2007

CONNECTIVE TISSUE GROWTH FACTOR IS REQUIRED FOR CHONDROGENESIS

Daphne Pala
Western University

CONNECTIVE TISSUE GROWTH FACTOR IS REQUIRED FOR CHONDROGENESIS

(Spine title: Connective Tissue Growth Factor is Required for Chondrogenesis)

(Thesis format: Monograph)

by

Daphne Pala

Graduate Program in Physiology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario, Canada
April 2007

ABSTRACT

Connective tissue growth factor (CTGF, CCN2) is expressed by mesenchymal cells during development. Mice genetically deficient in CCN2 display severe bone defects and die immediately after birth. The molecular basis of this defect is not known. We've used *in vitro* models of chondrogenesis to show that CCN2 expression is induced during chondrogenesis, paralleling induction of known early chondrogenic markers (sox9, sox6, l-sox5, type II collagen, aggrecan, decorin and link protein). Real-Time PCR, Western blot and Immunofluorescence reveal that CCN2 is required for the early stages of chondrogenesis, including expression of sox6 and aggrecan, but not type II collagen. Furthermore, focal adhesion kinase (FAK) was found to be an upstream mediator of CCN2 expression and the absence of FAK promotes the process of chondrogenesis. Therefore, CCN2 is required for early chondrogenic events, downstream of FAK and is responsible for regulating components of the chondrogenic extracellular matrix.

KEYWORDS

Chondrogenesis, Connective Tissue Growth Factor, Sox6, Aggrecan, Type II Collagen, Focal Adhesion Kinase, Gene Expression, Extracellular Matrix

"IF YOU CARE ABOUT SOMETHING, YOU HAVE TO PROTECT IT—IF YOU'RE LUCKY ENOUGH TO FIND A WAY OF LIFE YOU LOVE, YOU HAVE TO FIND THE COURAGE TO *LIVE* IT"

- John Irving, A Prayer for Owen Meany

ACKNOWLEDGEMENTS

I would like to first and foremost thank my supervisors, Dr. Andrew Leask and Dr. Frank Beier for allowing me to join both of their labs. I would like to thank Andrew for being there on a daily basis to infuse me with enthusiasm about our research and for encouraging me to always set higher and higher goals for myself. I would like to thank Frank for being a constant source of guidance and wisdom, and for helping me face new and challenging obstacles head on. I have learned a great deal in these past two years from both Andrew and Frank, and I am forever grateful for the time and effort they have both spent on training me. I would also like show my appreciation for being given the opportunity to contribute to a number of scientific publications during my Masters degree.

I would like to thank both David Carter and Dr. Andrew Leask for sharing with me the *Ccn2*$^{+/+}$ and $^{-/-}$ MEF microarray data. I would also like to thank Dr. Shaoqiong Chen for contributing her beautiful western blot for the *FAK*$^{+/+}$ and $^{-/-}$ MEFs. I would like to express my gratitude to Greek Tom for his baklava and his expert microscope support. I am also appreciative to the funding agencies who supported this project: Ontario Graduate Scholarship in Science and Technology, Canadian Arthritis Society and Canadian Institutes of Health Research.

I would like to acknowledge all the members of both the Leask and Beier labs who have been a pleasure to learn from and work with. I would particularly like to thank Dr. Anita Woods who has been there from my first minute as a Western student as a mentor and a friend. I won't ever forget my trip to the "library", the purple paint or the about-town incident with Laura1. I will always cherish sharing a lab bench and my trip to

the zoo with Laura2. Also, I am forever grateful for the everyday comic relief and mental support provided by the Lauras and Mistre. I would like to give a special thanks to a few of my friends that have helped make the past two years memorable: Janet, Steph, Merritt, Tyler, Aran and Ruby. I would especially like to thank my best friend and boyfriend Will, who throughout the years has enriched my life with laughter, love and support.

I also owe a great deal of thanks to my Mom and Dad who have always supported me and believed in me. They have given me opportunities in my life that I know they only dreamed were possibilities. Their determination and drive has always inspired me to aim high. I love them and thank them for always being there for me. I feel so fortunate to be able to share this achievement with them.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

BMP-2	Bone Morphogenetic Protein-2
BSA	Bovine Serum Albumin
CTGF/CCN2	Connective Tissue Growth Factor
Cyr61/CCN1	Cysteine-Rich 61
CD	Campomelic Dysplasia
CMD	Cartilage Matrix Deficiency
DAB	3,3'-Diaminobenzidine
DAPI	4',6-Diamidino-2-Phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
Dpc	Days Post Coitum
ECM	Extracellular Matrix
ERK	Extracellular Signal-Regulated Protein Kinase
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-Phosphate-Dehydrogenase
HMG	High Mobility Group
HSPG	Heparin Sulphate Containing Proteoglycan
HRP	Horse Radish Peroxidase
KO	Knockout
MAPK	Mitogen Activated Protein Kinase

MMP	Matrix Metalloproteinases
N-CAM	Neural Cell Adhesion Molecule
NOV/CCN3	Nephroblastoma Overexpressed
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3 Kinase
PNA	Peanut Agglutinin
PP2	4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine
RIPA	Radioimmunoprecipitation Assay
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
RNA	Ribonucleic Acid
SEM	Standard Error of the Mean
TGF-β	Transforming Growth Factor-β
TNF-α	Tumour Necrosis Factor-α
Wisp-1/CCN4	Wnt-Induced Secreted Protein-1
Wisp-2/CCN5	Wnt-Induced Secreted Protein-2
Wisp-3/CCN6	Wnt-Induced Secreted Protein-3
WT	Wild-type

1.0 Introduction

1.1 Cartilage Biology

1.1.1 Introduction to cartilage biology

Cartilage is a connective tissue with a wide array of functions such as laying the framework of the skeleton during embryonic development and cushioning joints in adulthood. Chondrocytes, the cell type found in cartilage, are responsible for generating and maintaining the cartilaginous extracellular matrix (ECM), which is an essential component of chondrogenic development and homeostasis (Karsenty and Wagner, 2002). Chondrogenesis is the earliest process in skeletal development, preceding the formation of bone and resulting in formation of the growth plate that controls the longitudinal growth of the skeleton (Olsen *et al.*, 2000). Thus, cartilage can be considered the foundation for the majority of the skeleton in vertebrates (Karsenty and Wagner, 2002). In adult mammals, cartilage is integral to the process of fracture healing and, in joints, plays a central role in skeletal mobility (Kronenberg, 2003).

The endochondral skeleton constitutes most skeletal elements of the body and is formed in two main steps. The first step involves the formation of cartilage in the process of chondrogenesis, which is initiated when mesenchymal cells aggregate to form condensations that determine the shape and location of future bones (Karsenty, 2003; Kronenberg, 2003; Zelzer and Olsen, 2003). Cells in these condensations differentiate to chondrocytes, which create the anlagen of future bones. Endochondral ossification is the subsequent step, which involves the creation of bone tissue and bone utilizing the cartilage template (Karsenty, 2003).

1.1.2 Chondrogenesis and endochondral bone formation

Formation of the complete adult skeleton is achieved by two separate mechanisms: intramembranous and endochondral ossification (Kronenberg, 2003; Zelzer and Olsen, 2003). Both of these processes involve the same initial step in which mesenchymal cells aggregate to form condensations. Intramembranous ossification occurs when the cells in the mesenchymal condensations differentiate directly into osteoblasts, omitting the intermediate step involving chondrocytes. This process occurs in only a few skeletal structures including parts of the craniofacial skeleton and the clavicles (Hall, 1988). Most of the axial and appendicular skeleton develops by the two-step process of chondrogenesis and endochondral ossification (Kronenberg, 2003) (Fig. 1.1 and 1.2).

Chondrogenesis starts with the aggregation of mesenchymal cells (for example, from the lateral plate mesoderm) into condensations (Karsenty, 1999). This process is reliant on increasing cell-cell interaction and a high cell density which are responsible for allowing the propagation of signal transduction events necessary for the initiation of chondrogenic differentiation (Cohn, 1996). In this process, cells initiate the expression of cell-cell adhesion molecules such as n-cadherin, which is important in the initiation of the condensations (DeLise *et al.*, 2000a), and neural cell adhesion molecule (N-CAM), which appears to be critical for their maintenance (Oberlender and Tuan, 1994). Not only does the expression of genes change during commitment to the chondrogenic lineage, but cellular morphology shifts from fibroblastic cells to spherical cells, and secretion of a chondrogenic ECM occurs (Wagner and Karsenty, 2001). Cells

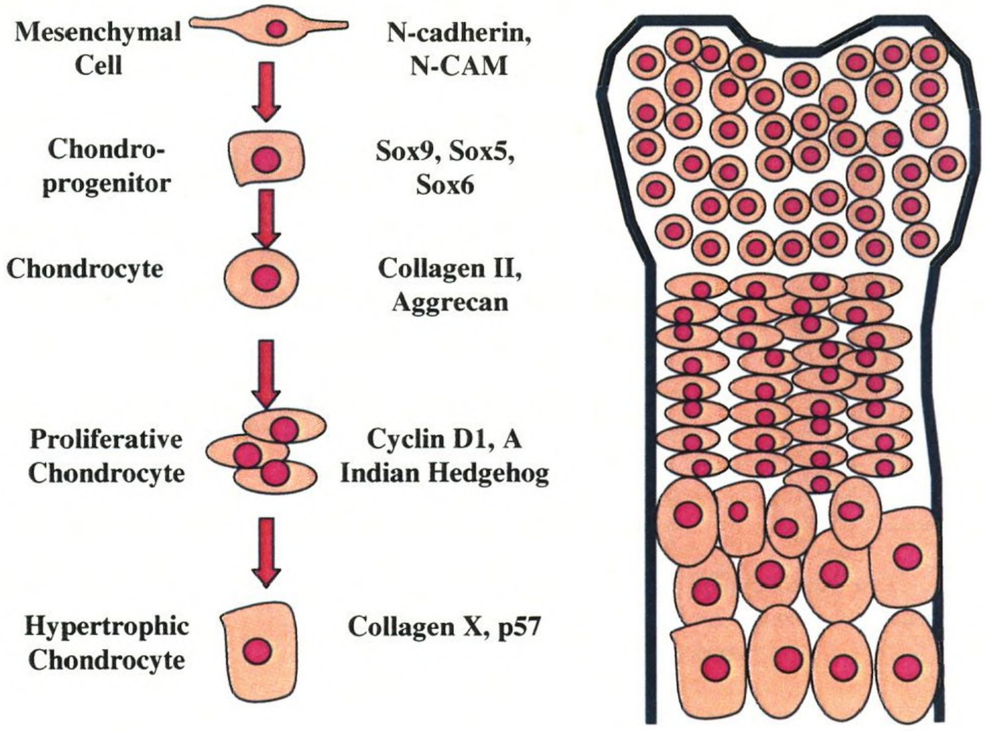


Figure 1.1 The process of chondrogenesis. Chondrogenesis is initiated with the aggregation of mesenchymal cells to condensations. Cells in these condensations grow in size and eventually proliferate and differentiate to terminal hypertrophy. Each stage of chondrogenesis is characterized by different molecular markers. *Adapted from (Stanton et al., 2003).*

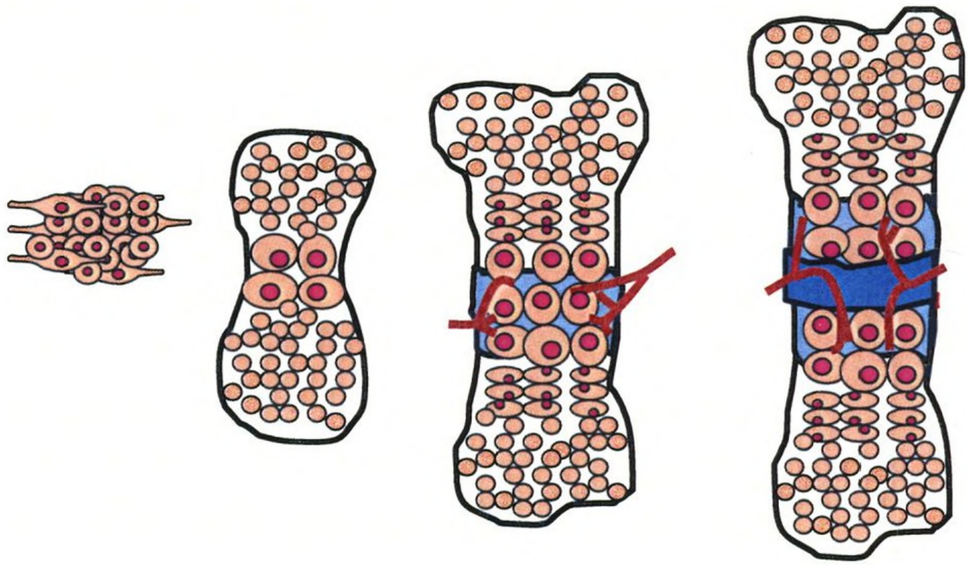


Figure 1.2 Endochondral ossification. Once the mesenchymal cells form the condensations, they develop in the growth plate. Through proliferation and differentiation events, the chondrocytes develop to hypertrophy, are eventually vascularized and replaced by bone. *Adapted from (Stanton et al., 2003).*

surrounding the condensations are responsible for forming the perichondrium (Horton, 1993). The cells in the condensations begin to express markers that are typical of early chondrogenic cells such as the transcription factor sox9, type II collagen, and aggrecan (Stanton *et al.*, 2003). Visual discrimination (Fell, 1925) is one way to ascertain the amount of condensation occurring during the early stages of chondrogenesis. Another marker for cellular condensations is the ability of cells to bind peanut agglutinin (PNA), which is a lectin that recognizes the disaccharide Gal(β1,3)GalNAc in condensing cells (Tuan, 2004). Once mesenchymal cells commit to the chondrogenic lineage, the subsequent events of endochondral bone formation occur through the growth plate (Kronenberg, 2003).

