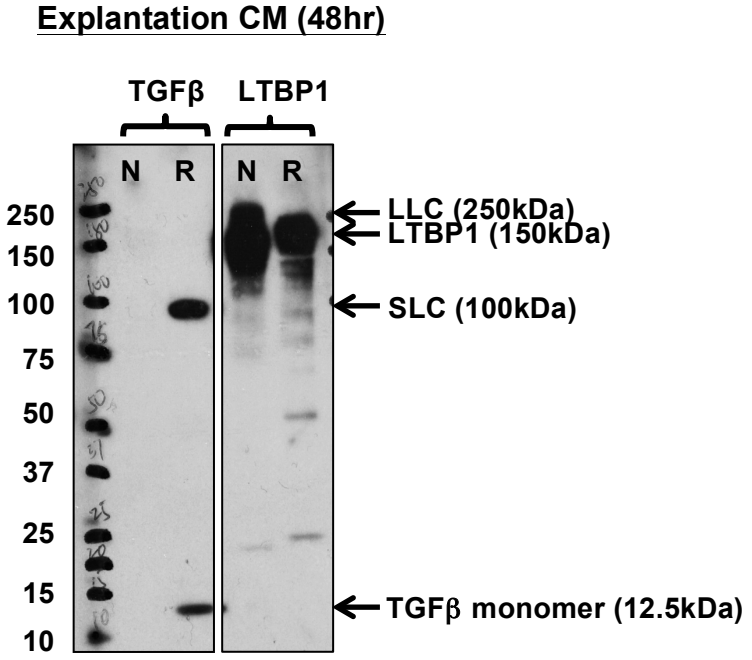


Figure 5.7 Non-reducing and reducing analysis of the explantation CM for TGFβ and LTBP1. Porcine cartilage explants were dissected in serum free medium and after 48hr the explantation CM was collected and western blotted under both non-reducing conditions (N) and reducing conditions (R) for TGFβ and LTBP1.

To confirm that the 150kDa band contained CTGF and LAP and to determine whether it also contained TGFβ, I extracted the protein from the gel piece containing the band and analysed it by western blot under reducing condition. The explantation (48hr) CM was electrophoresed under both reducing (R) and non-reducing (N) conditions. After electrophoresis, the gel was Coomassie-blue stained to visualise the bands of interest. I then cut off the gel piece around the 150kDa band (where CTGF and LAP co-migrate to under non-reducing conditions) from

the N lane and the 75kDa (LAP dimer), 37kDa (CTGF) and 12.5kDa (TGFβ) bands from the R lane. The gel pieces were transferred into new eppendorf tubes containing 100µl of extraction buffer (see 2.5.8). The gel pieces were minced using a scalpel and incubated in the buffer at 30°C overnight with shaking. The tubes were then centrifuged to spin down the gel pieces and the supernatant was collected and analysed by SDS-PAGE under reducing conditions. In figure 5.8, the left image shows the position where gel pieces were cut from the Coomassie-blue stained gel. The right image shows the western blot of the samples extracted from the gel pieces respectively (N1, R1, R2 and R3). The same blot was cut and probed for LAP, CTGF and TGFβ. As seen in figure 5.8, N1 contains a 100KDa protein that was also detected in R1 by anti-LAP1 antibody. N1 also contained a 37kDa protein that was detected in R2 by the anti-CTGF antibody. Despite seeing a clear TGFβ band in the reduced CM sample, the high molecular weight band did not appear to contain TGFβ. The 37KDa bands observed in N1 and R2 and the 12.5kDa band observed in R3 are CTGF and TGFβ respectively. The 100kDa picked up by the anti-LAP antibody has been seen in other experiments and may be the small latent complex of TGFβ although its mobility has not changed under reducing conditions. Overall, this experiment implies that the protein band around 150kDa observed on the non-reducing western blot contains CTGF, but it does not convincingly demonstrate the presence of small latent TGFβ.

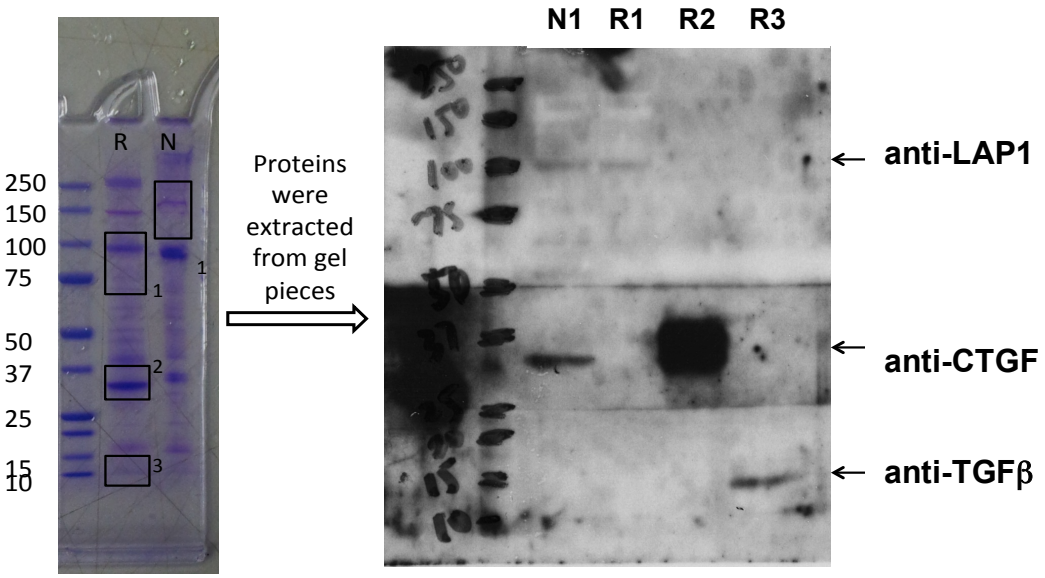


Figure 5.8 The high molecular weight protein in injury CM contains CTGF. Cartilage explantation CM was electrophoresed under both reducing (R) and non-reducing conditions (N). The gel was Coomassie- blue stained and bands of interest were cut at appropriate molecular weights (150kDa from N1, 100~75kDa from R1, 37kDa from R2 and 12.5kDa from R3) (left image). Protein extracted from these gel pieces and analysed under reducing conditions by western blot for LAP, CTGF and TGFβ (right image).

5.2.5 Purification of CTGF/TGFβ SLC complex by chromatography

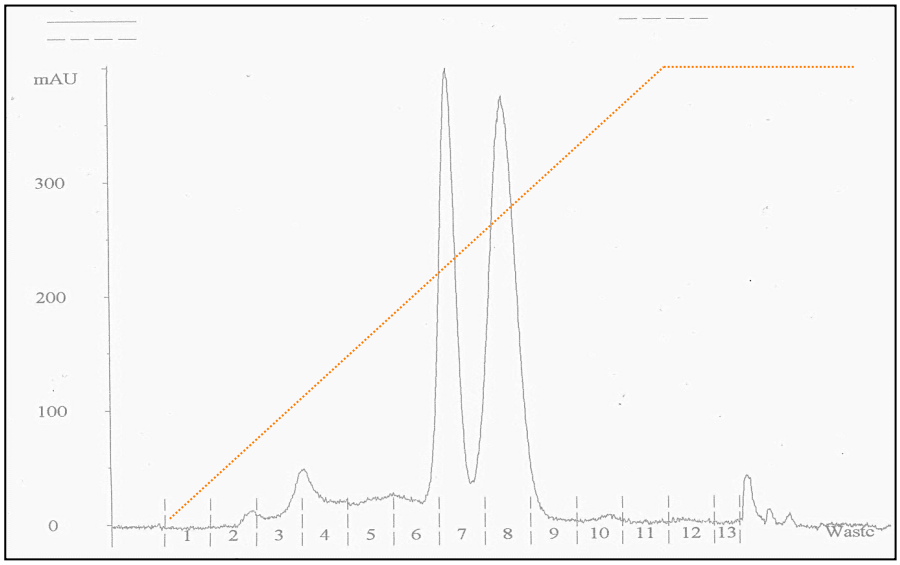
I next attempted purification of the complex to determine whether CTGF and the SLC would co-purify. I used 2 methods of purification. The first method was ion exchange chromatography that separates proteins according to their charge. The second method was size exclusion chromatography which separates proteins according to size.

5.2.5.1 Ion exchange chromatography

For ion exchange, I used a 1ml HiTrap SP HP column which is an cation exchange column containing negatively charged resin that interacts with positively charged protein molecules. After removing debris by centrifugation, 1ml explantation (48hr) CM was diluted in 4ml binding buffer (1:5) and passed through the column. After adequate washing, resin bound proteins were eluted in 1 ml size fractions with increasing concentrations of NaCl. Figure 5.9A is the profile of the ion exchange assay showing two large absorbance (280nm) peaks

between fractions 6 to 8 and several small peaks at fractions 3 to 4. The fractions (2 to 10) were analysed, alongside the starting material before (CM) and after (SM) 5 folds dilution in binding buffer. Western blots for CTGF and TGFβ (under reducing conditions) (Figure 5.9B) and LAP (under non-reducing conditions) (Figure 5.9C) are shown. As shown in the blot, CTGF (37kDa) could be detected in both the CM and SM (5 fold less concentrated than CM) and in the fractions of 3, 4, 5, 6 and 7 amongst which fractions 4, 5, 6 showed more CTGF. This detection correlated with that of TGFβ (12.5kDa), which was also observed in CM, SM and fractions 4, 5 and 6. The non-reducing analysis of the fractions showed LAP1 (150kDa) in fractions 3, 4, 5 and 6. Although the positive fractions are slightly shifted in position, strong detection for LAP1, CTGF and TGFβ could be seen in fractions 4 and 5. The pI (pH of isoelectronic point) values of porcine CTGF (Kozlowski LP. 2007-2013 Isoelectric Point Calculator) and latent TGFβ1 (Beatson *et al.* 2011) are 8.0 and 5.5 respectively. At the working pH 7.4 (i.e. the pH of the binding buffer), only the positively charged CTGF is likely to bind to the negatively charged column while the negatively changed latent TGFβ is supposed to be washed through. Therefore, the detection of both proteins in the same fractions suggests that they may associate in a single complex.

A **Chromatographic profile of ion exchange of porcine cartilage explantation CM**



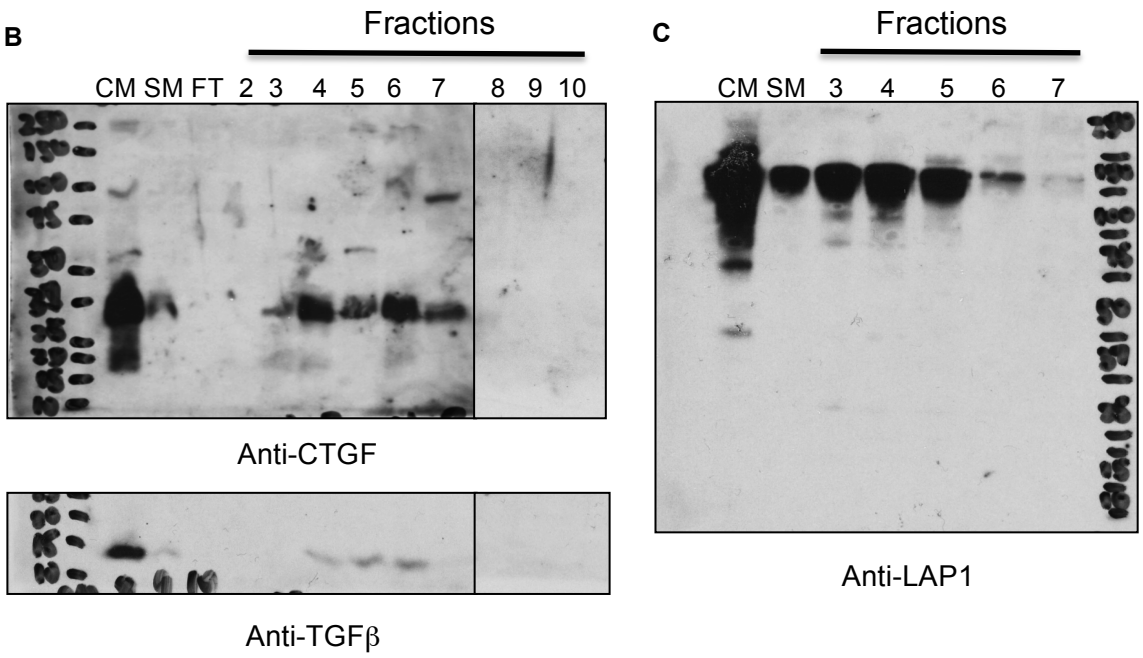


Figure 5.9 Purification of CTGF/TGFβ SLC by cation exchange chromatography 1ml of explantation (48hr) CM was centrifuged and diluted in 4ml of binding buffer (SM). SM was then passed through a 1ml cation exchange HiTrap SP HP column and the flow through (FT) was collected in a new tube. Following several washes the bound protein was eluted in 1ml fractions using NaCl in a linear gradient from 0 to 1M (A). The Fractions were then electrophoresed under reducing conditions for CTGF and TGFβ1 (B) and non-reducing conditions for LAP1 (C).