The epiphyseal growth plate is found at the distal ends of long bones between the epiphysis and metaphysis (van der Eerden *et al.*, 2003). The growth plate is comprised of three regions: a region of resting chondrocytes, a region of proliferating chondrocytes, and a region of hypertrophic chondrocytes (Chen *et al.*, 1995). These populations of cells are arranged in organized zones that are distinguishable structurally and by different molecular markers (Wagner and Karsenty, 2001). Within the growth plate the proliferation of the chondrocytes occurs in a unidirectional manner, resulting in longitudinal bone growth (Karsenty, 2001). As they develop in the growth plate, chondrocytes produce a specific ECM based on what stage of maturation they have acquired.

1.1.3 The extracellular matrix

The ECM of chondrocytes is the molecular material that is secreted by, and surrounds, the cells (Hacker *et al.*, 2005). It allows for the organization of tissues and

mediates many cellular activities, such as locomotion, storage of growth factors and cell shape (Hacker *et al.*, 2005). Generally, there are three main components of the ECM: structural proteins, proteoglycans and adhesive glycoproteins. The two components that are most essential for the chondrocyte matrix are the structural proteins and the proteoglycans (Ruoslahti, 1988). The structural proteins are comprised of collagens and elastins. Collagens are essential in the chondrocyte ECM because they provide tensile strength, while elastins provide flexibility (Scott, 2003). Proteoglycans are proteins with polysaccharide side-chains that constitute the matrix in which the structural proteins are embedded. Adhesive glycoproteins include fibronectin and laminin which allow for cellular attachment. The ECM not only plays an integral structural role in chondrocyte biology, but is also responsible for transducing signals and for the storage and release of a number of growth factors (Ruoslahti, 1988). Given that the ECM is essential to chondrocyte functions, slight changes in its composition during development are expected to result in significant changes to its ability to provide signaling cues to its resident cells. For example, an absence of the proteoglycan aggrecan results in cartilage matrix deficiency or nanomelia which are embryonic lethal diseases (Krueger *et al.*, 1999; Li *et al.*, 1993; Watanabe *et al.*, 1994). Similarly, if the amount of type II collagen is lessened in the ECM, this can result in a number of collagenopathies ranging from lethal to mild (Chen *et al.*, 1981). Therefore it is essential to maintain coordinated production and remodeling of the ECM, including a proper proteoglycan-to-collagen ratio, during chondrogenic differentiation.

1.1.3.1 Collagen

The expression of type II collagen is critical during the initiation of chondrogenesis and is often used as a molecular marker for early chondrogenesis. As mesenchymal cells condense and become chondrocytes, type II collagen expression increases (Dessau *et al.*, 1980). Collagen expression shifts from type II collagen to type X collagen as the cells approach hypertrophy during chondrogenesis (Reichenberger *et al.*, 1991). The mechanical and tensile strength of the ECM can be attributed to type II collagen. The interaction of type II collagen with aggrecan is important to the composition of the ECM, since type II collagen and aggrecan are thought to be co-expressed and co-regulated by sox9 (Lefevbre *et al.*, 1997; Sekiya *et al.*, 2000).

Structurally, type II collagen is composed of a rigid triple helix and is a homotrimer of three α1 (II) chains encoded by the *Col2a1* gene (Dessau *et al.*, 1980). Sox9 binds to a 48 base pair enhancer element in the *Col2a1* gene to stimulate its transcription (de Crombrugghe *et al.*, 2000). It is thought that an interaction of l-sox5 and sox6 with sox9 is necessary for maximal activation of *Col2a1* transcription (Smits, 2001).

The requirement for type II collagen as a component of early chondrocyte matrix is demonstrated by biological pathologies known as collagenopathies. Alterations to the *Col2a1* gene can result in an array of developmental abnormalities in humans, ranging from severe and lethal to mild and sometimes treatable. Achondrogenesis type II or Langer-Saldino achondrogenesis is an autosomal dominant disorder (Chen *et al.*, 1981). The disease is not, however, passed onto offspring since patients usually die at or shortly after birth due to respiratory failure. This disease is characterized by irregular cartilage that is gelatinous in appearance and affected individuals show abnormalities in

ossification of endochondral bones. Comparably, a severe phenotype is seen in mice with a null mutation in the *Col2a1* gene (Li *et al.*, 1995). These mice resemble human achondrogenesis type II and display perinatal lethality and many morphological and histological malformations of their skeleton (Li *et al.*, 1995). *Col2a1* gene expression is needed for normal chondrogenic and osteogenic development; however, in the complete absence of type II collagen some bone develops. When there is no type II collagen in the ECM, there is compensation by type I collagen and type III collagen (Chan *et al.*, 1995). An example of a milder collagenopathy is the heterogenous disorder, Stickler syndrome. In the case of a mutation in the *Col2a1* gene, the disease involves the onset of osteoarthritis at an early age, hyperextensible joints and in some cases, cleft palate (Chen *et al.*, 1981; Eyre *et al.*, 1986).

1.1.3.2 Aggrecan

Proteoglycans in general play an integral organizational role in chondrocyte biology by acting as a storage area for growth factors. Structurally proteoglycans aid chondrocyte development owing to their extensive hydration which acts to provide cushioning support for the ECM (Hardingham and Fosang, 1992). Aggrecan is the major proteoglycan of the ECM of chondrocytes (Watanabe *et al.*, 1998). The core size of aggrecan is 225-250 kDa, consisting of a core protein with many covalently bound glycosaminoglycan (GAG) side-chains (Hardingham and Bayliss, 1990). GAGs on aggrecan are comprised of mainly chondroitin sulphate and fewer keratin sulphate chains (Watanabe *et al.*, 1998). The GAG attachments are composed of a backbone of repeating disaccharide units incorporating amino sugars (Hardingham and Bayliss, 1990). The GAG chains have an overall anionic charge which acts as an attractant of water, which

allows proteoglycans to keep the ECM hydrated and provides resistance to compression (Krueger *et al.*, 1990).

Aggrecan has three domains and is connected via link protein to hyaluronan, which forms large multi-molecular aggregates in the cartilage ECM (Hascall and Heinegard, 1974). Cartilage matrix deficiency (CMD) or nanomelia are diseases that result in embryonic lethality in mice and chickens and malformation and shortening of limbs due to a mutation in the *Agc* gene (Krueger *et al.*, 1999; Li *et al.*, 1993; Watanabe *et al.*, 1994). The matrix of these mice shows a great reduction in the amount of aggrecan and a general disorganization of the ECM. In humans, the group of diseases known as Spondyloepiphyseal dysplasia have been found to be caused by point mutations in the *Agc* gene (Gleghorn *et al.*, 2005). It is thus apparent that not only chondrocytes are responsible for normal skeletal development, but interplay of a number of factors involving both the chondrocytes and their ECM. For example, it is known that the Ras/MEK/ERK cascade is involved in pro-adhesive events with heparin sulphate containing proteoglycans (HSPGs). Syndecan-4, an HSPG, is also known to be involved in pro-adhesive signaling of fibroblasts including ERK activation in fibroblasts (Chen *et al.*, 2004). In order to confirm the involvement of proteoglycans in adhesive signaling cascades, our laboratory has shown that heparin treatment of fibroblasts from patients with fibrosis results in a reduction in ERK activation and pro-adhesive gene expression (Shi-Wen *et al.*, 2006). In summary, an alteration in any number of components found within the ECM can have a large impact on development.

1.1.3.3 Coordinated regulation of collagen and aggrecan expression: The sox trio

Because of the apparent necessity for coregulation of aggrecan and type II collagen expression, it is *a priori* not surprising that similar transcription factors regulate their gene expression. Sox9, l-sox5 and sox6 are transcription factors which are primarily responsible for the regulation of chondrocyte ECM genes by controlling the expression of type II collagen, type IX collagen, type XI collagen and aggrecan (Okazaki and Sandell, 2004). The sox family of transcription factors are characterized by high mobility group (HMG) box DNA binding domains (Ikeda *et al.*, 2005). Sox9 is essential for the initiation of the differentiation process from mesenchymal cells to chondrocytes (Wright *et al.*, 1995). Sox9 has a transcription activation domain and binds to a specific sequence in the minor groove of DNA (Ikeda *et al.*, 2005; Wright *et al.*, 1995). It has also been found that the transcriptional targets of sox9 need other factors in addition to sox9 in order to produce maximal transcription (Tan *et al.*, 2003).

A physiological role for sox9 was first described in Campomelic Dysplasia (CD). CD is a developmental disease caused by heterozygous loss-of-function mutations in the *Sox9* gene and is characterized by hypoplaysia of most skeletal elements that are derived by endochondral ossification, resulting in dwarfism and bowing of long bones (Foster, 1994; Wagner *et al.*, 1994). CD demonstrates the requirement for sox9 in the formation of a cartilage template for proper development of bones. This disease was phenocopied by heterozygous *Sox9*-deficient mice that displayed delayed mesenchymal condensations and premature endochondral ossification (Bi, 2001). Mice chimeric for the *Sox9* gene were created, where *Sox9* homozygous mutant cells were unable to differentiate to

chondrocytes or to express any of the early chondrogenic markers. Therefore, absence of *Sox9* results in the blockage of chondrogenic differentiation (Bi *et al.*, 1999).

Sox9 acts together with l-sox5 and sox6 to regulate the expression of the two main chondrocyte matrix components, type II collagen and aggrecan (Lefevbre *et al.*, 1997). Except for the HMG-box, l-sox5 and sox6 do not have sequence similarity to sox9, and therefore they are thought to have complimentary function (de Crombrugghe *et al.*, 2000; Lefebvre *et al.*, 1998). Both l-sox5 and sox6 have a highly conserved coiled-coil domain and can form homo- or hetero-dimers (Lefebvre *et al.*, 1998). Structurally, l-sox5 and sox6 do not have their own transcription activation domain and are thought to act to aid the actions of sox9 (Lefebvre *et al.*, 1998). The inactivation of *L-Sox5* and *Sox6* genes was achieved via homologous recombination. The individual knock-out animals were born with mild skeletal defects (Lefebvre *et al.*, 2001). However, the double knock-out of *L-Sox5* and *Sox6* is characterized by generalized chondrodysplasia, a deficient cartilage matrix and embryonic lethality at 16.5 days post coitum (dpc) caused by cardiovascular failure from compression of internal organs due to an underdeveloped skeleton (Lefebvre *et al.*, 2001; Smits, 2001). The knockout model implicates the necessity for l-sox5 and sox6 in the development of normal cartilage and bones.

The sox trio is therefore integral for the initiation of chondrogenesis and development of chondrocytes that are capable of producing a normal cartilage ECM. Sox9 also regulates the transcription of *L-Sox5* and *Sox6* (Akiyama *et al.*, 2002a) which after translation, then, in turn, aid sox9 in the activation of other sox9-dependent genes. However, evidence still needs to be generated on the individual activities and functions of l-sox5 and sox6 in chondrogenesis.

1.1.3.4 Adhesive signaling and chondrogenesis

The early stages of chondrogenesis, specifically the formation of condensations and cartilage nodules, require remodeling of the actin cytoskeleton, in an integrin/focal adhesion kinase (FAK)-dependent fashion (Bang *et al.*, 2000). Focal adhesions form upon clustering and activation of integrin receptors, and FAK is necessary for the turnover of focal adhesions (Parsons *et al.*, 2000; Wozniak *et al.*, 2004). In a micromass model of early chondrogenesis, depolymerization of the actin cytoskeleton reduces expression of l-sox5 and sox6, but not sox9, resulting in a decrease of sox-dependent transcription of chondrocyte-specific matrix genes, emphasizing the importance of l-sox5 and sox6 in sox-dependent transcription (Woods and Beier, 2006). Proper early chondrogenic development is thus achieved by the interplay between the sox family of transcription factors and many other molecules. Despite what is known about the sox trio and their apparent cooperation with each other for co-regulation of aggrecan and type II collagen, much remains unknown about their specific and individual contributions to the induction of these genes. Therefore, the interplay between the activation and regulation of these two essential ECM components, aggrecan and type II collagen during the early chondrogenic program still remains poorly understood.

1.1.4 Clinical relevance and disease

Cartilage forms the initial skeletal framework in the embryo, directs the patterning of head, trunk and limb development, and functions throughout life to protect organs and aid in joint mobility (Karsenty and Wagner, 2002). The early stage of chondrogenesis is absolutely essential to lay the foundation for proper skeletal formation. The mammalian geneticist Hans Gruneberg found a multitude of mutations affecting skeletal development

acting at the early chondrogenic condensation formation stage. He termed the condensations the "membranous skeleton", that precedes the cartilaginous and osseous skeleton (Gruneberg, 1963), which emphasizes that the early framework of chondrogenesis is vital to the development of the skeleton. In addition, structures composed of cartilage in the adult, such as the larger airways, joints, and ears, are imperative for respiration, mobility and hearing (Olsen *et al.*, 2000). Disturbances in the development of cartilage via mutations, hormonal or environmental changes causing malformation of structures can thus have severe consequences for affected individuals (Kornack and Mundlos, 2003). Not only is functionality of structures affected in pathological conditions involving cartilage, but quality of life is compromised as well.