5.2.5.2 Gel filtration chromatography

Gel filtration column contains polymer beads with pores of different sizes. Large molecules cannot pass through the small pores so they run straight down the column, while smaller molecules enter the pores between the beads so travel slowly through the column. As a result, protein molecules with large molecular weight are eluted first from the column, followed by smaller molecules. The protein (CTGF/TGFβ SLC) I aimed to purify is between 100 and 250kDa, so I used a Superose 12 PC3.2/30 column, which works in a SMART system (for small volume samples) with a separation range of 1 to 300kDa.

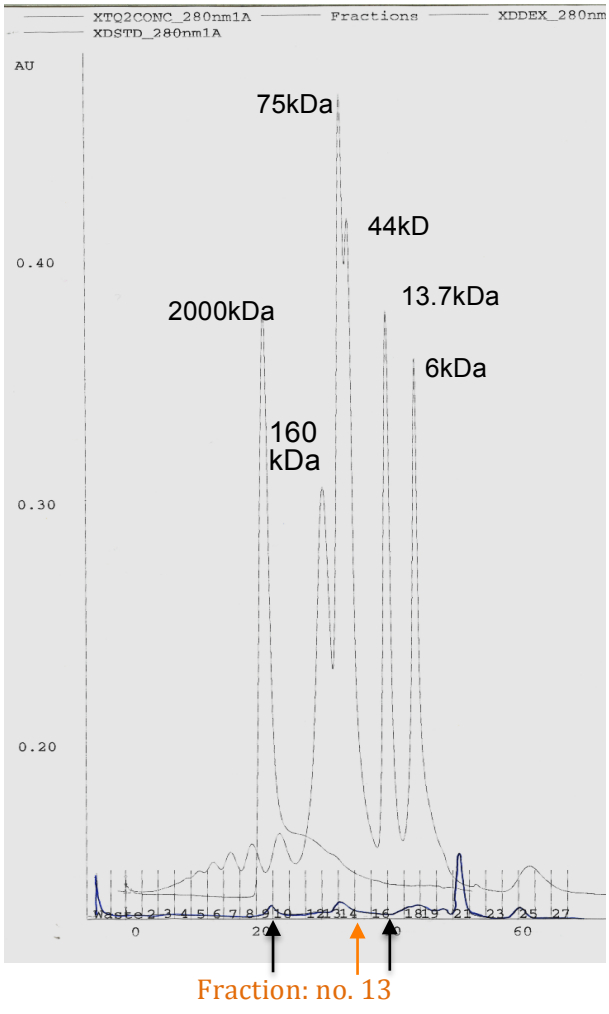
I pooled the positive fractions 3, 4, 5 and 6 from ion exchange together and concentrated the sample down to 50µl for size exclusion chromatography to determine whether these proteins

were still running in a high molecular weight complex. Before loading the sample onto the gel filtration column, I first carried out a standard run using 15µl of mixed standard proteins of various molecular weights (Figure 5.10A). The dotted line on the profile shows the separation of the 6 standard proteins, represented as 6 sharp peaks. The tested sample was then passed through the same column and collected in 100µl fractions (No.1 to 27). The profile generated from this test run (blue solid line) was overlapped with that of the standard proteins to identify approximate molecular weights of the complex of interest. The magnitude of peaks of tested protein is small compared with the standard peaks suggesting low protein concentration. CTGF/SLC was expected to have a molecular weight of around 150kDa, so I analysed the fractions No. 8 to 15 containing peaks between 2000kDa and 44kDa of the standard profile (Figure 5.10B). The samples were western blotted alongside the SM, for CTGF under both reducing and non-reducing conditions and TGFβ under reducing conditions only. As shown in figure 5.10C, a high molecular band (between 150 and 250kDa) was detected on the non-reducing blot by the anti-CTGF antibody in fraction 13. From the profile of the standard proteins, fractions 13 should correspond to proteins at around 160kDa. Proteins migrate differently on column compared with SDS-PAGE, in which denaturing agents (SDS and boiling) linearise proteins to cause retardation in gel. However, under reducing conditions, no CTGF or TGFβ was detected in fraction 13 nor in the other fractions. This may be because the protein concentration in the starting material was low, likely lost when the sample was concentrated using a spin cut-off column.

A

Standard proteins	Molecular weight	Concentration
Blue Dextran 2000	>2000kDa (eluted in void volumn)	50mg/ml
γ Globulin	160kDa	50mg/ml
Conalbumin	75kDa	50mg/ml
Ovalbumin	44kDa	50mg/ml
Ribonulease A	13.7kDa	50mg/ml
Aprotinin	6.5kDa	10mg/ml

B



C

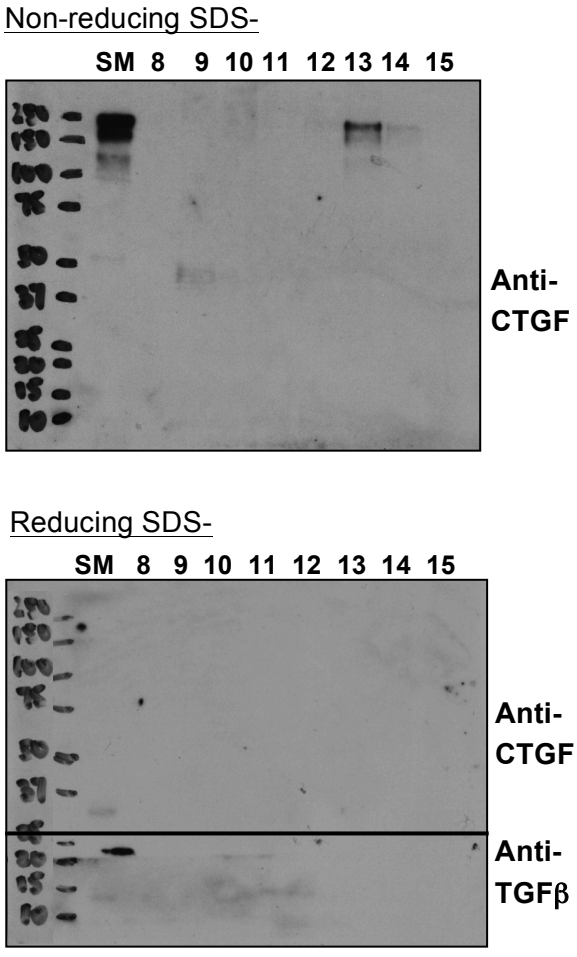


Figure 5.10 Gel filtration analysis of the ion-exchanged sample containing CTGF, TGFβ and LAP1. Positive ion exchange fractions (3 to 6) were pooled together and concentrated down to 50μl (~0.2mg/ml). A mixture of standard proteins (A) was first passed through the Superose 12 PC3.2/30 column. The profiles from standard and test samples were overlapped (B). Fractions (8 to 15) (black arrows indicated) containing a peak around 160kDa were western blotted for CTGF and TGFβ under either non-reducing or reducing conditions (C). n=2

Crude injury CM was also analysed by gel filtration chromatography. In order to minimise abundant plasma proteins and matrix macromolecules, I used recutting (24hr) CM, which was generated after the cartilage explants had been rested in serum free medium for 48hr. Crude recutting CM was first centrifuged to remove insoluble material and injected onto the column. The profile of the test sample was again compared to a standard sample containing known proteins (Figure 5.10A). In this experiment several peaks were generated by the CM with higher protein absorbance, suggesting a higher concentration of proteins present in the sample (Figure 5.11A). I chose to analyse fractions 8 to 15 covering the two short peaks positioned between 2000kDa and 44kDa. Each fraction was western blotted alongside the SM for both CTGF and TGFβ under the reducing conditions. In the SM lane, both CTGF and TGFβ were detected with strong intensity (Figure 5.11B). Fractions 9, 10 and 11 contained an unknown band at 45kDa showing immunoreactivity with the anti-CTGF antibody. Proteins in fractions 9 and 10 eluted from the column in the void volume, suggesting that they were aggregated in a large mass that was unable to enter the resin of the column. The weak band detected in fraction 14 migrated at the correct position for monomeric CTGF (just below the 37kDa). TGFβ1 was also detected in this fraction as well we fraction 13. From the standard profile, fraction 14 suggested a molecular weight between 160kDa and 75kDa. This coincides with the expected molecular weight of CTGF/SLC complex. These results support the hypothesis that CTGF released in the injury CM is associated with the SLC of TGFβ. The definitive way to determine whether molecules are in stable complexes is to immunoprecipitate one protein and look for whether the others are present in the precipitated sample.

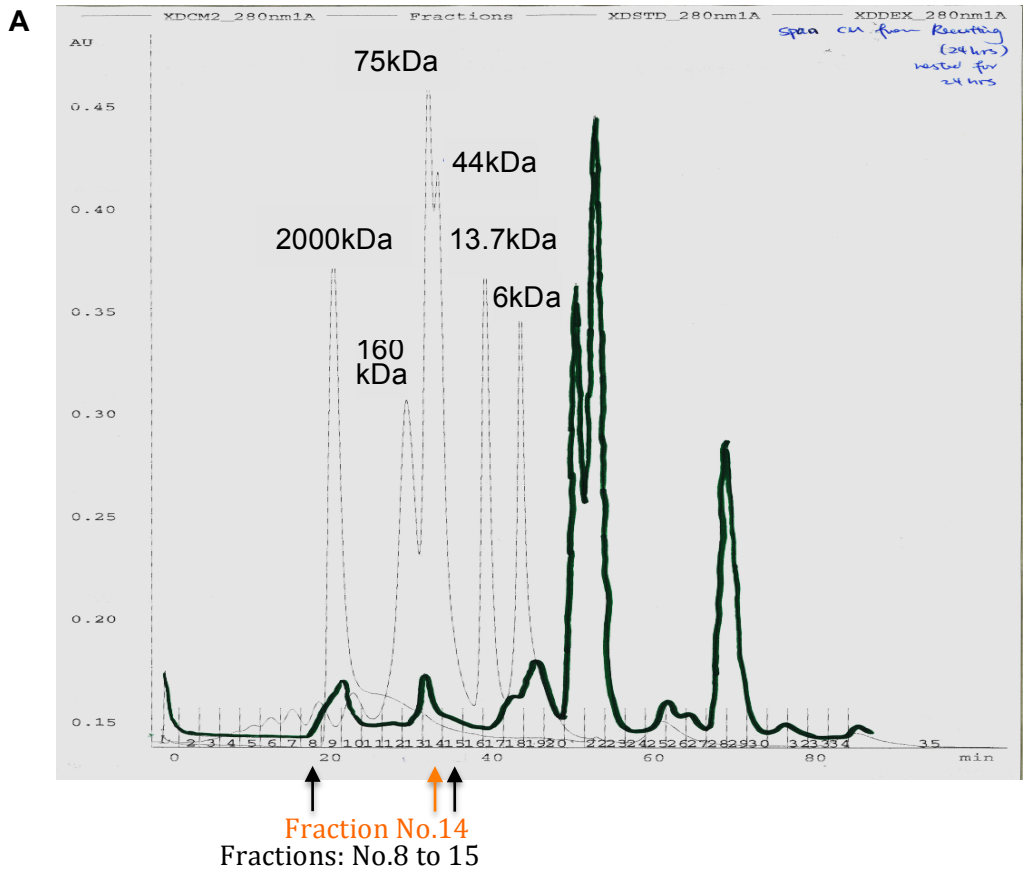
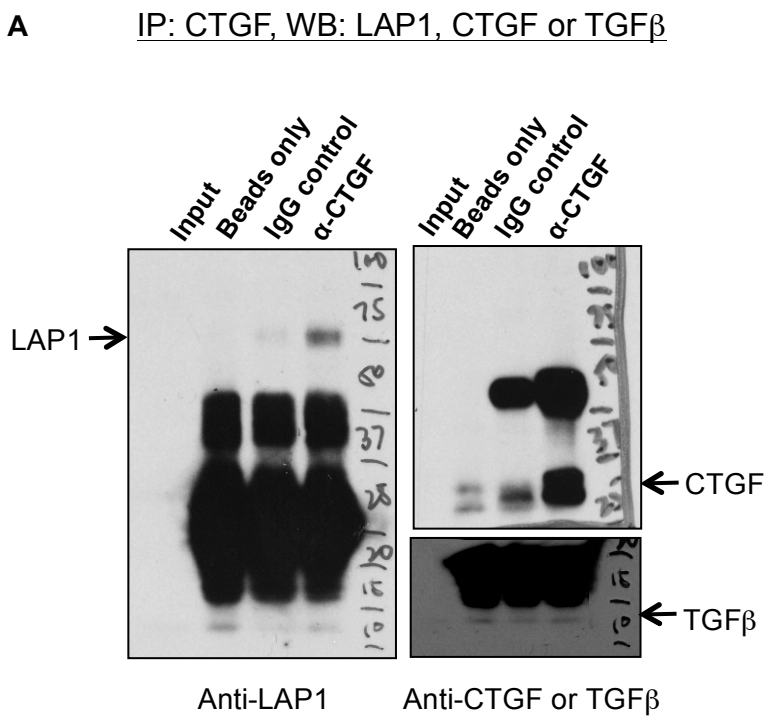