Fibroblast growth factor (FGF) signaling pathways are involved in the longitudinal growth of bones through regulation of chondrocyte proliferation and differentiation in the growth plate. Deregulation of these pathways is involved in pathological disease states. Research has shown that specific inactivation of fibroblast growth factor receptor 3 results in overgrowth of long bones (Ornitz and Marie, 2002). In contrast, activating mutations in the human *Fgfr3* gene result in dwarfism, specifically achondroplasia, the most common form of human dwarfism (Shiang, 1994). Disorders affecting sox9 have a large impact on chondrogenesis since sox9 is expressed in the mesenchymal condensations. Mutations of the *Sox9* gene cause CD, as described above (Foster, 1994; Wagner, 1994). Most of the affected infants die shortly after birth because of respiratory distress caused by a small rib cage, narrow airways, and hypoplastic lungs (Kornack and Mundlos, 2003). In addition, mutations in cartilage ECM genes cause numerous diseases as described in detail above.

A greater understanding of the molecular regulatory mechanisms and genes involved in the development of cartilage in both healthy states and pathological disease conditions will allow efforts to be directed towards new preventative strategies and treatment options for skeletal diseases. Many genes are involved in the development of the human skeleton, and increasing our knowledge on the roles of these genes and their signaling pathways will allow for a more comprehensive understanding of embryonic development of the skeleton and management of diseases. One gene (*Ctgf/Ccn2*) that has recently been identified as a crucial regulator of cartilage development, encodes the protein connective tissue growth factor.

1.2 Connective tissue growth factor

1.2.1 CCN family

The CCN (CYR61, CTGF, NOV) family consists of pro-adhesive matricellular regulatory factors and contains 6 members in humans (Bork, 1993; Leask and Abraham, 2006; Perbal, 2004). The members are: cysteine-rich 61 (CYR61, CCN1), connective tissue growth factor (CTGF, CCN2), nephroblastoma overexpressed (NOV, CCN3), and Wnt-induced secreted proteins-1 (WISP-1, CCN4), -2 (WISP-2, CCN5) and -3 (WISP-3, CCN6) (Brigstock, 2003). Structurally, the CCN proteins are characterized by the presence of an N-terminal secretory signal, followed by as many as 4 predicted modules and 38 cysteine residues that are largely conserved among members of the family (Lau and Lam, 1999). To date there has not been any three-dimensional biochemical validation of these predicted modules. Module 1 is an insulin-like growth factor (IGF) binding domain; module 2 is a Von Willebrand type C domain; module 3 is a thrombospondin-1 domain; and module 4 is a C-terminal domain containing a putative

cysteine knot (Bork, 1993; Brigstock, 2003; Perbal, 2004) (Fig. 1.3). Perbal (2004), hypothesized that the individual modular domain structure of the CCN family may be a basis for the function of these proteins since each individual domain is thought to partake in different cellular interactions (Perbal, 2004).

The CCN proteins were first discovered approximately 15 years ago (Bradham *et al.*, 1991; Brigstock, 1999; Lau and Lam, 1999; Perbal, 2001). The CCN family has been proposed to be responsible for the stimulation of a myriad of processes including mitosis, adhesion, apoptosis, ECM production, growth arrest and migration of multiple cell types and to play a role in mediating angiogenesis, tumour growth, placentation, implantation, embryogenesis and endochondral ossification (Brigstock, 2003; Lau and Lam, 1999; Leask and Abraham, 2006; Moussad and Brigstock, 2000; Perbal, 2004). Precise functional data linking CCN family members to specific biological responses, however, is scant. Attempts have been made to identify a specific signal transducing receptor for this family, but none has yet been discovered (Lau and Lam, 1999). Rather, CCN molecules appear to act as matricellular proteins which allow communication between ECM associated proteins and their cell surface receptors, resulting in amplification of a variety of signaling responses (Lau and Lam, 1999; Leask and Abraham, 2006; Moussad and Brigstock, 2000; Perbal, 2004). Genetic studies involving CCN family members, especially CCN1 and CCN2, have demonstrated that these proteins play an essential role in blood vessel and bone formation during development (Ivkovic *et al.*, 2003; Mo *et al.*, 2002).

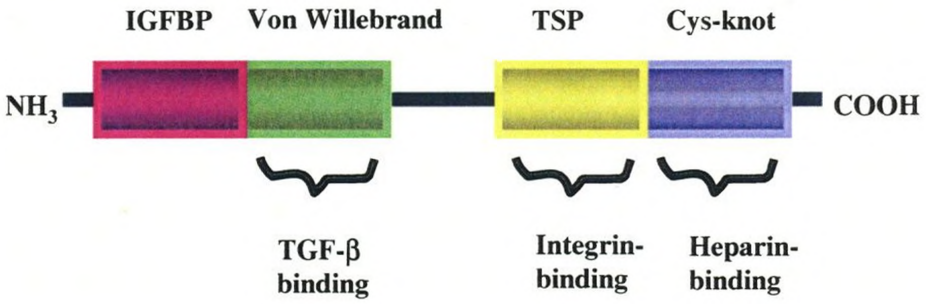


Figure 1.3 Typical Structure of CCN family proteins. The structure of connective tissue growth factor follows this pattern. The first module is an insulin-like growth factor binding domain, the second is a Von Willebrand type C domain involved in TGF-β interactions, the third is a thrombospondin-1 domain associated with integrin-binding and the fourth is a putative cysteine knot largely linked with heparin interactions. *Adapted from (Leask and Abraham, 2003).*

1.2.2 Connective tissue growth factor structure

Bradham et al. (1991), identified human CCN2 as a 38-kDa polypeptide growth factor secreted by human endothelial cells that could stimulate DNA synthesis and chemotaxis in fibroblasts (Bradham *et al.*, 1991). CCN2 is a cysteine-rich pro-adhesive matricellular protein that plays a role in the formation of blood vessels, bone and connective tissue. After synthesis, CCN2 is secreted via the Golgi apparatus and it is a glycosylated protein, but this modification is not necessary for its secretion and does not seem to serve any specific function (Chen *et al.*, 2001). The secretion of CCN2 is highly dependent on an N-terminal amino acid sequence (Chen *et al.*, 2001).

1.2.3 Connective tissue growth factor expression

CCN2 is primarily expressed during conditions of tissue remodeling and repair. As such, it is expressed during development and in wound healing and overexpressed in fibrotic pathologies; however, it is not normally expressed in adulthood (Leask and Abraham, 2006). We believe that CCN2 plays an important role in developmental biology since it is highly expressed in developing mesenchyme, the glomerulus in the developing kidney and connective tissue surrounding muscle and in hypertrophic chondrocytes in the growth plate of cartilage (Friedrichsen *et al.*, 2003). *Ccn2* is also found at the mRNA level in layer VII neurons (Friedrichsen *et al.*, 2003). CCN2 expression has been detected during female reproduction, such as during embryo implantation (Surveyor *et al.*, 1998) and in ovarian follicogenesis (Slee *et al.*, 2001). CCN2 is overexpressed in various pathological conditions, including scarring and fibrosis (Leask *et al.*, 2004), cancer progression (Brigstock, 1999) and atherosclerosis of blood vessels (Oemar and Luscher, 1997).

Igarashi et al. (1993), found that CCN2 was a serum- or transforming growth factor-beta (TGF-β)-inducible immediate early gene (Igarashi *et al.*, 1993). Indeed, CCN2 has long been hypothesized to be a potential mediator of TGF-β action *in vivo* (Grotendorst, 1997). Also, Mori and colleagues (1999) found that CCN2 acted with TGF-β to promote sustained fibrotic responses *in vivo* (Mori *et al.*, 1999) and appears to be a physiologically relevant co-activator of TGF-β-mediated adhesive signaling (Shi-wen *et al.*, 2006). However, CCN2 expression can occur through both TGF-β-dependent and -independent mechanisms (Blom *et al.*, 2001). In particular, TGF-β activates *Ccn2* gene expression through TGF-β response element including functional binding motifs for Smad3 and Ets-1, and through protein kinase C (PKC) and ras/MEK/ERK (Holmes *et al.*, 2001; Leask and Abraham, 2003; Shi-Wen *et al.*, 2006; Van Beek *et al.*, 2006). Endothelin-1 further activates *Ccn2* gene expression through a distinct element in the *Ccn2* promoter (Shi-Wen *et al.*, 2004). CCN2 is expressed independently of TGF-β and the TGF-β response element in embryonic and fibrotic fibroblasts, likely through an endothelin-1-dependent mechanism (Holmes *et al.*, 2001; Leask and Abraham, 2006; Shi-Wen *et al.*, 2004; Shi-Wen *et al.*, 2006).

1.2.4 Connective tissue growth factor function

That CCN2 is expressed both dependent upon and independent of TGF-β *in vitro*, implies that CCN2 has independent activity. Indeed, it is unclear to what extent CCN2 acts alone or in concert with TGF-β, or additional growth factors, *in vivo*. By itself, CCN2 has potent adhesive ability (Chen *et al.*, 2002; Shi-Wen *et al.*, 2006; Yang *et al.*, 2005). CCN2 can promote cell survival by stimulating cell proliferation, inducing DNA synthesis, modulating ECM production and supporting angiogenesis; however, in most

studies these activities are modest and rely on a co-factor for potent responses (Brigstock, 1999). For example, CCN2 promotes adhesion, migration and cell survival in endothelial cells in an integrin-$\alpha_v\beta_3$ dependent mechanism (Babic *et al.*, 1999). However, CCN2 is also recognized as an apoptotic factor. In the human breast cancer cell line MCF-7, transient overexpression of CCN2 resulted in a significant increase in apoptosis (Hishikawa *et al.*, 1999). Also in fibroblasts CCN2 has the ability to unmask the cytotoxic effects of tumour necrosis factor-α (TNF-α) which leads to apoptosis through integrin-$\alpha_v\beta_5$, integrin-$\alpha_6\beta_1$ and syndecan-4 mediated mechanisms (Chen *et al.*, 2007). Furthermore, it was described that CCN2 does not promote angiogenesis but is rather anti-angiogenic. Inoki et al. (2002), discovered the anti-angiogenic property of CCN2 by screening a chondrocyte cDNA library to find inhibitors against vascular endothelial growth factor (VEGF) using a yeast two-hybrid system (Inoki *et al.*, 2002). Some factors contributing to the diverse and sometimes conflicting roles of CCN2 can be attributed to a lack of both readily available commercial reagents and highly purified recombinant CCN2 which contribute to inconsistent model systems, and to varying assay conditions used between laboratories (Moussad and Brigstock, 2000). These problems leave gaps in the knowledge about CCN2 signal transduction pathways and activities.

1.2.5 Connective tissue growth factor receptors

One feature of the field of CCN2 research is the fact that no specific CCN2 receptor has been identified (Moussad and Brigstock, 2000). It was initially proposed that CCN2 was binding to platelet derived growth factor (PDGF) receptors (Bradham *et al.*, 1991); however, this has proved to be incorrect. Subsequently, cell surface integrins were proposed to be possible receptors for CCN2 (Lau and Lam, 1999). Indeed, it is now

accepted that integrins and HSPGs are the functional receptors for CCN2 (Chen *et al.*, 2001a). The integrins and HSPGs that CCN2 signals through vary depending on the cell type and assay examined. For example, in human foreskin fibroblasts it was found that CCN2 acts through integrin-$\alpha_6\beta_1$ to promote adhesion (Babic *et al.*, 1999). In contrast, CCN2 promotes adhesion in human platelets through integrin-$\alpha_{IIb}\beta_3$, endothelial cells by integrin-$\alpha_V\beta_3$ and blood monocytes by integrin-$\alpha_M\beta_2$. As mentioned above, CCN2 also acts via integrin-$\alpha_v\beta_5$ and integrin-$\alpha_6\beta_1$ and the HSPG syndecan-4 mediated mechanisms to promote apoptosis in fibroblasts (Chen *et al.*, 2007). In the chondrosarcoma-derived cell line HCS-2/8, CCN2 promotes proliferation through the HSPG perlecan, primarily during the later stages of chondrogenesis (Nishida *et al.*, 2003). CCN2 has also been observed to bind to lipoprotein receptor related protein (LRP), which is 620-kDa in size and is principally involved with CCN2 turnover (Chen *et al.*, 2001b; Segarini *et al.*, 2001). LRP may also be implicated in cell adhesion (Babic *et al.*, 1999; Lillis *et al.*, 2005; Segarini *et al.*, 2001). Nishida and colleagues detected a novel 280-kDa binding protein for CCN2 specifically in chondrocytes and osteoblasts (Nishida *et al.*, 2000). However, the techniques used to identify this novel binding protein were the same as those which identified the CCN2/LRP interaction; therefore, it is highly likely that the proteins detected by Nishida and colleagues (2000) and Segarini and colleagues (2001) are similar, if not identical.

1.2.6 The role of connective tissue growth factor in skeletal development

Owing to the unresolved issues in the CCN field described above, relating to the lack of a specific signaling receptor and the conflicting views about CCN2 function *in vitro*, genetic models to elucidate the function of CCN family members, including CCN2,

are essential. Ivkovic and colleagues (2003), found that CCN2 is essential for normal endochondral ossification. Mice homozygous for a deletion in the *Ccn2* gene had expanded hypertrophic zones in long bones and an underdeveloped rib cage and died soon after birth due to an inability to breath (Ivkovic *et al.*, 2003). These mice were also unable to produce cartilage-specific ECM components such as aggrecan and link protein and displayed an inability of chondrocytes to proliferate (Ivkovic *et al.*, 2003). Conversely, a gain of function model for CCN2 function *in vivo* was also developed. Transgenic mice overexpressing CCN2, developed under the control of the type XI collagen promoter show decreased bone density and exhibited dwarfism within a few months of birth (Nakanishi *et al.*, 2001). These *in vivo* loss and gain of function models suggest that CCN2 is important in processes involved in remodeling of the skeletal ECM.

Most of the *in vitro* studies to date have been primarily focused on the role of CCN2 in the late stages of chondrogenesis. A gene that is predominantly expressed in hypertrophic chondrocytes was isolated from a human chondrosarcoma-derived cell line, HCS-2/8, and it was found that it encoded CCN2 (Nakanishi *et al.*, 1997). They further found that this gene, named *Hcs24* (hypertrophic chondrocyte specific gene product No. 24), was highly expressed in hypertrophic chondrocytes and up-regulated in HCS-2/8 chondrosarcoma cells by TGF-β and bone morphogenetic protein-2 (BMP-2), both of which are known regulators of endochondral ossification (Nakanishi *et al.*, 1997). Takigawa and coworkers (2003), observed that CCN2 had the ability to promote proliferation, maturation and hypertrophy of HCS-2/8 chondrosarcoma cells (Takigawa, 2003). They also found that CCN2 stimulated the proliferation and differentiation of

osteoblastic cells. They established that CCN2 was produced by osteoblasts in primary spongiosa but at a lower level than in the hypertrophic cell. The current *in vitro* studies of CCN2 function pertaining to bone formation are therefore primarily based on evidence obtained from cell lines, such as the chondrosarcoma-derived chondrocytic cell line HCS-2/8, or in mature osteoblasts which is perhaps more relevant in revealing roles for CCN2 in cancer or later stages of development, respectively, rather than chondrogenesis or osteogenesis *per se* (Nakanishi *et al.*, 1997; Takigawa, 2003; Takigawa *et al.*, 2003).

The *in vivo* genetic evidence that CCN2 is essential for bone development (that is, chondrogenesis and osteogenesis) therefore currently lacks an appropriate mechanistic *in vivo* or *in vitro* context. As a specific example, it is interesting to note that despite the fact that CCN2 is a known inducer of type X collagen in the HCS-2/8 cells, there is no difference in this hypertrophic cartilage marker between the *Ccn2*$^{-/-}$ and *Ccn2*$^{+/+}$ mice (Ivkovic *et al.*, 2003; Nakanishi *et al.*, 2000). Therefore, the lack of proper endochondral ossification in *Ccn2*$^{-/-}$ mice could be due to disturbances at any stage in bone formation, including improper early chondrogenic events (Fig. 1.4), leading to the development of a malformed chondrocyte template and subsequently malformed bone. Detailed mechanistic investigations into the stages of endochondral ossification regulated by CCN2 are therefore required. For example, disruption of early chondrogenesis could result in a failure of the improperly formed chondrocyte template to activate osteogenic signals for normal bone formation. Furthermore, analyses of the cartilage phenotype in *Ccn2*-deficient mice suggest alterations in chondrocyte ECM synthesis, but this has not been studied in a detailed and quantitative manner. Overall, an examination of the precise

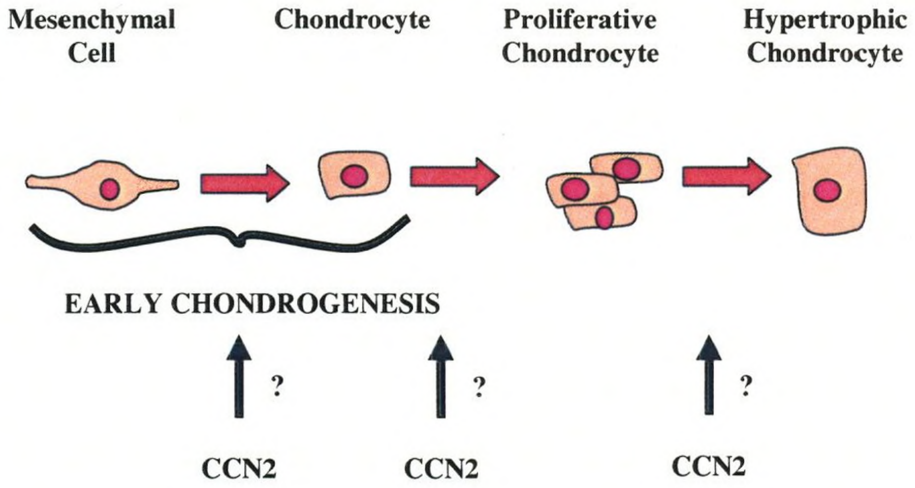


Figure 1.4 Potential roles of connective tissue growth factor in chondrogenesis. It is not known what stage of chondrogenesis CCN2 is exerting its effects. It is also not known by which signalling mechanism it is taking action.

molecular mechanism by which CCN2 exerts its effects in bone formation has not yet been undertaken and is required.

1.3 Rationale, Objectives and Hypothesis

Based on results obtained using *Ccn2*$^{-/-}$ mice, CCN2 plays a role in skeletogenesis (Ivkovic *et al.*, 2003). From the existing, albeit problematic, *in vitro* studies, it may be inferred that CCN2 is involved in the late stages of chondrogenesis, in hypertrophic chondrocyte maturation. However, as discussed above, the precise observed phenotype of the *Ccn2*$^{-/-}$ mice may arise due to defects at any step in bone formation.

There is indeed evidence providing further support to the notion that the phenotype of the C*cn2*$^{-/-}$ mice may arise due to defects occurring at earlier stages in chondrogenesis. The overall kinked appearance of the long bones in the *Ccn2*$^{-/-}$ mice may, in fact, implicate a defect in the formation of the cartilage anlagen occurring at the early stages of chondrogenesis, which could result in subsequent improper development. The chondrocyte template is essential for proper bone formation, and lack of proper initiation of chondrogenesis can result in a number of skeletal abnormalities, as exemplified by CD, caused by mutations in the *Sox9* gene (Foster, 1994; Wagner *et al.*, 1994). It is therefore fair to speculate that in the *Ccn2*$^{-/-}$ mice, a similar defect early in chondrogenesis may contribute to the bone phenotype. Moreover, CCN2 is generally expressed in mesenchymal cells during development (Friedrichsen *et al.*, 2003; Holmes *et al.*, 2001) and is essential for adhesive signaling and migration of these cells (Chen *et al.*, 2004; Kennedy *et al.*, 2007; Song *et al.*, 2007). Also, CCN2 was found to be expressed in the mesenchymal cell condensations and the perichondrium of developing Meckel's cartilage *in vivo* and recombinant CCN2 treatment promoted adhesion and migration of

Meckel's cartilage progenitor cells *in vitro* (Shimo *et al.*, 2004). Early chondrogenic events such as the aggregation of mesenchymal cells to form condensations involve adhesive signaling and migration (Hall and Miyake, 2000). It is plausible, therefore, that CCN2 is involved in these steps since CCN2 is involved in adhesive signaling and migration (Babic *et al.*, 1999). Furthermore, data from our earlier microarray analyses of $Ccn2^{+/+}$ and $Ccn2^{-/-}$ cells indicates changes of known early chondrogenic genes (Table 1). Our array data, in conjunction with established data on the connection between early chondrogenesis and skeletal defects, provides ample rationale to pursue a study of CCN2 gene expression in the early stages of chondrocyte differentiation.

Based on these above observations, I hypothesized that CCN2 is required for ECM production in early chondrogenesis. To test my hypothesis, I compared the abilities of $Ccn2^{+/+}$ and $Ccn2^{-/-}$ mouse embryonic fibroblasts (MEFs) and $Fak^{+/+}$ and $Fak^{-/-}$ MEFs to undergo early chondrogenic differentiation. Since, it is known that adhesive signaling through FAK is necessary for the recruitment and migration of mesenchymal cell precursors to form condensations and initiate chondrogenesis (Bang *et al.*, 2000), I wanted to look at the effects of FAK expression on CCN2 with the use of the $Fak^{+/+}$ and $Fak^{-/-}$ MEFs. To perform this study I used the micromass method of cell culture that allows for the recapitulation of a physiologically relevant *in vivo* three-dimensional environment *in vitro*. Chondrogenesis is highly reliant on high cell density which allows for a large degree of cell-cell contact (Ahrens *et al.*, 1977; DeLise *et al.*, 2000b). The micromass model system encourages cell differentiation along the chondrogenic lineage (Ahrens *et al.*, 1977; Lengner *et al.*, 2004), by increasing cell-cell

Table 1.1 Microarray of *Ccn2*$^{+/+}$ and $^{-/-}$ MEFs shows down-regulation greater than 2 fold in CCN family members and some known early chondrogenic genes.

Gene	Full Name
Ctgf, Ccn2	Connective tissue growth factor*
Wisp2, Ccn5	Wnt-induced secreted protein-2 **
Cyr61, Ccn1	Cysteine-rich 61*
Agc	Aggrecan*
Hapln	Link protein **
Dcn	Decorin **
Sox6	Sex determining region Y (SRY)-box 6 **

* Published data (Kennedy et al., 2007)
** Unpublished data Leask lab.

contact and reducing the amount of interaction cells have with the culture plate (Ahrens *et al.*, 1977; DeLise *et al.*, 2000b).

My aim was to elucidate upstream regulators of CCN2, to identify what particular aspects of early chondrogenesis require CCN2 and to determine downstream targets of CCN2 in chondrogenesis. Therefore my **overall objective** was to determine whether CCN2 mediates particular events during early chondrogenesis and to determine potential upstream mediators or downstream effectors of CCN2 activity. My **hypothesis** was that CCN2 is required for ECM production in early chondrogenesis. My thesis provides novel insights into the mechanism underlying the role of CCN2 in early stages of chondrogenic differentiation from mesenchymal cells, and thus provides key insights to understanding the essential role of CCN2 in skeletal development.

2.0 Materials and Methods

2.1 Cell culture

2.1.1 Monolayer cell culture

Mouse embryonic fibroblasts (MEFs), (13.5 dpc from *Ccn2*$^{+/+}$ *and* *Ccn2*$^{-/-}$ mice from the lab of Karyn Lyons), were isolated from total embryos and cultured as previously described (Chen *et al.*, 2004; Ilic *et al.*, 1995; Ivkovic *et al.*, 2003; Shi-Wen *et al.*, 2006) in Dulbecco's Modified Eagle's Medium (DMEM), 10% Fetal Bovine Serum (FBS) (Invitrogen), 1% penicillin/streptomycin/amphotericin B (Invitrogen). Monolayer cultures where plated at high density at 2.5 x10^5 cells/well in a 6 well dish (Nunc).

2.1.2 Micromass cell culture

MEFs, (13.5 dpc from *Ccn2*$^{+/+}$ and *Ccn2*$^{-/-}$ mice), and (8.5 dpc *Fak*$^{+/+}$ and *Fak*$^{-/-}$ mice from American Type Culture Collection), were isolated from embryos and cultured as previously described (Chen *et al.*, 2004; Ilic *et al.*, 1995; Ivkovic *et al.*, 2003; Shi-Wen *et al.*, 2006) in DMEM, 10% FBS (Invitrogen), 1% penicillin/streptomycin/amphotericin B (Invitrogen). Briefly, MEFs were isolated from embryos, heads were removed and mechanically digested as well as enzymatic digestion with trypsin EDTA. MEFs were plated at a density of 1x10^5 10 µl droplets into each well of a 24-well tissue culture plate (Nunc) and let to adhere for an hour. Once adhered, micromasses were given 1ml of medium. Micromass cultures were grown for a period of six days, and medium was replenished daily (Ahrens *et al.*, 1977; Lengner *et al.*, 2004). The micromass medium used was 60% F12, 40% DMEM, 10% FBS, supplemented with 0.25% Penicillin/Streptomycin and 0.25% L-glutamine (Invitrogen). Micromass cultures were used for RNA isolation, protein isolation, imaging and staining. For experiments involving inhibitor treatment, starting on Day 0, the day of plating, medium was

supplemented with 10 μM of FAK/Src inhibitor PP2 in dimethyl sulfoxide (DMSO) (Calbiochem), whereas control cultures were supplemented with the vehicle DMSO only. Medium with inhibitor and control was replenished daily. The high density culture system allows for the recapitulation of the *in vivo* high density environment during chondrogenesis which allows for a high degree of cell-cell contact (Ahrens *et al.*, 1977; James *et al.*, 2005; Lengner *et al.*, 2004; Stanton *et al.*, 2004; Woods and Beier, 2006; Woods *et al.*, 2005).