Figure 5.11 Gel filtration analysis of crude injury CM. Crude recutting (24hr) CM was centrifuged and passed through the Superpose 12 PC3.2/30 column. The profiles from standard (dotted line) and test samples (green solid line) were overlapped (A). Fractions (8 to 15) (black arrows indicated) containing two peaks between 2000kDa and 44kDa were western blotted for CTGF and TGFβ under reducing conditions (B). Fraction 14 (in red) shows detection of both CTGF and TGFβ, suggesting the possible association between the two protein molecules. n=1

5.2.6 Immunoprecipitation of CTGF an LAP from the injury CM

I carried out immunoprecipitation (IP) assays to pull out CTGF or LAP from the injury CM and then western blot to see if LAP or CTGF was associated with each other. Protein A/G agarose beads were incubated with the explantation (48hr) CM in the presence or absence of polyclonal goat anti-CTGF or anti-LAP1 antibody while goat IgG was used as an isotype control. Incubated beads were then washed, spun down and analysed under reducing conditions. Figure 5.12A shows the IP assay for CTGF, western blotted for both LAP1 and CTGF. In order to reduce non-specific background, protein G-HRP was used instead of IgG HRP. Protein G-HRP detects exclusively the Fc region of intact antibodies from various species (Lal *et al.* 2005). This can avoid the detection of antibody used for IP as those molecules have been denatured during sample preparation by boiling. Even so, non-specific bands were still detected in these blots including the heavy (50kDa) and light (25kDa) chains of the IP antibody but luckily this did not interfere with the detection of proteins of interest. Following immunoprecipitation by anti-CTGF, western blotting for LAP1 showed the usual immunoreactive band at 75kDa in the 'anti-CTGF' fraction, which is likely to be the LAP dimer. This band was absent in the 'beads only' and 'IgG control' lanes showing that it is specific for CTGF (Figure 5.12A). The band was also absent in starting material (input). This was most likely because the concentration of LAP1 is very low relative to the concentrated IP fraction. The membrane was stripped and re-blotted for CTGF and TGFβ. A strong CTGF band can be seen in the 'anti-CTGF' fraction with less in the 'IgG control' and 'beads only' samples, demonstrating effective pull-down. A usual 12.5kDa anti-TGFβ immunoreactive band was seen in all fractions, not correlating with either LAP or CTGF. This may be non-specific detection as a similar molecular weight band (12.5kDa) was also seen in the anti-LAP blot. I was unable to immunoprecipitate successfully using the LAP antibody; CTGF was present in all fractions (Figure 5.12B).



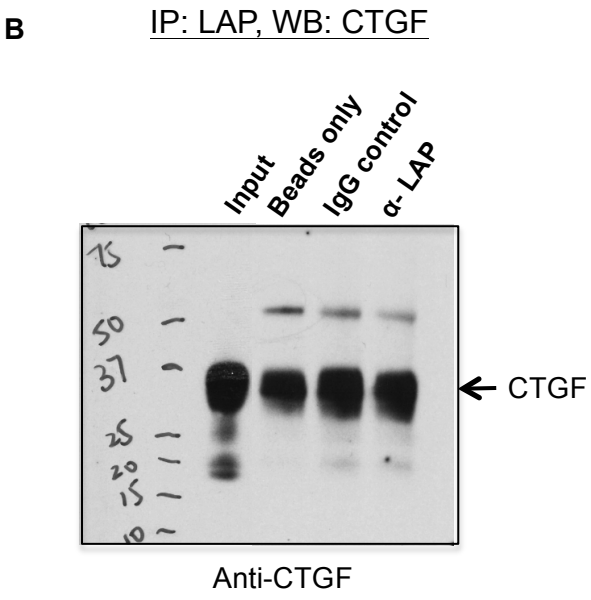


Figure 5.12 Immunoprecipitation of injury CM for CTGF and LAP to determine their interaction. Explantation (48hr) CM was immunoprecipitated with protein G agarose beads and anti-CTGF or anti-LAP1 antibodies; goat IgG was used as an isotype control. The pull-down was western blotted for CTGF and/or LAP.

5.2.7 Localisation of latent TGFβ in human articular cartilage

I next determined the localisation of latent TGFβ in articular cartilage. Frozen normal human articular cartilage was processed as described before (section 2.3) and then immunostained for LAP1 and type VI collagen as a marker of the PCM. As shown in figure 5.13, the immunofluorescent staining of LAP1 co-localised significantly with that of type VI collagen, suggesting that latent TGFβ is also sequestered in the PCM in normal human articular cartilage.

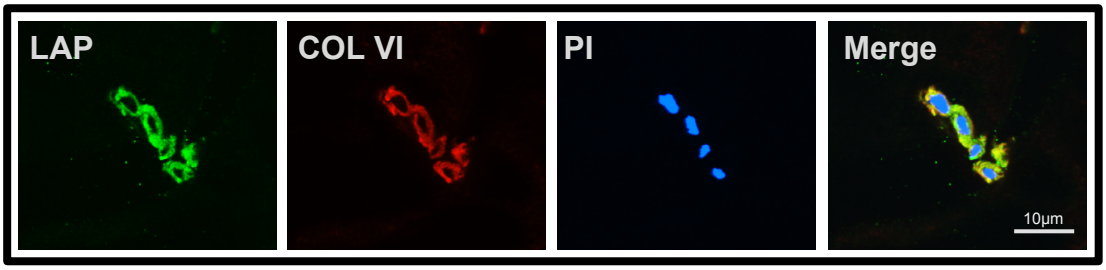


Figure 5.13 Immunofluorescent staining of LAP1 in human articular cartilage. Frozen sections (7µm) of normal human articular cartilage were pre-treated with chondroitinase ABC (10mU/ml for 120min). Sections were stained with primary antibodies to LAP1 (1:500) (green) and type VI collagen (1:1000) (red). Sections were washed and incubated with secondary FITC-labeled antibodies (1:1000) and propidium iodide (PI) (1:2000) (blue). Fluorescence signals were detected by UltraView confocal microscopy (x60 oil) n=3

5.3 Discussion

Intensive mechanical injury predisposes cartilage to OA. However, there is substantial evidence that cartilage attempts to repair damaged tissue. One injury-induced response in cartilage that might trigger this repair is release of FGF2. Release of such growth factors from the PCM appears to act as a 'mechanotransducer' converting mechanical stimuli into cellular responses in articular cartilage (Vincent *et al*. 2002). This mechanism of bioavailability now also appears to be true for CTGF and indirectly TGFβ. CTGF is rapidly released from porcine cartilage explants and accumulated in the CM within the first 5

minutes upon cartilage injury. The rapidity of CTGF release, and the observation that the protein synthesis inhibitor CHX could not abolish the initial accumulation of CTGF supports its release from a matrix, rather than cellular source. Such a mechanism allows for very rapid responses following injurious mechanical insults. The binding of CTGF into the heparan sulphate within the PCM and its rapid extracellular clearance through the LRP receptor means that bioavailable CTGF is present only in short bursts at time of injury.

In cartilage, TGFβ is known to stimulate extracellular matrix production such as collagen and proteoglycan (Redini *et al*. 1988). Increased synthetic activity in early OA is accompanied by an up-regulation of TGFβ expression (Goldring *et al*. 2004). In disease, it is generally felt that the increased anabolic activity generated by TGFβ cannot compensate for the overall catabolic insult to cartilage. Western blot analysis of the injury CM revealed the release of TGFβ protein from injured cartilage. Unlike CTGF, TGFβ builds up in the CM over time following injury. The addition of CHX does not prevent the build-up of TGFβ protein in the CM, suggesting no active protein synthesis event is involved. As mentioned in the introduction, TGFβ is normally synthesised and secreted in a latent complex containing LAP. In a SLC, a disulfide-bonded dimer of LAP encloses a disulfide-bonded dimer of mature TGFβ through a non-covalent interaction. When I analysed the injury CM in the absence of reducing agent (the non-reducing condition), TGFβ was not detected by western blot. This happens regardless whether samples had been boiled. In theory, boiling should break the non-covalent interaction between TGFβ and LAP to liberate the dimer of TGFβ. However western blotting under these conditions was unable to detect any form of TGFβ in the injury CM. This is consistent with most of the TGFβ molecules released from injured cartilage being in an unusually stable latent form. It is generally accepted by others in the field that the antibody cannot access its epitope when the TGFβ dimer is enclosed by LAP.

Although the injury CM apparently contains only the latent form of TGFβ, this medium was able to activate SMAD2 in rested porcine chondrocytes, which was inhibitable by a TGFβ neutralising antibody. This suggests that in chondrocytes, like in many other cells, latent TGFβ is activated on the cell surface. A number of mechanisms involving activation of TGFβ have been reported. Tests using inhibitors GM6001 or RGD peptide implies that TGFβ activation in cartilage is not solely mediated by MMPs or integrins respectively. Under reducing conditions, LAP was predominately detected in the CM at early time point following recutting, correlating with the dynamic of CTGF release rather than TGFβ (slow accumulation at later time points). Under non-reducing conditions, both CTGF and LAP were detected in a large (~150kDa) complex within 5min following injury. These together suggest that both CTGF and SLC are released as an early response to cartilage injury.

Consistent with what I found using recombinant CTGF (chapter 4), a new model of CTGF-induced activation of TGFβ following cartilage injury emerges. CTGF may be involved in both the release of SLC from matrix pool and the liberation of active TGFβ from the SLC at cell surface. This model predicts a direct interaction between CTGF and the SLC. CTGF is able to interact with a number of molecules through its cysteine rich (CR) von Willebrand type c (VWC) domain, including active TGFβ and BMP protein (Abreu *et al*. 2002). The VWC domain of CTGF has 10 cysteine residues that are evolutionally conserved amongst the CR domains of von Willebrand factor, thrombospondin (TSP), procollagens I and II and chordin (Abreu *et al*. 2002). SLC and LTBP are joined by disulfide bonds at one of CR domains of LTBP (Munger and Sheppard 2011). A similar association may be apparent between CTGF and SLC through the homologous CR domain.

I attempted 3 further methods to determine a direct interaction between CTGF and TGFβ/LAP. Firstly, protein extracted from the gel piece containing the 150kDa band was re-run under reducing conditions revealed both CTGF and LAP were present within these

samples. TGFβ was absent from this band, but this may have been due to low abundance of the starting material. The second method involved purification of the 150kDa complex by a combination of ion exchange chromatography and gel filtration. These experiments were challenging but the ion exchange chromatography was at least supportive of a CTGF complex which containing TGFβ and LAP. Finally and most definitively I was able to show immunoprecipitation of LAP from the CM (explantation) using a CTGF antibody. This strongly supports direct binding of CTGF to the SLC of TGFβ or possibly LLC. We are exploring this further with Prof. Cay Kielty's group at University of Manchester to determine whether CTGF binds to TGFβ, LAP or LTBP directly (by BIAcore).