2.2 RNA isolation and Real-Time RT (reverse transcription) PCR

RNA extraction was performed with the QIAGEN RNeasy kit according to the manufacturer's protocol (Qiagen Inc.). RNA was collected from micromass cultures on days 1, 3, 6 of differentiation from $Ccn2^{+/+}$ and $Ccn2^{-/-}$ MEFs and on day 6 from $Fak^{+/+}$ and $Fak^{-/-}$ MEFs. RNA concentration was determined using a spectrophotometer (Beckman Coulter). 25 ng of RNA/reaction was used for Real-Time RT-PCR according to established protocols (James *et al.*, 2005; Stanton *et al.*, 2004; Woods *et al.*, 2005). A total 15 μl reaction volume was used containing the TaqMan one-step master mix kit (Applied Biosystems), gene-specific target primers and probes [FAM (6-carboxyfluorescein) dye layer] and endogenous reference primers and probes (VIC dye layer). The FAM dye layer yields quantification of the target genes, whereas VIC yields simultaneous quantification of glyceraldehyde-3-phosphate-dehydrogense (GAPDH) as an internal control. Relative gene expression was determined by measuring *Col2a1, Agc, Dcn, Hapln, L-Sox5, Sox6, Sox-9, Ccn1, Ccn2, and Ccn5,* using 40 cycles on the ABI Prism 7900 HT sequence detector (Perkin Elmer Life Sciences). All samples were amplified in three parallel reactions per trial, and three independent trials were performed

2.3 Western blot analysis

Harvesting protein was performed using Radioimmunoprecipitation Assay (RIPA) lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% Triton-X, 1% deoxycholate, 0.1% SDS, 2 mM EDTA), 50 mM NaF and 1 mM Na_3VO_3 (Sigma), supplemented with a protease inhibitor mini complete tablet (Roche). Protein concentration was quantified by a kit (BCA, Sigma) as described by the manufacturer's instructions. Equal amounts of cell lysate (40 µg), and of concentrated medium (20 µl) were subjected to SDS/PAGE and transferred to nitrocellulose (Biorad). Membranes were blocked for 1 hour in 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.01% Tween-20, and incubated with anti-sox6 (1:200; Sigma), anti-aggrecan (1:500, R&D Systems), anti-CCN2 antibody (1:2000; FibroGen) or anti-FAK antibody (1:2000; Cell Signaling) overnight at 4°C. Blots were washed 3 times for 5 minutes with Tris-buffered saline with 0.01% Tween-20, followed by the application of appropriate horse radish peroxidase (HRP)-conjugated secondary antibodies and detection of proteins using ECL™ Western blot detection reagents (Amersham Bioscience) according to the manufacturer's instructions and visualized using ChemiImager™5500 (AlphaInnotech Inc.).

2.4 Immunofluorescence

Ccn2$^{+/+}$ and *Ccn2*$^{-/-}$ MEFs were plated in monolayer at a density of 12,000 cells/well in a 24 well dish (Falcon) on glass coverslips. Cells were harvested and fixed in 4% paraformaldehyde (PFA) for 30 minutes at 4°C. Cells were washed in phosphate buffered saline (PBS), incubated for 5 minutes with 0.1% Triton-X in PBS and rinsed again in PBS. Cells were then incubated in blocking solution containing goat serum (Sigma) in PBS, 1:20, for 30 minutes at room temperature. Primary antibodies directed to

sox6 were diluted in blocking solution at a concentration of 1:200 and incubated with coverslips for one hour at room temperature. Coverslips were rinsed in PBS and then incubated with an Alexa-Fluor® 488–conjugated secondary antibody, diluted 1:300 in PBS, for one hour at room temperature in the dark. Following another wash in PBS, coverslips were mounted in VectaShield anti-fade mounting medium containing DAPI. Images were taken with a Zeiss Axiophot microscope using Northern Eclipse software (Empix) and exported into Adobe Photoshop.

2.5 Images

Brightfield images and peanut agglutinin (PNA) stained images of day 6 $Fak^{+/+}$ and $Fak^{-/-}$ MEF micromass cultures were captured with both a Nikon SMZ1500 microscope at .75 times magnification using a Photometrics coolSNAP-cf colour digital camera (Nikon) and PTI ImageMaster 5 software (Photon Technology International).

2.5.1 Peanut agglutinin (PNA)

Micromass cultures of $Fak^{+/+}$ and $Fak^{-/-}$ MEFs were cultured as above. Cultures were fixed on day 6 of culture in 4% PFA at 4°C for 30 minutes. Cells were rinsed with PBS and then incubated for 2 hours in 50 µg/ml PNA diluted in PBS. Cultures were washed again with PBS, and PNA was detected colorimetrically by DAB (Dako Cytomation). Images were captured as described above.

2.6 Statistical Analysis

Data collected from Real-Time RT- PCR are an average of three trials of samples run in triplicate. Means were quantified relative to GAPDH and then data were normalized to day 1 of control per trial, or in the case where there is only one day being examined, data were normalized to the control sample. Statistical significance was

determined by Student's paired t-test or two-way ANOVA, with a level of significance defined as $p < 0.05$ with Bonferroni post test using GraphPad Prism version 4.00 for Windows, (GraphPad Software).

3.0 Results

3.1 Loss of *Ccn2* disrupts early chondrogenic gene expression

Previous *in vivo* studies have revealed that $Ccn2^{-/-}$ mice have severe skeletal defects and die upon birth due to an inability to breathe (Ivkovic *et al.*, 2003). The mechanisms underlying the phenotype of these mice remain unknown. It is also known that CCN2 is highly expressed during embryonic development in mesenchymal tissue (Friedrichsen *et al.*, 2003; Kireeva *et al.*, 1997). Furthermore, our microarray data of $Ccn2^{+/+}$ and $^{-/-}$ MEFs demonstrated that many early chondrogenic genes, such as *Agc, Dcn, Hapln* (the gene encoding link protein) and *Sox6* showed a greater than two-fold reduction in knockout cells (Table 1.1). This suggests that a defect in early chondrogenesis underlies the phenotype of $Ccn2^{-/-}$ mice. Therefore, I first investigated gene expression of some CCN family members that were down regulated in the microarray and some known early chondrogenic markers using $Ccn2^{+/+}$ and $Ccn2^{-/-}$ MEFs plated in monolayer culture for 48 hours. I examined the difference in expression of genes between the wild-type and knockout cells by Real-Time RT-PCR, partially to confirm expression patterns observed in the microarray study. As expected, *Ccn2* mRNA expression was observed in wild-type, but not in knockout cells (Fig. 3.1a). However, knockout cells also showed a significant decrease in the transcript levels for two other CCN family members, *Ccn1* and *Ccn5* (Fig. 3.1b, c). Moreover, mRNA levels for genes encoding the transcription factor *Sox6* (Fig. 3.2a) were significantly reduced in knockout cells, while there was a significant increase in *L-Sox5* gene expression (Fig. 3.2b) and no change in *Sox9* (Fig. 3.2c). Gene expression of the proteoglycans *Agc, Dcn* and the gene encoding for link protein *Hapln* (Fig. 3.3a, b, c) were significantly reduced in knockout

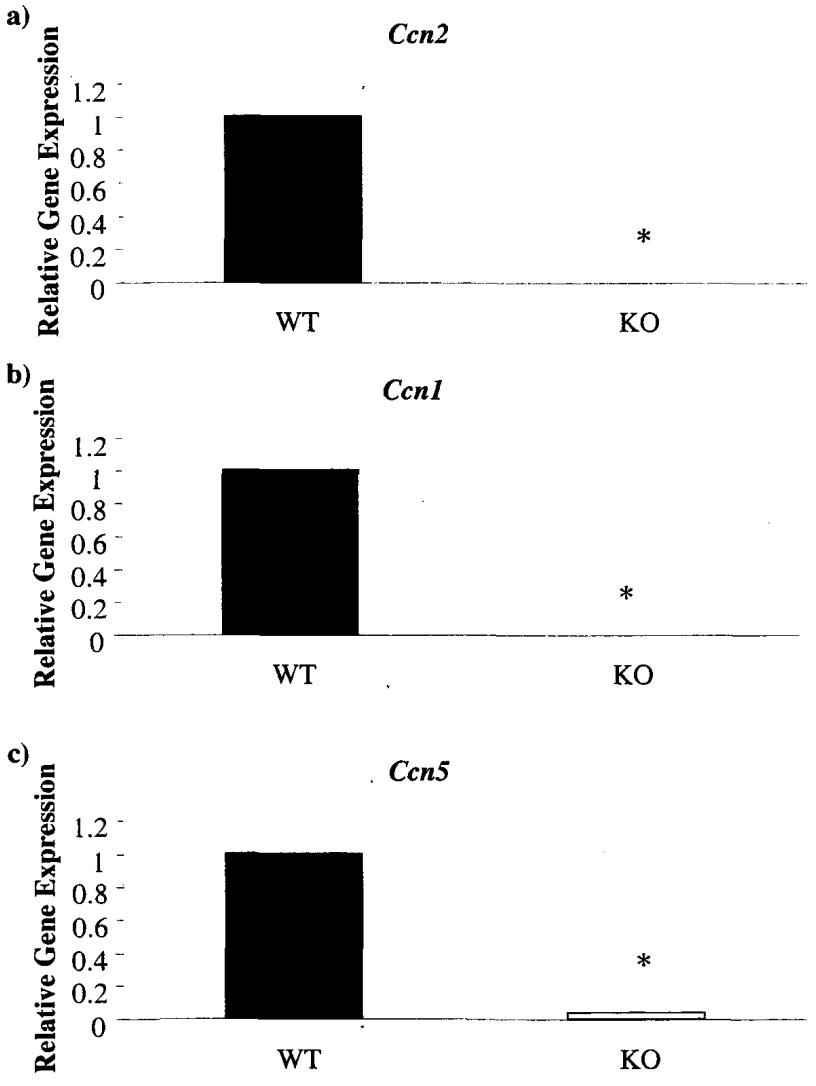


Figure 3.1 Loss of *Ccn2* results in reduced mRNA expression of CCN Family Members *Ccn1* and *Ccn5*. *Ccn2* $^{+/+}$ (WT) and $^{-/-}$ (KO) mouse embryonic fibroblasts (MEFs) were cultured for 48 hours in monolayer, and gene expression was analyzed by Real-Time RT-PCR. a) *Ccn2* mRNA expression is absent in *Ccn2*$^{-/-}$ MEFs. b) *Ccn1* mRNA gene expression is significantly reduced in *Ccn2*$^{-/-}$ MEFs. c) *Ccn5* mRNA expression is significantly reduced in *Ccn2*$^{-/-}$ MEFs. Data shown represent means relative to GAPDH $\pm$ SEM from three independent experiments and are normalized to control sample (each performed in triplicate) (* $p < 0.05$ using a Student's paired t-test).

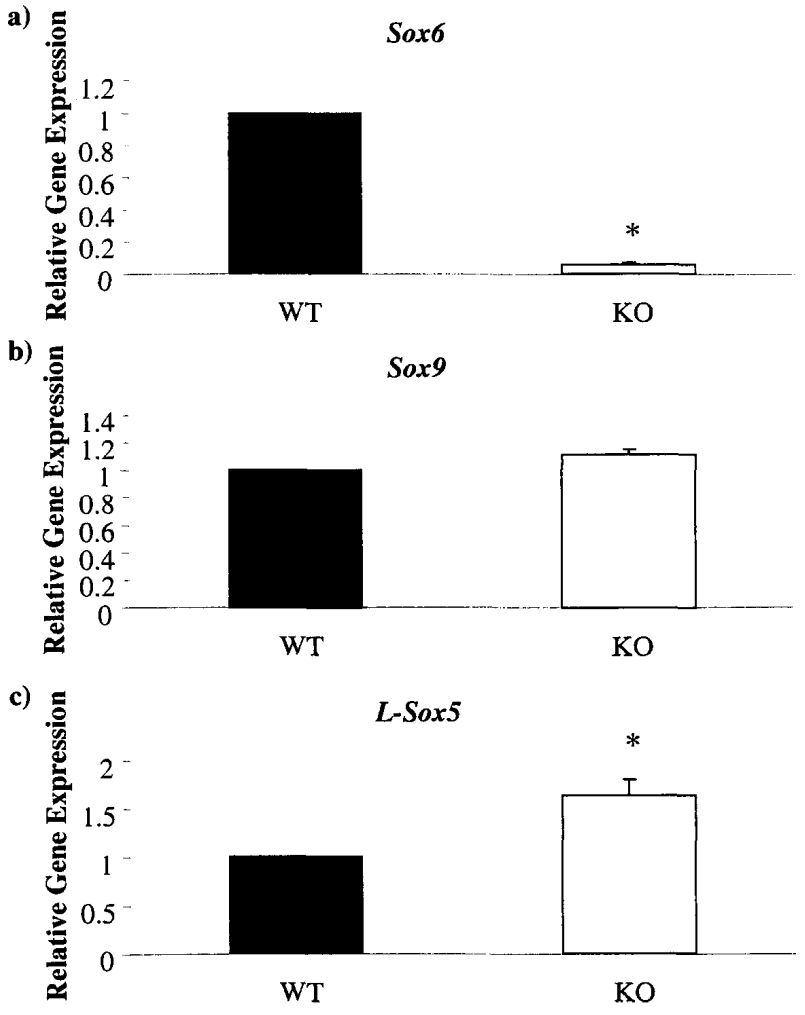


Figure 3.2 Loss of *Ccn2* reduces *Sox6* mRNA expression. *Ccn2* $^{+/+}$ (WT) and $^{-/-}$ (KO) mouse embryonic fibroblasts (MEFs) were cultured for 48 hours in monolayer, and gene expression was analyzed by Real-Time RT-PCR. a) *Sox6* mRNA expression is significantly reduced in *Ccn2*$^{-/-}$ MEFs. b) *Sox9* mRNA expression is not altered upon loss of *Ccn2*, whereas c) *L-Sox5* mRNA expression increases upon loss of *Ccn2*. Data shown represent means relative to GAPDH $\pm$ SEM from three independent experiments and are normalized to control sample (each performed in triplicate) (* $p < 0.05$ using a Student's paired t-test).