Studies for the activation of TGFβ in cartilage have been very limited. The studies in Albro's group highlighted the importance of mechanical force -induced TGFβ activation in cartilage (Albro *et al*. 2012; Albro *et al.* 2013) This includes synovial fluid shearing and mechanical loading. However, their recent study of mechanical loading did not successfully demonstrate TGFβ activation in bovine cartilage explants. This may be because that the mechanical forces introduced in these studies are not sufficient to induce TGFβ activation, which requires additional interaction with other molecules or that activator is restricted to the pericellular space and cannot be easily measured in culture medium. My studies using a much cruder method of mechanical injury have revealed a potentially novel CTGF-dependent mechanism for TGF activation.

Part II: Study of injury response using CTGF knockout murine cartilage

5.4 Introduction

Another system used to study the role of CTGF is the CTGF knockout mouse (*ctgf*$^{-/-}$*).* The purpose of using the knockout mouse is first to study *in vitro* injury response in cartilage tissue that depleted of CTGF; second is to study the *in vivo* role of CTGF in response to surgically induced joint destabilisation, a validated model of OA. CTGF is essential for chondrogenesis; constitutive CTGF knockout mice die at birth from respiratory failure due to axial skeletal defects. Osteogenesis is impaired due to compromised chondrogenesis and growth plate angiogenesis (Ivkovic *et al.* 2003). Due to the embryonic lethality, we generated inducible conditional CTGF knockout mice models using *Cre*-mediated recombination under the control of the ubiquitin promoter. CTGF floxed mice (*ctgf*$^{flox/flox}$) were acquired through collaboration with Prof. David Abraham at University College London and Prof. George Bou-Gharios at the University of Liverpool. Crossing the floxed mice with the Ubiquitin (Ub) Cre ERT2 generated pan tissue CTGF deleted mice (*ctgf*$^{flox/flox}$ *ERT2*$^{cre/+}$).

5.4.1 Ubiquitin Cre-Lox P knockout system

The Cre–loxP recombination system is well characterised and widely used as a simple tool for genetic modification (Sauer and Henderson 1988). *Cre* (*c*yclization *re*combination) gene encodes a 38kDa site-specific DNA recombinase (Cre), which recognises the 34bp loxP (*lo*cus of *X*-over of *P*1) site, and catalyses both intra- and intermolecular recombination between two loxP sites. The loxP site consists of an 8bp core region flanked by two 13bp inverted repeats (Deng 2012). Cre-loxP-mediated recombination between two directly placed loxP sites excises all DNA sequences located within the two sites as a covalently closed circle (Figure 5.14).

The Ub-*Cre/ERT2* transgenic mouse line was purchased from the Jackson Laboratory and originally made by Eric Brown at the University of Pennsylvania using a self-inactivating lentiviral vector containing the human ubiquitin promoter sequence upstream of a *Cre-ERT2* fusion gene. The Cre-ERT2 fusion protein consists of Cre recombinase fused to a triple mutant form of the human estrogen receptor, which does not bind its natural ligand (17β-estradiol) at physiological concentrations but will bind the synthetic estrogen receptor ligand hydroxytamoxifen (Brocard *et al*. 1998). *Cre-ERT2* can only gain access to the nuclear compartment after exposure to tamoxifen. When these *Cre-ERT2* mice are bred with mice containing a *loxP*-flanked sequence (exon4) of *ctgf* i.e. $ctgf^{flox/flox}$, tamoxifen-inducible Cre-mediated recombination results in the deletion of *ctgf* exon 4 in widespread tissues and the gene becomes unstable for protein synthesis (Araki *et al*. 1997). Figure 5.14 shows the mechanism of Cre mediated deletion of *ctgf* in a schematic diagram.

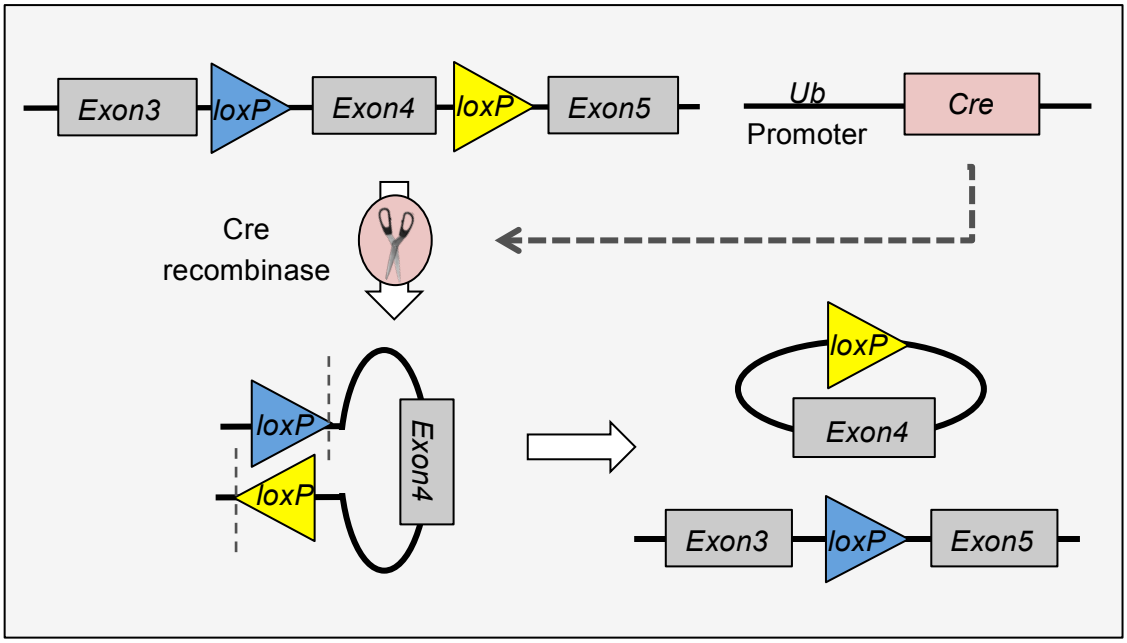


Figure 5.14 The schematic diagram of *ctgf* exon 4 deletion by the Lox-Cre recombination system.

5.5 Results

5.5.1 Generation of homozygous *ctgf*$^{flox/flox}$ /*cre* mice

Homozygous *ctgf*$^{flox/flox}$ mice were crossed with mice that carrying the *Cre-ERT2* insert (Cre+ve) to give rise to the first generation (F1) of heterozygous *ctgf*$^{flox/+}$/Cre+ve or *ctgf*$^{flox/+}$/Cre-ve offspring. Those *ctgf*$^{flox/+}$/Cre+ve litters in F1 were then inter-bred to produce the second generation (F2) of mice with mixed genotypes including homozygous *ctgf*$^{flox/flox}$, heterozygous *ctgf*$^{flox/+}$ and wild-type *ctgf*$^{+/+}$ either carrying or not carrying the *Cre* gene. The genotypes of the litters were determined by genotyping using primers that were designed to amplify across the loxP sites and as a result the floxed allele will generate a longer PCR product compared with the wild type *ctgf* allele. The resultant PCR products of different lengths can be differentiated on agarose gel, thus determining the presence of one, two or absence of the floxed allele. Ear biopsy tissue was obtained from mice at the age of 4 weeks, and from which genomic DNA was extracted (method described in 2.8.6). Figure 5.15 shows the genotyping result from F2 mice that were generated by the F1 cross (*ctgf*$^{flox/+}$/*Cre*+). There are three genotypes generated in F2: homozygous (HO) *ctgf*$^{flox/flox}$, heterozygous (HE) *ctgf*$^{flox/+}$ and wild-type (WT) *ctgf*$^{+/+}$, and these mice were either Cre+ve or Cre-ve. The DNA samples from the mice (ID: 304/0-5 and 305/4&5) were amplified by PCR using primers for *ctgf* or *Cre* gene. The resultant PCR products were electrophoresed on 1.5% agarose gel, and visualised in the Gel-doc UV system. The PCR products of the heterozygous *ctgf*$^{flox/+}$ DNA show two bands on the gel at 1000 basepairs (bp) and 800bp, while the homozygous *ctgf*$^{flox/flox}$ and the wild-type *ctgf*$^{+/+}$ produce single bands at 1000bp and 800bp respectively. For the presence of *Cre* gene, a band at 400bp can be seen on the gel. In this way, the genotype can be determined for each individual mouse. The presence of the Cre band does not allow distinction between hemizygous and homozygous expression. This is because the exact site and copy number of Cre insert in this line is unknown. The HO *ctgf*$^{flox/flox}$ /Cre+ mice were then bred to give rise the F3 generation of *ctgf*$^{flox/flox}$ /Cre+ mice, which were

induced for *ctgf* deletion. It is important to bear in mind that as the Cre may be hemizygous rather than homozygous, the F3 generation may be Cre-ve. Genotyping was checked on every mouse for all experiments.

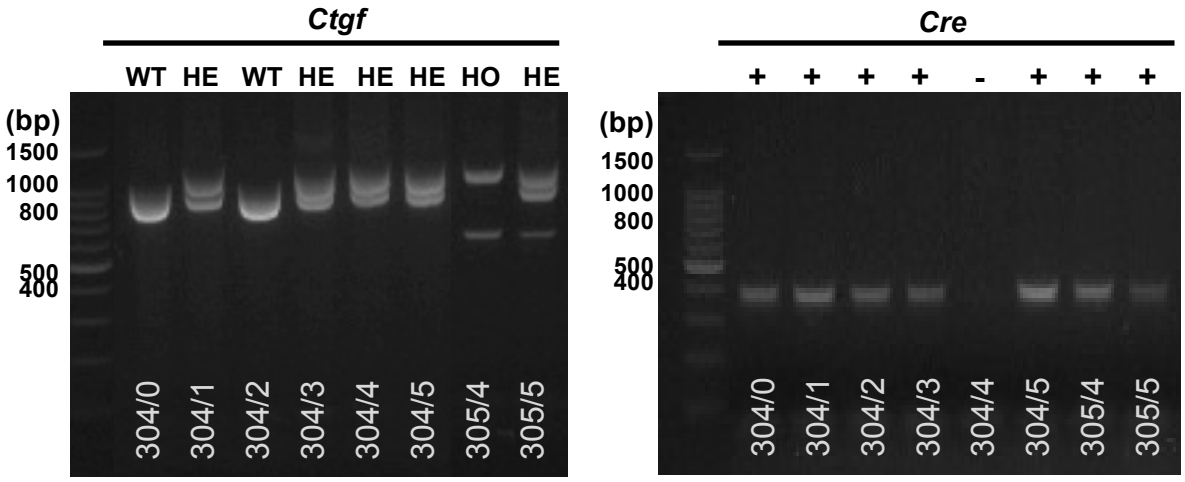


Figure 5.15 Genotyping results of mice (F2) generated from inbred *ctgf*$^{flox/+}$ Cre+ mice. PCR products were electrophoresied on 1.5% agarose gel and bands were visualised using GelDoc system for WT *ctgf*$^{+/+}$ (single band at 800bp), HE *ctgf*$^{flox/+}$ (double bands at 1000bp and 800bp) or HO *ctgf*$^{flox/flox}$ (single band at 1000bp) with either positive (band at 400bp) or negative (absent) Cre. The number under each lane is the ID (case No./litter No.) of the mouse from which DNA was obtained.

5.5.2 Deletion of *ctgf* gene by tamoxifen induction

Mice from the F3 generation at age of 4 weeks were injected with three doses of tamoxifen at 50mg/kg (100ul of 50mg/ml in corn oil) on three consecutive days to induce the deletion of the *ctgf* gene. The genotypes of these mice were confirmed before the tamoxifen treatment, and after two weeks of the injection, mice were culled for tissues. Hip cartilage was collected by avulsing the hip cap off the head of femur from both hip joints of 6-week-old mice (before ossification) using forceps and cultured separately in serum free medium. The conditioned medium from the injured cartilage explants was analysed for released proteins (i.e. CTGF and TGFβ) and the difference in protein intensity compared between the WT and knockout mice. Figure 5.16 shows the genotyping results of F3 mice (ID: 372/0-1 and 373/0-5) using

DNA extracted from their ear biopsy tissue. All these analysed mice from the F3 generation were homozygous for the floxed *ctgf* allele but not every mouse carried the *Cre* allele.

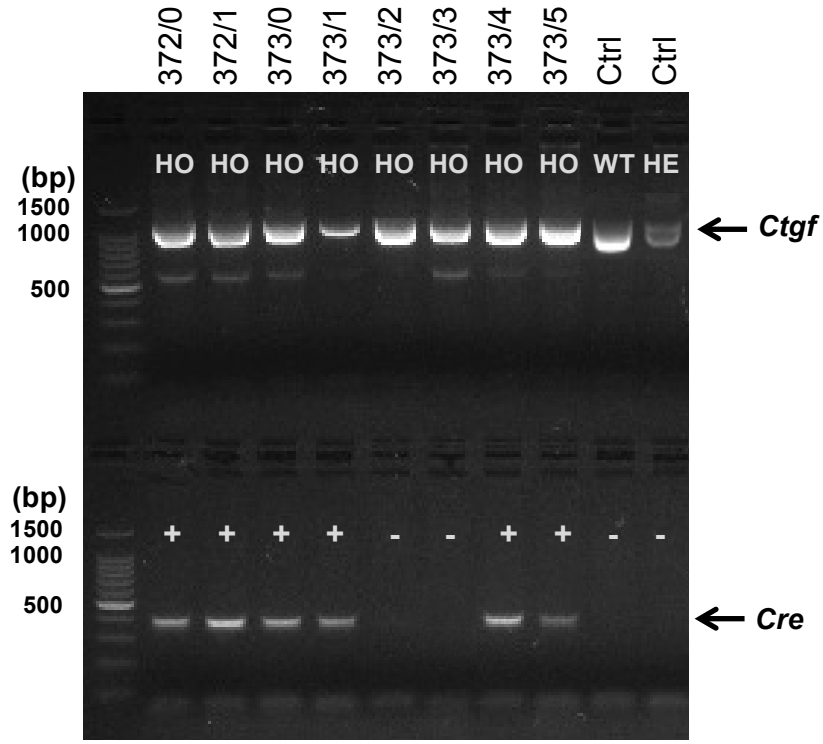


Figure 5.16 Genotyping results of mice (F3) generated from breeding *ctgf*$^{flox/flox}$ Cre+ mice. PCR products were electrophoresed on 1.5% agarose gel and bands were visualised using GelDoc system for WT *ctgf*$^{+/+}$ (single band at 800bp) (control), HE *ctgf*$^{flox/+}$ (double bands at 1000bp and 800bp) (control) or HO *ctgf*$^{flox/flox}$ (single band at 1000bp) with either positive (band at 400bp) or negative (absent) Cre. The number above each lane is the ID (case No./litter No.) of the mouse from which DNA was obtained.

5.5.3 Determination of CTGF and TGFβ protein level in the knockout mice

CTGF is described as a matricellular protein; down-regulation in the mRNA level may not necessarily reflect the depletion of protein already made and stored in the matrix before deletion. Therefore it was important to determine the knockout efficiency at protein level. The released CTGF as well as TGFβ were analysed in the conditioned medium of cartilage tissue harvested from the knockout mice. F3 generation mice with ID number 373 were injected with tamoxifen as described above whereas mice with ID 372 were used as untreated controls. Hip cartilage explants from each mouse (372/0 & 1 and 373/0-5) were

cultured in serum free medium for 24hr, and the CM was western blotted for CTGF and TGFβ under reducing conditions (Figure 5.17). The two control mice (372/0 &1) and three of the tamoxifen treated mice (373/1, 2 & 3) showed high levels of CTGF protein, two of these (373/2 & 3) were negative for the *Cre* gene. The third one (373/1) had reduced amount of CTGF, compared with the controls (Figure 5.17). The other three tested mice (373/0, 373/4 and 373/5) had very weak detection of CTGF protein. Three out of the four Cre+ve mice showed nearly 100% depletion of CTGF compared with the control mice, implying high knockout efficiency at protein level. The same CM was also western blotted for TGFβ. Interestingly, levels of TGFβ did not correlate with that of CTGF nor with genotype (Figure 5.17).

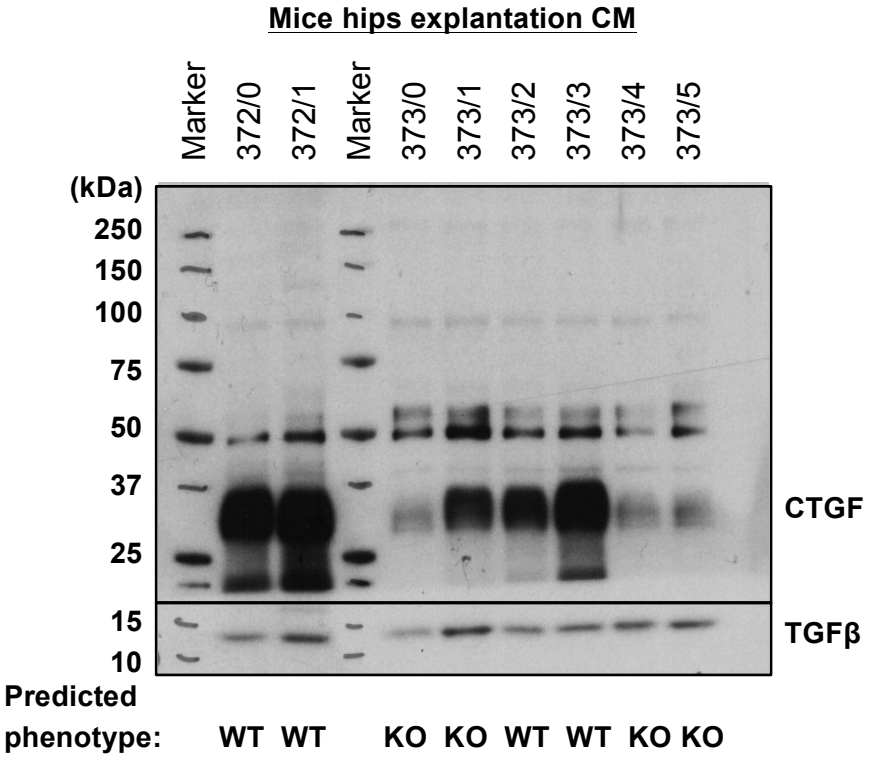


Figure 5.17 Western blot of CM from mice hip cartilage explants (ID 372 and 373). The CMs of mice (372 and 373) hip cartilage explants were western blotted for CTGF and TGFβ under reducing conditions. From the protein level of CTGF, it shows good knockout efficiency in three out of four tamoxifen treated mice that were positive for Cre. However, the levels of TGFβ protein did not correlate with that of CTGF.

Further *ctgf* knockout mice were generated, and their genotypes were confirmed before tamoxifen treatment. Hip cartilage explants were again cultured and the CM was analysed for CTGF and TGFβ. Figure 5.18 shows the western blot result from another set of *ctgf* knockout mice (427/4, 428/1 and 431/1-4). To control for possible Cre leakage, the control mice used here are litters from the pure *ctgf*$^{flox/flox}$ line (116/1 and 118/3 & 4) (not carrying the *Cre* allele). All mice were treated with tamoxifen. As shown in figure 5.18, WT mice (116/1, 118/3 & 4 and 431/1) showed strong CTGF protein detection in the CM. Out of the five Cre+ve mice, four (427/4, 428/1, 431/2 and 431/4) showed nearly complete CTGF knockdown at protein level, while one (431/3) showed 50% reduction compared with the controls. When the same CM samples were western blotted for TGFβ, the protein abundance for TGFβ again did not correlate with that of CTGF protein levels (Figure 5.18). The genotypes and predicted phenotypes are indicated for each mouse below the western blot result.

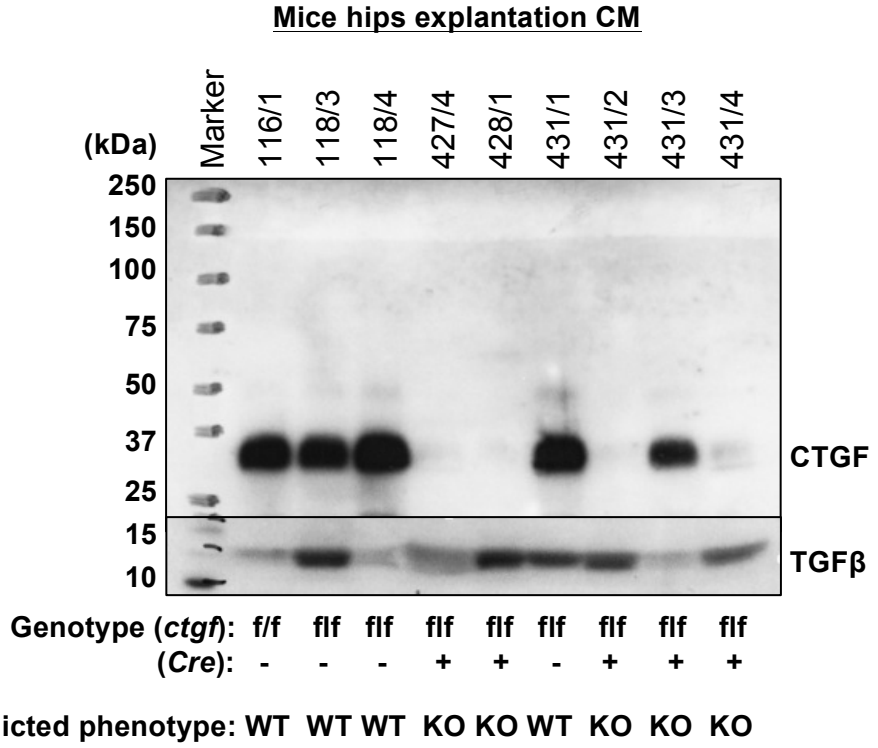


Figure 5.18 Western blot of CM from mice hip cartilage explants (ID 427, 428 and 431). The CMs of mice (116,118, 427,428 and 431) hip cartilage explants were western blotted for CTGF and TGFβ under reducing conditions. From the protein level of CTGF, it shows approximately 100% of knockout efficiency in four out of five Cre+ tested mice. However, the abundance of TGFβ protein does not correlate with that of CTGF.

The protein bands of CTGF and TGFβ on each blot were quantified using ImageJ which calculates pixel value statistics in each selected area that above the threshold of the background. The relative levels of CTGF or TGFβ were calculated using the pixel value of each band of the knockout mouse against that of the wild-type mouse on the same blot. 19 knockout and 15 wild-type mice were analysed for CTGF protein while 14 knockout and 14 wild-type mice were analysed for TGFβ protein. The difference between the wild-type and knockout groups were statistically tested. The p values shown in these tests suggest that the protein level of CTGF was significantly reduced by 75% in the knockout mice group whereas TGFβ protein showed no significant difference between the knockout and wild-type groups (Figure 5.19).

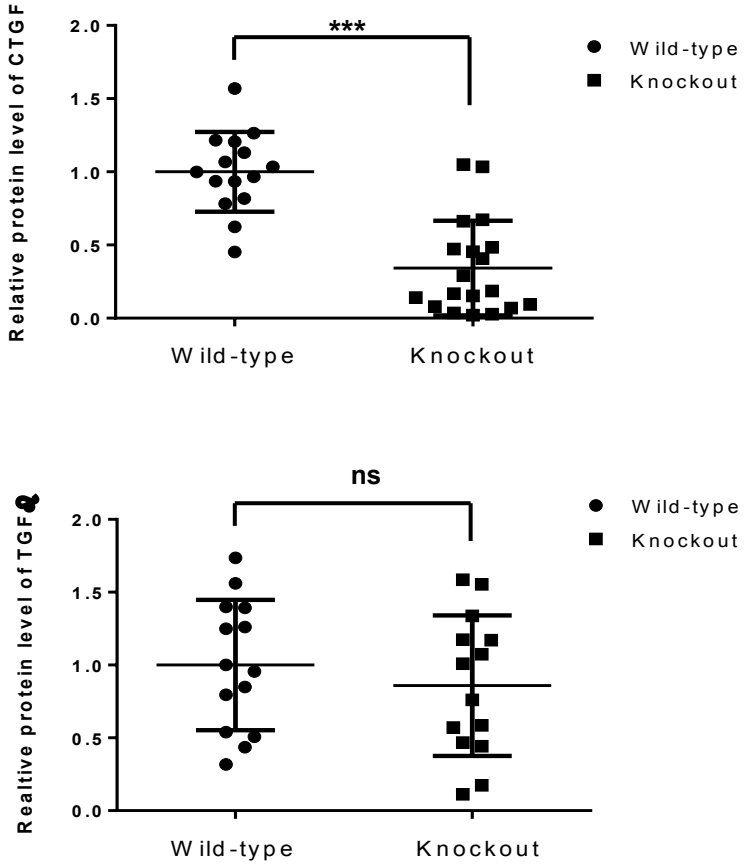


Figure 5.19 Quantification of CTGF and TGFβ protein bands from western blot. Protein bands were quantified using ImageJ and the pixel values from knockout mice were compared against the wild-type mice on the same blot. The relative protein levels for both CTGF (A) and TGFβ (B) were calculated. ***p<0.001 ns=not significant.