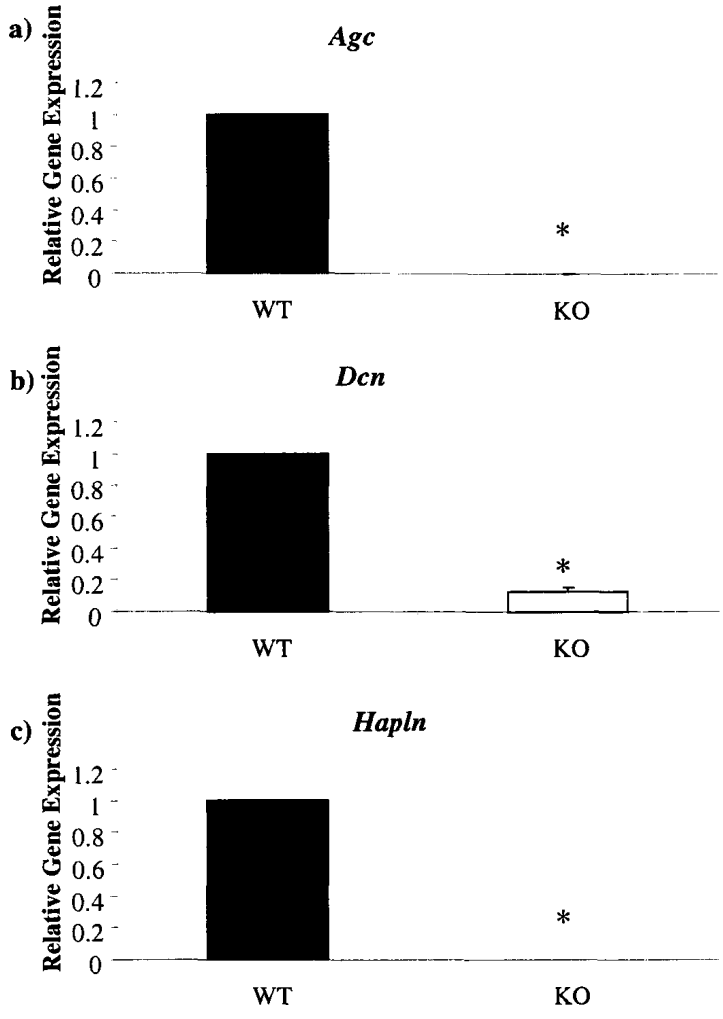


Figure 3.3 Loss of *Ccn2* reduces mRNA expression of chondrogenic extracellular matrix components *Agc, Dcn, Hapln.* *Ccn2* $^{+/+}$ (WT) and $^{-/-}$ (KO) mouse embryonic fibroblasts (MEFs) were cultured for 48 hours in monolayer, and gene expression was analyzed by Real-Time RT-PCR. a) *Agc*, b) *Dcn* and c) *Hapln* mRNA levels show a significant decrease in gene expression in the *Ccn2* $^{-/-}$ MEFs. Data shown represent means relative to GAPDH $\pm$ SEM from three independent experiments and are normalized to control sample (each performed in triplicate) (* $p < 0.05$ using a Student's paired t-test).

cells. No change in mRNA transcript levels for *Col2a1* or *Col10a1* (Fig. 3.4a, b) was observed in *Ccn2*$^{-/-}$ deficient MEFs. Furthermore, the mRNA expression of the cell adhesion protein *N-cad* (Fig. 3.5) was reduced in knockout cells. These data demonstrate that the expression of some, but not all chondrogenic genes are dependent on the expression of CCN2.

3.2 Sox6 and aggrecan are down-regulated in the absence of *Ccn2*

I next decided to confirm reduced expression of sox6 and aggrecan at the protein level, using *Ccn2*$^{+/+}$ and $^{-/-}$ MEFs plated in monolayer culture for 48 hours. Traditionally, sox6 in conjunction with sox9 and l-sox5 is thought to control the expression of aggrecan and type II collagen (Lefebvre *et al.,* 1998; Lefevbre *et al.,* 1997; Okazaki and Sandell, 2004; Smits, 2001). By Western blot analysis, I determined that protein expression of both sox6 and aggrecan was down-regulated in the *Ccn2*$^{-/-}$ MEFs (Fig. 3.6a). To quantify this difference, densitometry was performed relative to β-actin control, demonstrating a significant reduction in aggrecan and sox6 expression in the *Ccn2*$^{-/-}$ cells (Fig. 3.6b, c). Additional evidence to support the down-regulation of sox6 in the *Ccn2*$^{-/-}$ cells was seen by sox6 immunofluorescence analyses of *Ccn2*$^{+/+}$ and $^{-/-}$ MEFs. Sox6 was localized around the nuclei and markedly reduced in knockout cells (Fig. 3.7). These data demonstrate that sox6 and aggrecan are expressed in a CCN2-dependent fashion.

3.3 Loss of *Ccn2* results in a reduction in mRNA gene expression of *Ccn1* and *Ccn5*

I next analysed specific effects of loss of *Ccn2* during early chondrogenesis using the micromass culture system that provides a more authentic model of chondrogenesis

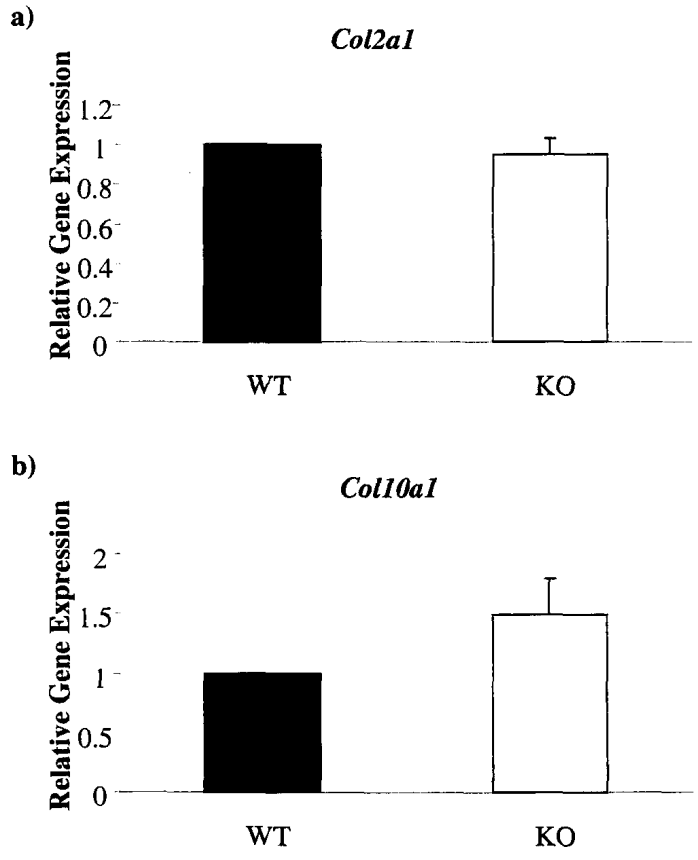


Figure 3.4 Loss of *Ccn2* does not decrease mRNA collagen levels in the extracellular matrix. *Ccn2* $^{+/+}$ (WT) and $^{-/-}$ (KO) mouse embryonic fibroblasts (MEFs) were cultured for 48 hours in monolayer, and gene expression was analyzed by Real-Time RT-PCR. a) *Col2a1* and b) *Col10a1* expression does not change between cell types. Data shown represent means relative to GAPDH ± SEM from three independent experiments and are normalized to control sample (each performed in triplicate) (* $p < 0.05$ using a Student's paired t-test).

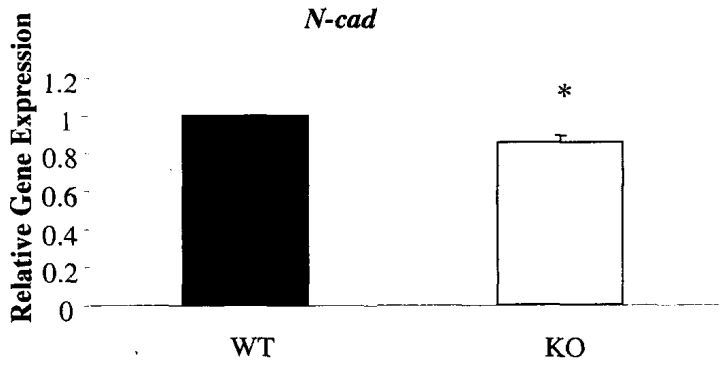


Figure 3.5 Loss of *Ccn2* lowers *N-cad* mRNA expression. *Ccn2* $^{+/+}$ (WT) and $^{-/-}$ (KO) mouse embryonic fibroblasts (MEFs) were cultured for 48 hours in monolayer, and gene expression was analyzed by Real-Time RT-PCR. *N-cad* mRNA levels show a decrease between the *Ccn2* $^{+/+}$ and $^{-/-}$ MEFs, as shown by Real-Time RT-PCR. Data shown represent means relative to GAPDH $\pm$ SEM from three independent experiments and are normalized to control sample (each performed in triplicate) (* $p < 0.05$ using a Student's paired t-test).

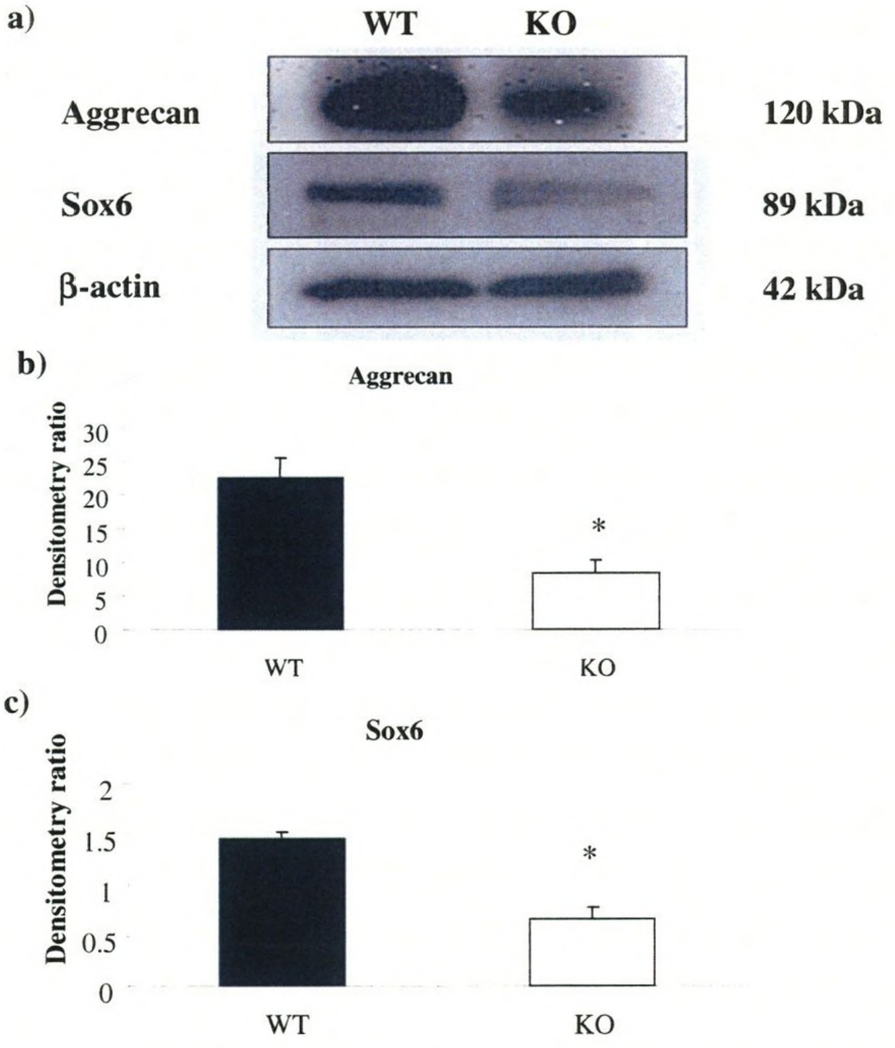


Figure 3.6 In the absence of *Ccn2*, levels of early chondrogenic matrix associated proteins sox6 and aggrecan are decreased: Western blot analysis. a) Protein isolated from *Ccn2* $^{+/+}$ (WT) and $^{-/-}$ (KO) mouse embryonic fibroblasts (MEFs) were cultured in monolayer for 48 hours. Conditioned media and protein extracts were examined for aggrecan and sox6, respectively, by western blot analysis. Both sox6 and aggrecan expression are down-regulated in the *Ccn2* $^{-/-}$ MEFs. β-actin was used as a loading control. b) Densitometry analysis of aggrecan relative to β-actin shows a significant difference between samples. c) Densitometry analysis of sox6 relative to β-actin shows a significant difference between samples. Data shown represent means ± SEM from three trials (* $p < 0.05$ using a Student's paired t-test).

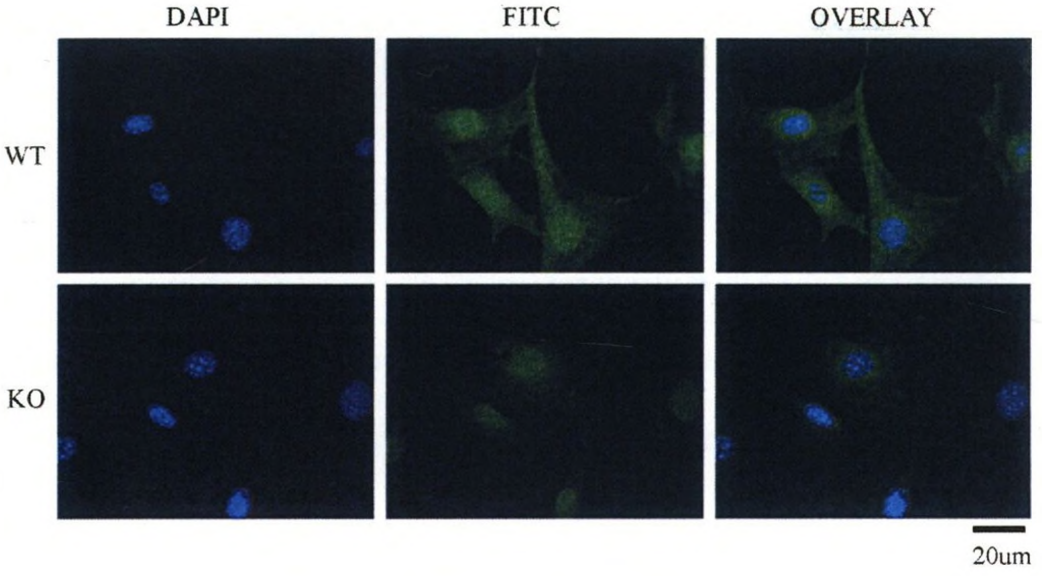


Figure 3.7 Loss of *Ccn2* results in decreased expression of sox6: Immunofluorescence analysis. *Ccn2* $^{+/+}$ (WT) and $^{-/-}$ (KO) mouse embryonic fibroblasts (MEFs) were cultured in monolayer for 48 hours. Cells were fixed in paraformaldehyde and stained with FITC labeled antibody for sox6 and with DAPI for nuclei. Sox6 is localized around the nuclei in the *Ccn2* $^{+/+}$ MEFs, whereas expression is markedly decreased in the *Ccn2* $^{-/-}$ MEFs. Six different fields were examined. A representative field is shown.

(Ahrens *et al.*, 1977; James *et al.*, 2005; Lengner *et al.*, 2004; Woods and Beier, 2006; Woods *et al.*, 2005). *Ccn2*$^{+/+}$ and $^{-/-}$ MEFs were plated in high density micromass cultures and cultured for up to 6 days. RNA was harvested on days 1, 3 and 6, and transcripts were analyzed by Real-Time RT-PCR. In wild-type MEFs, *Ccn2* was expressed at constant levels throughout the time course; as expected, it was undetectable in knockout cells (Fig. 3.8a). Previous studies have shown that CCN1 has a high degree of functional similarity and is expressed simultaneously with CCN2 in a number of tissue types (Brigstock, 2002; Chen *et al.*, 2001a; Kireeva *et al.*, 1997; Schober *et al.*, 2002). Therefore it was important to examine the expression of this gene in the absence of *Ccn2*. It is interesting to note that *Ccn1* mRNA expression was significantly lower in knockout cells on days 1 and 3 of culture, but reached similar expression levels in both cell types by the end of the culture period (Fig. 3.8b). mRNA expression of *Ccn5* increased significantly during differentiation of wild-type cells, but was significantly lower in knockout cells on all six days (Fig. 3.8c). These data document the dependence of these family members on *Ccn2* in early chondrogenesis.

3.4 mRNA expression of *Sox6*, but not *Sox9* or *L-Sox5*, is reduced in the absence of *Ccn2*

I next asked whether the difference in expression of Sox6 seen in the monolayer experiments persisted in the micromass culture. *Ccn2*$^{+/+}$ and $^{-/-}$ MEFs were plated in high density micromass cultures for up to 6 days. RNA was harvested on days 1, 3 and 6, and transcripts were analyzed by Real-Time RT-PCR. Previous studies have shown that that l-sox5 and sox6 cooperate with sox9 to control chondrogenesis and are themselves

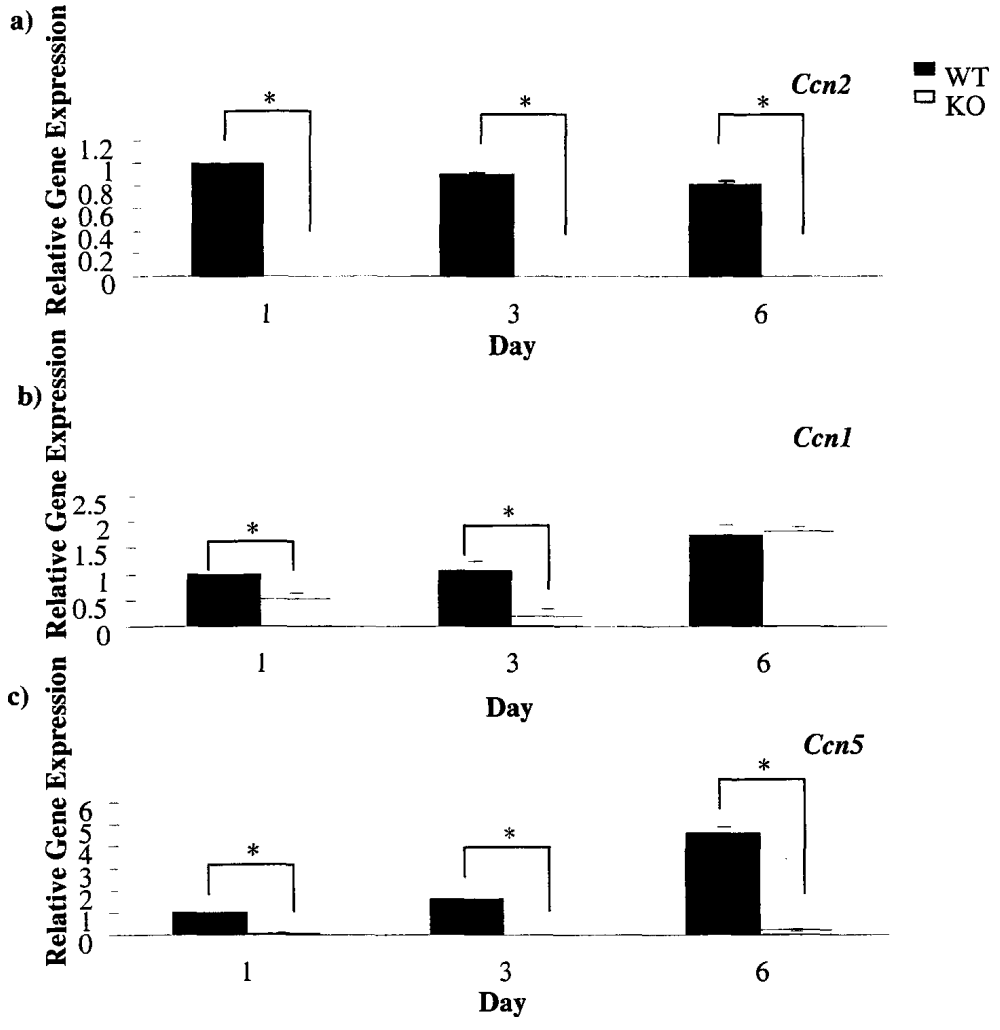


Figure 3.8 mRNA gene expression of CCN family members *Ccn1* and *Ccn5* in micromass cultures is regulated by *Ccn2*. $Ccn2^{+/+}$ (WT) and $^{-/-}$ (KO) mouse embryonic fibroblasts (MEFs) were plated in micromass cultures for 6 days. RNA was harvested on days 1, 3 and 6, and transcripts were analyzed by Real-Time RT-PCR. a) *Ccn2* mRNA expression illustrates significantly elevated levels in $Ccn2^{+/+}$ MEFs and in the $Ccn2^{-/-}$ MEFs there is no *Ccn2* mRNA detected. b) *Ccn1* mRNA expression shows significant down-regulation in $Ccn2^{-/-}$ MEFs on days 1 and 3, with a return to $Ccn2^{+/+}$ levels on day 6. c) *Ccn5* expression is significantly reduced in the $Ccn2^{-/-}$ MEFs on all six days. Data shown are relative to GAPDH and represent means $\pm$ SEM from three independent experiments and are normalized to day 1 control sample (each performed in triplicate) (* $p < 0.05$ using a two-way ANOVA).

under the transcriptional control of sox9 (Akiyama et al., 2002b; Lefebvre et al., 2001; Lefebvre et al., 1998). In knockout cells, *Sox6* expression was significantly reduced throughout the 6 days in culture (Fig. 3.9a), whereas *Sox9* and *L-Sox5* showed no difference on days 1 and 3 and displayed a significant increase in expression in knockout cells on day 6 (Fig. 3.9b, c).

3.5 Loss of *Ccn2* reduces expression of proteoglycans within the extracellular matrix

I next examined expression levels of chondrogenic ECM components in micromass culture. *Ccn2* $^{+/+}$ *and* $^{-/-}$ MEFs were plated in micromass cultures for 6 days. RNA was harvested on days 1, 3 and 6, and transcripts were analyzed by Real-Time RT-PCR. It is known that the two main chondrocyte ECM components, aggrecan and type II collagen, are co-expressed and co-regulated (Okazaki and Sandell, 2004). However, my data show that the mRNA expression of proteoglycans, *Agc* and *Dcn* and ECM protein, *Hapln* were significantly reduced in *Ccn2*$^{-/-}$ micromass cultures, whereas *Col2a1* was significantly increased throughout the 6 days of culture (Fig. 3.10a - d). These data indicate that the absence of *Ccn2* alters the balance of collagen and proteoglycans in the cartilage ECM.

3.6 *Loss* of *Fak* promotes expression of CCN2 in early chondrogenesis

Overall, the molecular events responsible for driving early chondrogenesis are not completely understood. Previous studies showed that *Fak*$^{-/-}$ MEFs, exhibit a rounded morphology, increased focal adhesion formation, decreased migration, and decreased actin stress fibre formation (Ilic et al., 1995; Sieg et al., 1999; Sieg et al., 1998). Since rounded cell morphology typifies a cell undergoing chondrogenesis

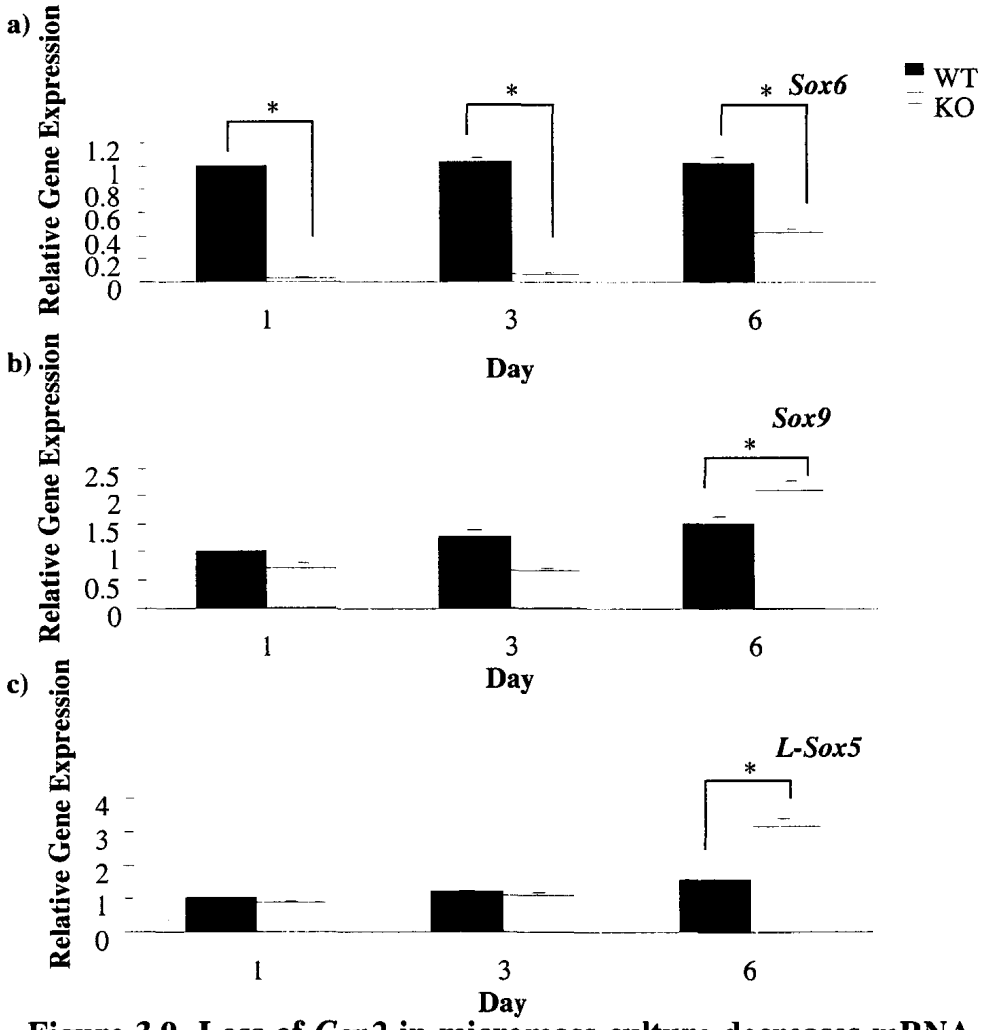


Figure 3.9 Loss of *Ccn2* in micromass culture decreases mRNA gene expression of the transcription factor *Sox6* but not *Sox9* or *L-Sox5*. *Ccn2*$^{+/+}$ (WT) and $^{-/-}$ (KO) mouse embryonic fibroblasts (MEFs) were plated in micromass cultures for 6 days. RNA was harvested on days 1, 3 and 6, and transcripts were analyzed by Real-Time RT-PCR. a) *Sox6* mRNA expression illustrates significantly reduced levels in the absence of *Ccn2* on all six days. b) *Sox9* and c) *L-Sox5* mRNA expression increase over time in culture, and are both significantly higher in the knockout MEFs on day 6. Data shown are relative to GAPDH and represent means $\pm$ SEM from three independent experiments and are normalized to day 1 control sample (each performed in triplicate) (* $p < 0.05$ using a two-way ANOVA).