5.5.4 Determining the activity of the injury CM from CTGF knockout mice

The injury CM generated from porcine cartilage explants contained both CTGF and TGFβ protein, which was able to activate SMAD2 in isolated porcine chondrocytes. The injury medium conditioned from CTGF knockout mice hips showed reduced amount of CTGF protein compared with that of wild-type mice while the level of TGFβ does not correlate. Therefore it was interesting to investigate whether CTGF depletion affects the SMAD2 activity of the injury CM. Mice carrying *ctgf*$^{flox/flox}$ Cre+ve alleles were generated and their genotypes were confirmed before tamoxifen treatment as before. Two hips from each mouse were cultured separately in serum free medium for 24hr. One hip was further recut in fresh serum free medium whilst the other was kept intact as a control. After 24hr, these control/recut CM were collected and used to stimulate isolated serum starved porcine chondrocytes. SMAD2 activation was then analysed in these cells by western blot. Litters (112/1, 2 & 3) from the pure *ctgf*$^{flox/flox}$ line were used as control and tested against litters (408A/1, 2 & 3 and 408B/1 & 2) generated from the Cre crossed line. Three (408A/1, 2 & 3) mice were Cre+ve and two (408B/1 & 2) were Cre-ve. All mice were treated with tamoxifen at age of week 4 and culled after 2 weeks of tamoxifen induction. In the western blot of CTGF (Figure 5.20A), the WT mice (112/1, 2 & 3 and 408B/1 & 2) showed high levels of CTGF protein compared with the three Cre+ve KO mice (408A/1, 2 & 3). Although some level of CTGF could be detected in one of the Cre+ve mice (408A/1), the significant reduction of CTGF protein in the Cre+ve mice suggests good knockout efficiency. In this experiment, unlike the previous ones, the western blot of TGFβ showed strong bands in the control mice and weaker intensity bands observed for the CTGF depleted ones. Having determined the level of CTGF and TGFβ protein in these mice, it was decided to further analyse the CM of the control mouse 112/3 and KO mice 408A/1, 2 & 3 (indicated in red) for SMAD2 activation. Both the control (non recut) and recut CM generated from the left and right hip of each mouse were used to treat isolated porcine chondrocytes and the level of

phospho-SMAD2 protein was compared by western blot. As shown in the figure 5.20B, the recut CM of the WT mouse (112/3) caused strong SMAD2 activation, and of the three KO hips tested, two out of three showed reduced SMAD2 activity. In this experiment the signal intensity of pSMAD2 did correlate reasonably well with CTGF but not with TGFβ protein detected in the explantation CM from these mice. Taken together these results suggest that CTGF is required for SMAD2 phosphorylation.

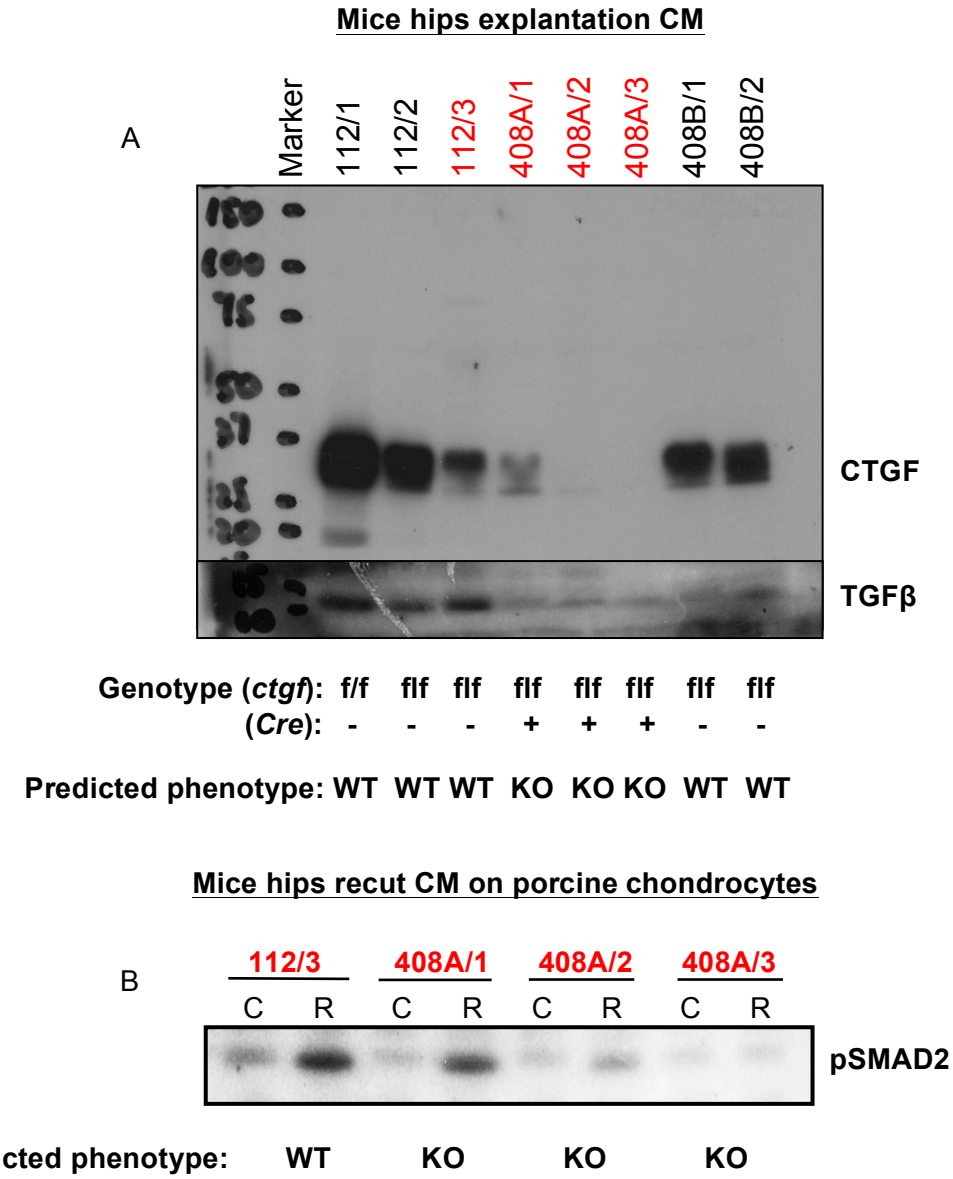
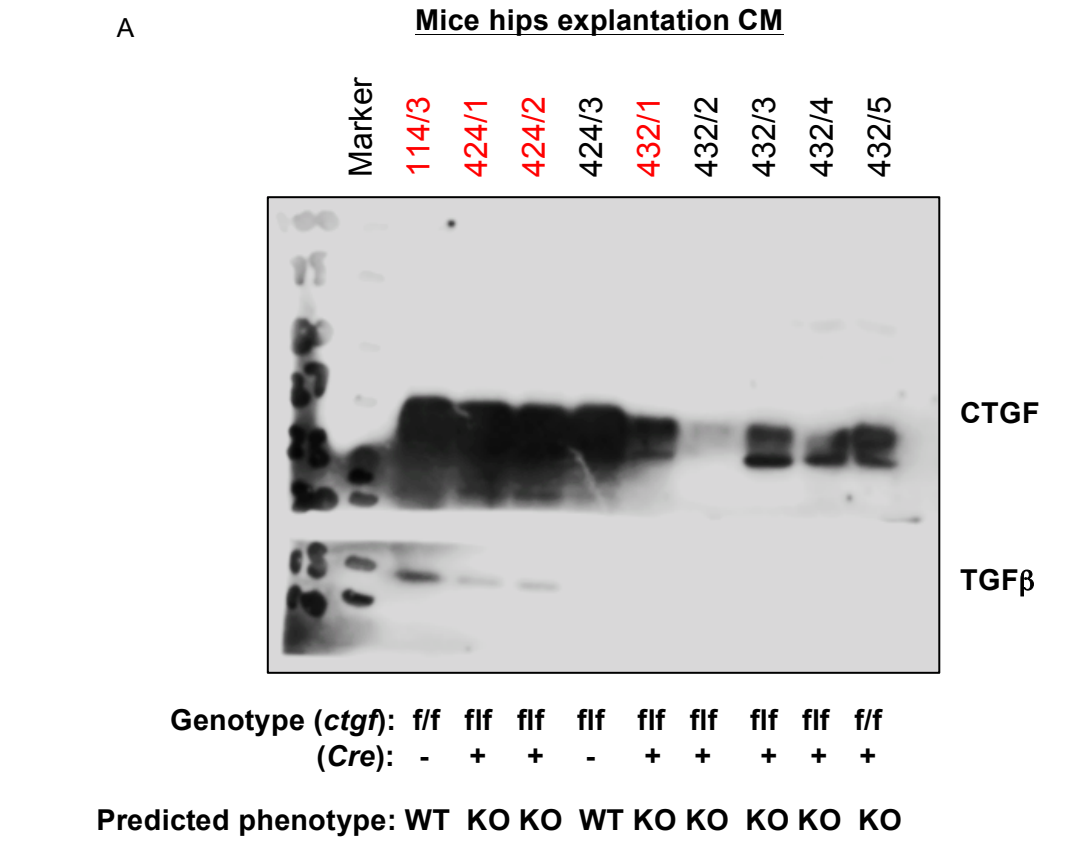


Figure 5.20 SMAD2 activity and CTGF/TGFβ levels in injured hips (ID112 and 408A). The hip cartilage explants from mice (116, 408A and 408B) were used to generate explantation CM (24hr) then re-cut to generate recut CM (over 24hr). The explantation CM was western blotted for CTGF and TGFβ (A). The recut CM (from 112/3, 408A1, 2 and 3) (indicated in red) was used to stimulate isolated porcine chondrocytes to induce SMAD2 activation (B).

This SMAD2 activation experiment was repeated three further times. Even though the knockout efficiency was variable in some of these experiments, all results reproducibly supported the role of CTGF in SMAD2 activation because phosphorylation of SMAD2 appeared to correlate with levels of CTGF in the medium. Figure 5.21, 5.22 and 5.23 show the western blot results of three further SMAD2 activation experiments demonstrating this.

In the experiment shown in figure 5.21A, the explantation CMs from tamoxifen treated mice (114/3, 424/1 - 3 and 432/1- 5) were western blotted for CTGF and TGFβ. Although the Cre gene had been detected in 424/1 & 2 and 432/1, strong detection of CTGF was seen in these mice with similar intensity observed in the control (114/3). The CM from other Cre+ve mice 432/3, 4 & 5 also contained CTGF protein but in lower amounts compared with the control. TGFβ was detected in the control 114/3 and two Cre+ve mice 424/1 and 424/2 with stronger intensity compared with other mice. The recut CM from mice 114/3, 424/1, 424/2 and 432/1 (indicated in red) were used for the pSMAD2 experiment. The CM from 424/1 and 424/2 mice (massive amount of CTGF was detected) caused strong activation of pSMAD2 while the CM with lower amounts of CTGF (432/1) had little pSMAD2 activity. Both CTGF and TGFβ could be detected in the explantation CM of the control mouse (114/3), however its recut CM could not activate SMAD2. Although the exact reason for this is not known, one thing to note here is that the measurements of CTGF and TGFβ were made on the initial explantation CM whereas SMAD2 activity was measured in the medium that had been generated when the same explants had be further recut. It is possible that the amount of protein released during recutting differs from explantation. Because the entire CM generated from recutting was used to treat cells for SMAD2 activation, it is not possible to determine the protein level in this material. However most of time the pSMAD2 signal activated by the recutting CM correlates with the CTGF signal detected in the explantation CM.



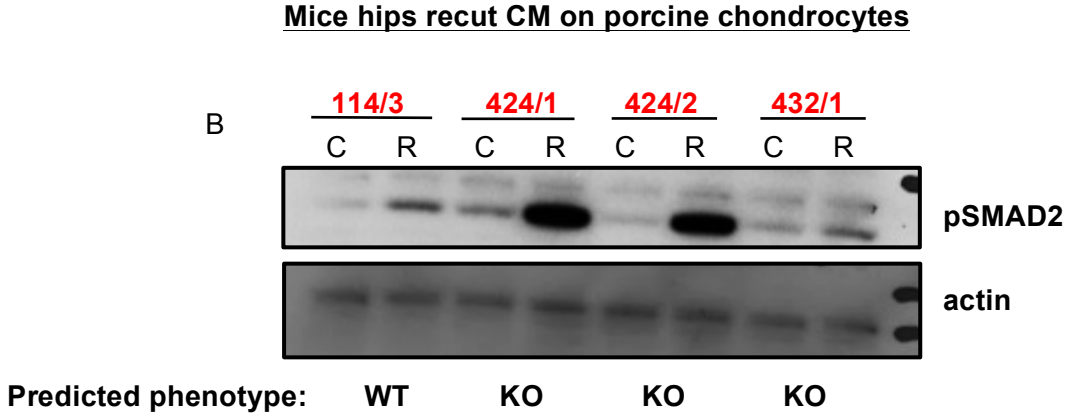


Figure 5.21 SMAD2 activity and CTGF/TGFβ levels in injured hips (ID 114, 424 and 432). The hip cartilage explants from mice (114, 424 and 432) were used to generate explantation CM (24hr) then re-cut to generate recut CM (over 24hr). The explantation CM was western blotted for CTGF and TGFβ (A). The recut CM (from 114/3, 424/1, 2 and 432/1) (indicated in red) was used to stimulate isolated porcine chondrocytes to induce SMAD2 activation (B).

Figure 5.22 shows the western blot result of SMAD2 activation using recutting CM harvested from another set of *ctgf* knockout mice. Mice 412B/1 and 412A/1-3 are all Cre+ve but 412B/1 was not treated with tamoxifen thus used as an untreated control. The pSMAD2 signal detected for the control mouse is again stronger than that of KO mice. However the protein levels of CTGF and TGFβ have not been analysed by western blot in these mice to confirm whether the pSMAD2 intensity correlates with that of the CTGF.

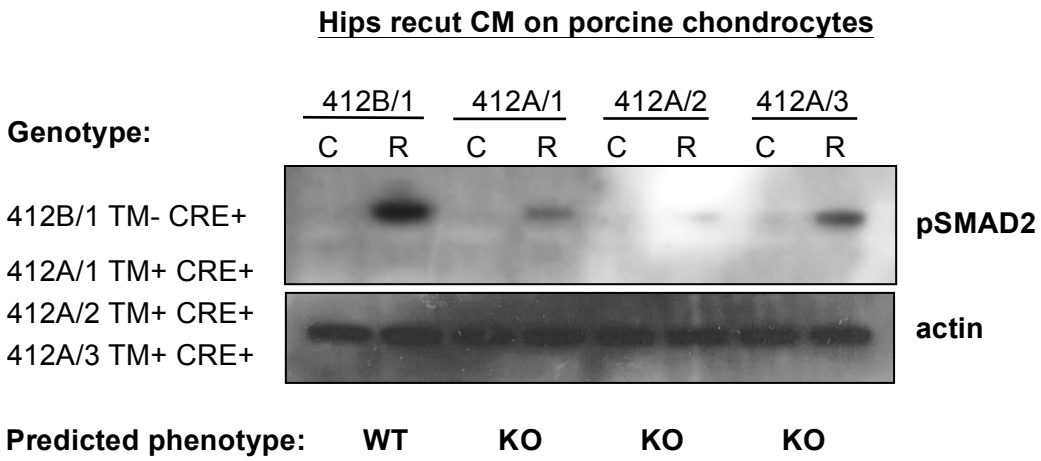


Figure 5.22 Inhibited pSMAD2 activation was observed for ctgf knockout mice (ID 412A and 412B). The hip cartilage explants from mice (412B untreated and 412A tamoxifen treated) were used to generate recut CM, which was used to stimulates isolated porcine chondrocytes to induce SMAD2 activation.

In the next experiment, explantation (rather than recut) CM was used to study SMAD2 activity. Figure 5.23 showed the pSMAD2 blot from cells that had been stimulated with the explantation CM harvested from mice 437/1-4. All four mice had been injected with tamoxifen but two (437/2 and 437/3) were Cre-ve while 437/1 and 437/4 are Cre+ve. As shown in the blot, the CM generated from the two Cre-ve mice induced SMAD2 activation while no/little activation was observed for the two Cre+ve mice. This again suggests that depletion of CTGF inhibits SMAD2 activity of injury CM.

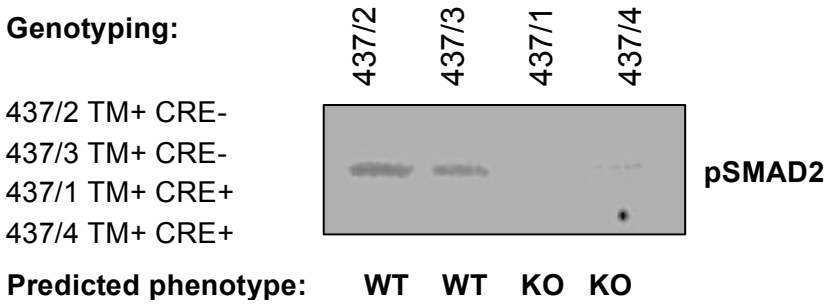


Figure 5.23 No/little pSMAD2 activation was observed for ctgf knockout mice (ID 437). The hip cartilage explants from mice (437/2 & 3 Cre- and 437/1 & 4 Cre+) were used to generate explantation CM, which was used to stimulates isolated porcine chondrocytes to induce SMAD2 activation. Activation could only be detected in the samples from Cre- mice.

The protein bands of phospho-SMAD2 on each blot were also quantified using ImageJ. 11 knockout mice and 4 wild-type mice were analysed. The level of phospho-SMAD2 resulted by the recut CM was first calculated relative to the uncut controls in both the wild-type and knockout groups. The relative level of phospho-SMAD2 in knockout mice was then calculated relative to the wild-type mice and the difference was statistically tested. Figure 5.24 shows a significant reduction of phospho-SMAD2 level in the knockout group.

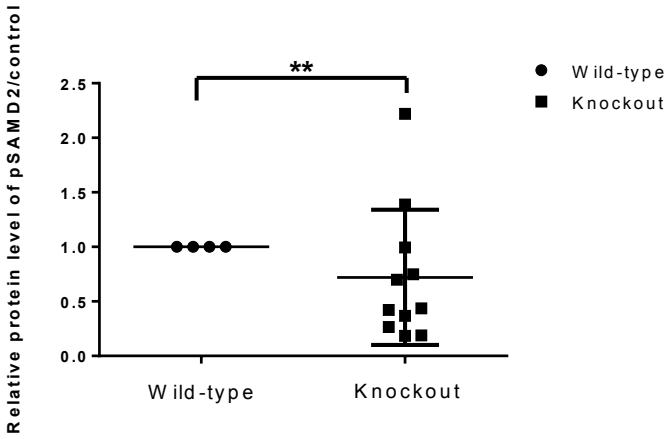


Figure 5.24 Quantification of pSMAD2 protein bands from western blot. Protein bands were quantified using ImageJ and the pixel values from recut group were calculated against the control group, and then the value of knockout mice were compared against the wild-type mice from the same blot. 11 knockout mice and 4 wild-type mice were analysed. ** $p<0.01$

5.6 Discussion

CTGF knockout mouse model was used to study the effect of *in vivo* CTGF depletion on the bioavailability and activity of TGFβ in injury-conditioned medium. Due to embryonic lethality, postnatal inducible knockout mice were generated by crossing homozygous *ctgf* floxed mice with ubiquitin Cre ERT2 mice. Upon tamoxifen injection, the expression of the Cre recombinase under the ubiquitin promoter leads to widespread deletion of CTGF. This allowed us to determine the importance and function of released pericellular CTGF from damaged hips cartilage explants.

The genotypes of the knockout mice were confirmed using conventional PCR primers that were specific for the floxed *ctgf* gene and the *Cre* gene. The PCR products generated from floxed allele and wild type *ctgf* allele have little difference in length (1000bp and 800bp). This difference sometimes is difficult to be visualised on agarose gel. Therefore, $ctgf^{flox/flox}$/Cre+ mice were continuously bred for several generations to ensure every mouse is homozygous for the *ctgf* floxed allele before experiments. The presence of the Cre allele was determined by the detection of the 400bp PCR product generated using Cre primers. Mice that did not

show the Cre band after genotyping were used as wild-type controls. However, sometimes the absence of the Cre band was due to technical errors, which did not reflect the true genotype of a mouse. Another type of wild-type control was to use mice that untreated with tamoxifen. However, Cre leakage was sometimes observed, where Cre fused estrogen receptor binds its natural ligand (17β-estradiol) to achieve deletion regardless the induction of tamoxifen. To avoid Cre leakage I decided to use the mice that were bred from the pure *ctgf*$^{flox/flox}$ line as wild-type controls. These *Cre-ve* wild-type mice as well as the *Cre+ve* mice were both treated with tamoxifen to ensure that the conditions applied to both groups are comparable.

Out of the 19 KO (*Cre+ve*) mice studied, 15 mice achieved at lease 50% CTGF protein reduction; 10 of these showed nearly complete depletion. This implies good knockout efficiency. However, this is also surprising because CTGF is a matricellular protein and there should be CTGF molecules that are already made and stored in the matrix of the cartilage explants before deletion was induced. It is not likely that 100% of pre-existing matrix bound CTGF had been turned over during the two weeks of tamoxifen induction. One may speculate that the reason for no or very little CTGF detection in the CM of knockout mice is because the medium analysed had been conditioned by the damaged cartilage explants for 24hr. The released CTGF protein during that period might have been internalised through the LRP receptor. A priority for future experiment is to culture the hip explants for shorter periods (e.g. 1hr), and then western blot the CM for CTGF to determine whether good knockout efficiency can still be achieved. Further tests are also required to confirm that the SMAD2 phosphorylating activity in the CM of CTGF knockout mice cartilage was mediated by TGFβ rather than other TGFβ family protein such as activin A. This can be achieved using specific neutralising antibodies.

Taken together where CTGF protein depletion is evident, there is quite consistent data that indicates the importance of CTGF in TGFβ activation in the injury CM. There is little correlation between TGFβ and CTGF release in the CM implying that CTGF may be more important in activating latent TGFβ than in causing release of latent complexes from the pericellular matrix. However, these experiments are not compared like with like; as mentioned previously, CTGF and TGFβ levels were measured in the explantation CM whereas activity was generally measured in the recut CM. Moreover the collections were all taken over a 24hr period. During this time it is possible that CTGF and/or TGFβ levels are being modulated by other cellular activities. Despite this, the activity of the CM for SMAD2 activation correlates well with the amount of CTGF in the CM, suggesting that CTGF may be required for its activation at the cell surface. This provides a novel role for CTGF in connective tissue and has important functional implication. It would also tie in nicely with data that link CTGF and TGFβ, for example, in the fibrosis literature (Leask and Abraham 2004; Gore-Hyer *et al.* 2002; Mori *et al.* 1999; Rachfal 2003). Whether CTGF is also involved in the release of TGFβ from the PCM remain unanswered although one hopes that this will become more apparent once experiments have been repeated at shorter time points. It is possible that CTGF has two roles: the first to liberate the SLC from its matrix-bound LLC; the second to activate the SLC at the cell surface, allowing TGFβ to signal through the canonical pathway.

Chapter 6

Final discussion

This project is a continuation of the work from a former PhD student Celia McLean who identified CTGF from a proteomic analysis of porcine articular chondrons and determined the localisation of CTGF in porcine cartilage by immunofluorescent staining. She also established a method to express and purify human recombinant CTGF protein from a stably transfected HEK293 cell line. I started this project by repeating these approaches to confirm the pericellular localisation of CTGF but this time in human articular cartilage; later I found that like FGF2, CTGF also binds to perlecan and is rapidly released from the PCM of cartilage upon mechanical injury. In the following years of research my main aim was to investigate the role of CTGF in articular cartilage.

CTGF has been reported to promote proliferation, maturation, adhesion and differentiation of chondrocytes and osteoblastic cells in culture (Yosimichi *et al.* 2001; Hoshijima *et al.* 2006; Nishida *et al*. 2003). Takigawa's group in Japan and Lyons's group in USA reported that CTGF was important in chondrogenesis and angiogenesis during skeletal development. In Lyons's study CTGF deficiency is perinatal lethal and led to skeletal dysmorphism in mice. These CTGF mutants have decreased expression of specific extracellular components and matrix metalloproteinases, suggesting that CTGF is a crucial regulator of cartilage extracellular matrix remodeling (Ivkovic *et al*. 2003).

CTGF is also important in damaged cartilage and may have repairing roles. Our group found that CTGF was one of the up-regulated proteins from OA cartilage (Hermansson *et al.* 2004). Injection of recombinant CTGF into the joints of mice with MIA-induced OA or in situ injured articular cartilage repaired the defects. CTGF treated wounds repaired with new

cartilage which wasn't evident in the vehicle-treated controls (Nishida *et al*. 2004). Another group implanted CTGF modified bone marrow mesenchymal stem cells into injured articular cartilage in rabbit knee joints and showed regeneration with hyaline like cartilage (Zhu *et al*. 2014).

These studies indicate that CTGF is an important molecule in cartilage, however the molecular mechanism by which CTGF exerts these effects was not known. Abreu *et al* shed light on the role of CTGF in modulating TGFβ signalling in epithelial cells. In their study, they reported that CTGF directly binds to TGFβ through its cysteine rich domain, which potentiated TGFβ's binding to its both type I and type II receptors and increased TGFβ-mediated SMAD2 activation in cultured epithelial cells (Abreu *et al*. 2002). This finding was attractive as it also explained the shared properties observed between CTGF and TGFβ in fibrosis. TGFβ and CTGF are both over expressed in various fibrotic diseases (Leask *et al.* 2002). TGFβ induced fibroblast proliferation and fibroblast collagen production was inhibited by CTGF antibodies and CTGF silencing (Duncan *et al*. 1999; Kothapalli *et al.* 1997). Subcutaneous injection of TGFβ and CTGF into neonatal mice caused similar fibrotic reactions, which was not mimicked by other growth factors (Frazier *et al.* 1996; Shinozaki *et al*. 1997; Ball *et al.* 2003). Although TGFβ and CTGF share pro-fibrogenic properties, anti-inflammatory and immunosuppressive properties are unique to TGFβ (Rachfal 2003).

My findings suggest that CTGF plays a role in TGFβ activation in response to cartilage injury. I propose a molecular mechanism by which TGFβ latent complex and CTGF are both sequestered in the PCM in rested articular cartilage and both are released upon injury. From

my experiments it is not clear whether there is a pre-existing association between CTGF and latent TGFβ or whether CTGF and latent TGFβ are released independently and rapidly bind to one another. Evidence that they are bound to one another in the CM comes from the observations that: both CTGF and LAP were detected as large complexes (150kDa) in the CM under non-reducing conditions following injury; SMAD2 phosphorylating activity was present within a high molecular fraction of the injury conditioned medium; immunoprecipitation experiments indicated that CTGF was bound to LAP in the conditioned medium.

The possibility that CTGF is already associated with TGFβ in the matrix is suggested by the observation that neither CTGF nor the SLC are detectable in the CM after injury in their predicted monomeric molecular weight forms. However, this does not sit comfortably with the observation that when CM time courses were run under reduced conditions, TGFβ levels in the medium accumulated slowly over time and did not correlate with CTGF levels. This may be because early released SLC saturates its activation sites on the cell surface of adjacent chondrocytes and then is internalised through its receptor. This would also fit with the detection of LAP in the CM at early time points following cartilage injury.

Using CTGF deficient tissue from CTGF conditional knockout mice I have been able to ask whether CTGF is important for release of TGFβ from the matrix upon injury and/ or is responsible for activation of the latent complex. The ability of CTGF to release TGFβ from the matrix was suggested by my experiments showing TGFβ release when cartilage explants were treated with exogenous recombinant CTGF. However, there appeared to be

poor correlation between the amount of TGFβ and CTGF in the CM when CTGF knockout hips were injured, despite demonstrating good knockdown of CTGF. As the conditioned medium in these experiments were collected over a 24hr period it is conceivable that there is residual CTGF release from the matrix early on that facilitates TGFβ release but is subsequently cleared. Repeating these experiments but examining the CM at early time points following injury will be a priority.

On the other hand, these experiments did suggest that CTGF is more likely to be involved in activating the SLC at the cell surface because the injury CM from CTGF deficient cartilage had reduced SMAD2 activity compared with that of wild-type hip cartilage despite having similar levels of latent TGFβ.

There are still remaining questions, which need to be answered with further investigation. These include whether CTGF binds to latent TGFβ through LAP, LTBP or through another unknown partner. LTBP has not been studied extensively in my research and may be involved in the interaction between CTGF and latent TGFβ when being sequestered in the cartilage matrix. Both CTGF and LTBP contain conserved cysteine rich domains, thus it is possible to form disulfide bonds between the two molecules. Binding assays (Biacore) between CTGF and LAP or LTBP are to be carried out in collaboration with Cay Kielty's group at Manchester. LTBP2 is one of the genes identified in response to CTGF and activin A stimulation. LTBP2, unlike other LTBP isomers, does not bind to TGFβ SLC and has been reported to compete with other LTBPs from binding to filibrillin and heparin (Hirani *et al.* 2007;

Sideek *et al.* 2014). It would be interesting to investigate whether LTBP2 is also involved in latent TGFβ liberation from cartilage matrix.

Another question is how CTGF assists the SLC to be activated at its cell surface. Molecules that have been reported to be involved in TGFβ activation at the cell surface include TSP1, integrin (mainly alpha V) and MMPs (Khalil 1999). I have briefly examined whether integrins and MMPs are involved in the CTGF-mediated TGFβ activation using integrin and MMPs inhibitors. However, these experiments have not been optimised in the cartilage system before, and thus need to be repeated. Integrin alpha v is thought to be very low in cartilage (Woods *et al.* 1994), which makes it unlikely to be involved in TGFβ activation in cartilage. The involvement of TSP1 also needs to be tested using its specific inhibitor. TSP1 and CTGF have homology in the conserved TSP1 domain (Ribeiro *et al.* 1999; Bork 1993), thus CTGF may be able to cause a conformational change in LAP to release active TGFβ like TSP1.

TGFβ is a critical molecule for normal skeletal growth and development. *In vivo* murine studies have provided important insights into the contribution of TGFβ to the processes of chondrogenesis, chondrocyte maturation and joint morphogenesis. Mice lacking TGFβ type II receptor in chondrocytes show defects in the postnatal regulation of chondrocyte maturation in the axial skeleton (Baffi *et al*. 2004). Deletion of TGFβ type II receptor in limb mesenchyme resulted delayed cartilage formation, reduced chondrocyte proliferation and malformation of joints in the digits (Seo & Serra 2007). Some of these observations are also reported in CTGF knockout mice, suggesting some shared activities in skeletal development.

In mature cartilage, TGF-β signalling is crucial for maintaining articular chondrocyte homeostasis in synovial joints (Serra *et al*. 1997). Loss of TGF-β signalling in cartilage induces chondrocyte hypertrophy, ultimately resulting in cartilage degeneration (Yang *et al.* 2001), and pharmacological activation of the TGF-β pathway has been proposed to preserve articular cartilage integrity during osteoarthritis (Blaney Davidson *et al.* 2007).

Conversely, recent studies are showing more interest in the catabolic effect of TGFβ in osteoarthritis. Zhen *et al* provided new evidence using several models of osteoarthritis to show that TGF-β is involved in aberrant bone remodelling and cartilage degeneration in osteoarthritis. Increased TGF-β activity in the subchondral bone may be an early key event in disease (Zhen *et al.* 2013). The opposite effects of TGFβ may have been attributed to the different signalling mediated through the different TGFβ type I receptors. Recent studies on endothelial cells have identified ALK1 as a second type I TGFβ receptor and have shown that ALK1 and ALK5 have opposing functions in these cells (Finnson *et al.* 2008). Inhibition of ALK5 using SB431542 in my study showed increased accumulation of large CTGF complex (150kDa) in the explantation CM (data not shown). This may imply that CTGF-mediated activation of SLC on the cell surface involves TGFβ receptor, specifically ALK5. Moreover, chondrocytes treated with recombinant CTGF in my experiment only showed activation of SMAD2 not SMAD1 and 5 which are downstream of ALK1 activation. Therefore CTGF may be important in controlling the direction (anabolic or catabolic) of TGFβ's activity in cartilage.

To summarise, my work has led to a new model, involving CTGF, for how TGFβ is activated upon cartilage injury:

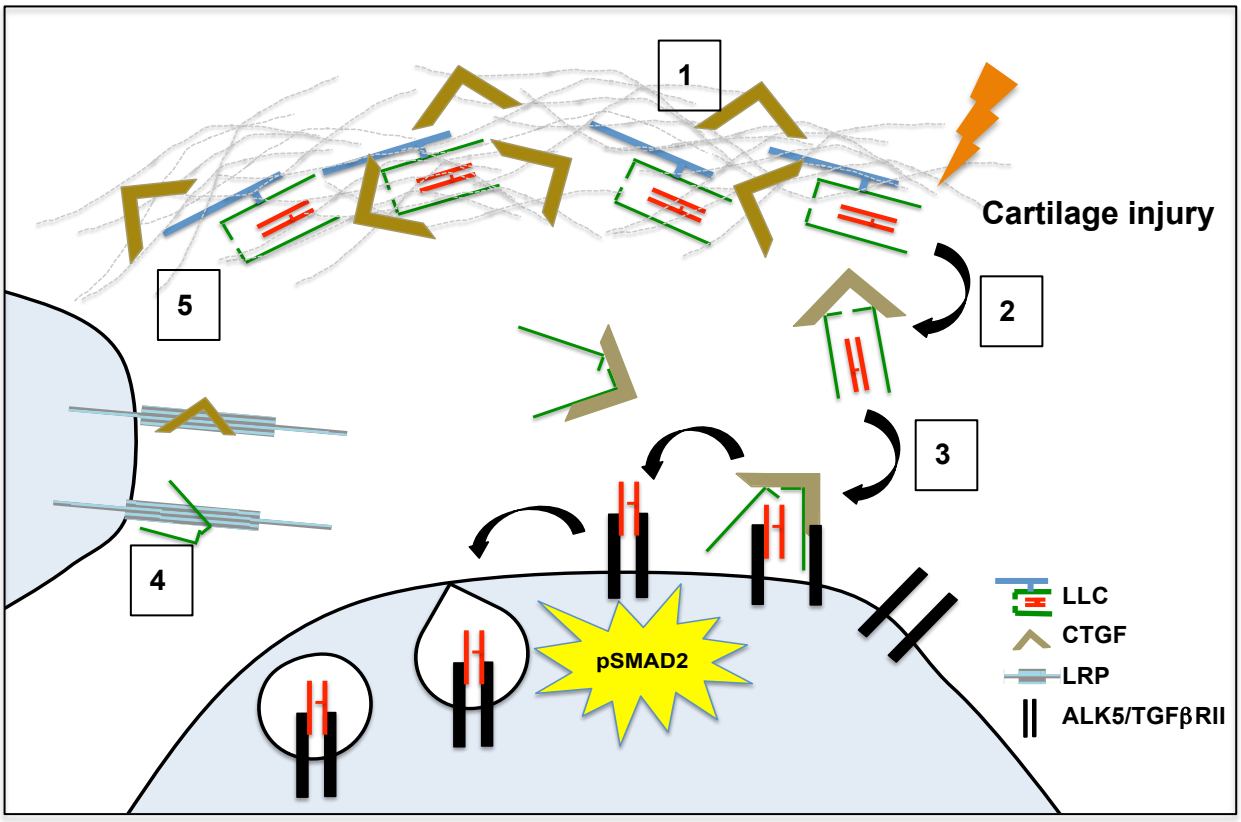


Figure 6.1 The schematic diagram of the proposed model, for how CTGF may be involved in TGFβ activation upon cartilage injury.

1. CTGF and LLCs are bound into the pericellular matrix of articular cartilage.
2. Upon injury, both become liberated where they rapidly bind to one another.
3. The CTGF/latent TGFβ complex binds to the cell surface where TGFβ is released (in a CTGF-dependent manner) from the latent complex, binds the ALK5 TGFβ receptor and is internalised.
4. Free CTGF and latent complexes are rapidly cleared by an LRP1-dependent manner.
5. Matrix bound stores are replaced by constitutively produced CTGF and LLCs until homeostasis is once again restored.

Chapter 7

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