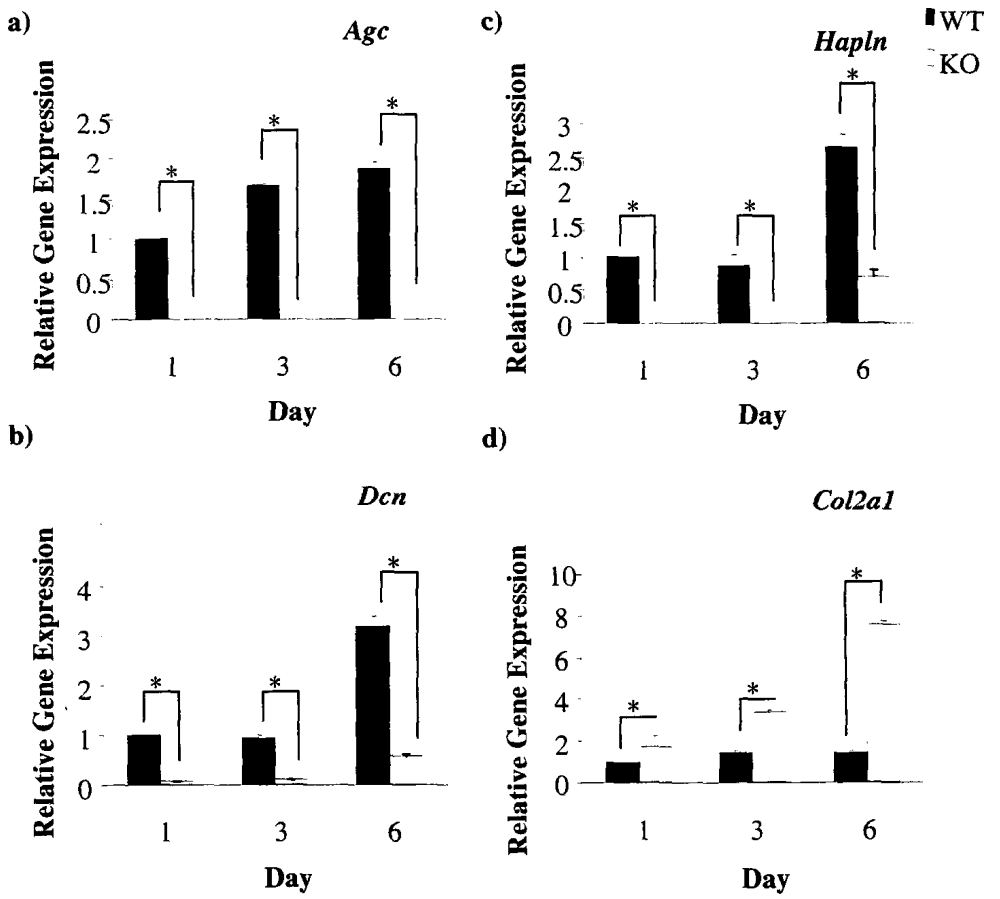


Figure 3.10 Loss of *Ccn2* decreases mRNA gene expression of *Agc*, *Dcn* and *Hapln* in micromass culture yet increases expression of *Col2a1*. *Ccn2*$^{+/+}$ (WT) and $^{-/-}$(KO) mouse embryonic fibroblasts (MEFs) were plated in micromass cultures for 6 days. RNA was harvested on days 1, 3 and 6, and transcripts were analyzed by Real-Time RT-PCR. a) *Agc* mRNA expression is significantly reduced in the *Ccn2*$^{-/-}$ MEFs on all six days. b) *Dcn* mRNA expression is significantly reduced in the absence of *Ccn2* on all six days. c) *Hapln* mRNA expression is significantly reduced in the absence of *Ccn2* on all six days. d) *Col2a1* expression is significantly increased in the *Ccn2*$^{-/-}$ MEFs on all six days examined. Data shown are relative to GAPDH and represent means ± SEM from three independent experiments and are normalized to day 1 control sample (each performed in triplicate) (* $p < 0.05$ using a two-way ANOVA).

(von der Mark *et al.*, 1977; Woods *et al.*, 2005), I speculated that *Fak*$^{-/-}$ cells should undergo enhanced chondrogenesis. CCN2 is a known regulator of adhesive signaling (Chen *et al.*, 2004; Gau and Brigstock, 2004). CCN2 is also known to be involved in migration and adhesion events in early chondrogenesis (Song *et al.*, 2007). Thus, I next addressed whether (a) FAK signaling was required for chondrogenic differentiation and (b) whether CCN2 was involved in FAK signaling during chondrogenesis.

I first used *Fak*$^{+/+}$ and *Fak*$^{-/-}$ MEFs in high density micromass cultures and incubated the cells for 6 days. Since *Fak*$^{-/-}$ animals die at embryonic day 8.5 (Furuta *et al.*, 1995; Ilic *et al.*, 1995), this precludes use of any type of cell at later stages. Therefore, I used MEFs at 8.5 dpc, which represent mesenchymal cells that have yet to differentiate (Hall and Miyake, 2000). To begin to evaluate the hypothesis that CCN2 might operate downstream of FAK in early chondrogenesis, I initially assessed the impact of loss of *Fak* on *Ccn2* gene expression. RNA was harvested on day 6, and *Ccn2* expression was analyzed by Real-Time RT-PCR. I found that in the absence of *Fak*, *Ccn2* mRNA expression on day 6 was significantly elevated (Fig. 3.11). I also looked at protein expression of FAK and CCN2 on day 6 (Fig. 3.12a). Densitometry analysis relative to β-actin loading control, confirms that CCN2 protein expression is significantly up regulated in the FAK knockout (Fig. 3.12b, c). These results demonstrate that FAK is genetically upstream of CCN2 and that the absence of FAK promotes the early stages of chondrogenesis through increased CCN2 levels.

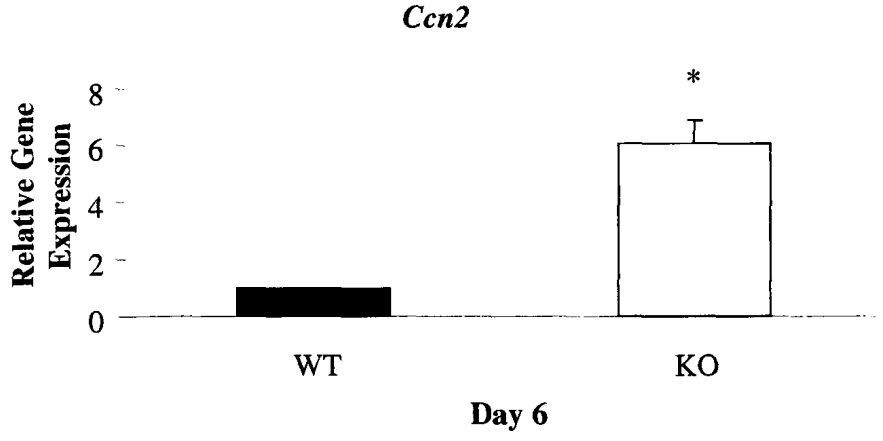


Figure 3.11 Loss of FAK in micromass culture increases *Ccn2* mRNA expression. *Fak*$^{+/+}$ (WT) and $^{-/-}$ (KO) mouse embryonic fibroblasts (MEFs) 8.5 dpc were plated in micromass cultures for 6 days. RNA was harvested on day 6, and transcripts were analyzed by Real-Time RT-PCR. *Ccn2* mRNA expression significantly increases in *Fak*$^{-/-}$ MEFs. Data shown are relative to GAPDH and represent means ± SEM from three independent experiments and are normalized to control sample (each performed in triplicate) (* $p < 0.05$ using a Student's paired t-test).

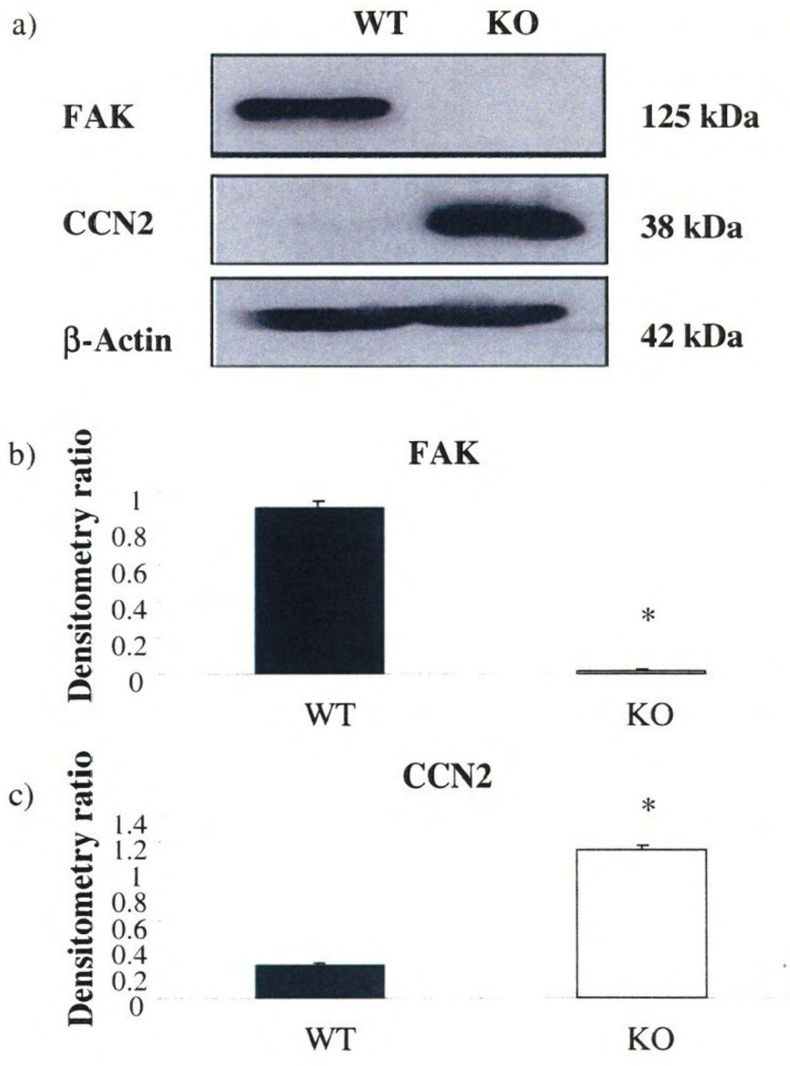


Figure 3.12 Loss of FAK in micromass culture increases CCN2 protein expression. a) *Fak*$^{+/+}$ (WT) and $^{-/-}$ (KO) mouse embryonic fibroblasts (MEFs) 8.5 dpc were plated in micromass cultures for 6 days. Protein isolated from MEFs on day 6 were examined for FAK and CCN2, by western blot analysis. FAK protein expression is absent in *Fak*$^{-/-}$ MEFs. CCN2 expression is up regulated in the *Fak*$^{-/-}$ MEFs. β-actin was used as a loading control. b) Densitometry analysis of FAK relative to β-actin shows a significant difference between samples. c) Densitometry analysis of CCN2 relative to β-actin shows a significant difference between samples. Data shown represent means ± SEM from three trials (* $p < 0.05$ using a Student's paired t-test).

3.7 *Fak*$^{-/-}$ mouse embryonic fibroblasts display an increased number of nodules in micromass culture

To obtain a better understanding of chondrogenesis in the absence of FAK, it was important to obtain visual evidence. Since CCN2 appears to promote chondrogenesis and FAK deficiency stimulates CCN2 expression, I speculated that FAK knockout cells should display enhanced chondrogenesis. *Fak* $^{+/+}$ and $^{-/-}$ MEFs were plated in micromass cultures for 6 days. On day 6 of culture, PNA stained images (Fig. 3.13a) and brightfield images (Fig. 3.13b) were taken of the micromass cultures. In the PNA stained micromass cultures the FAK $^{-/-}$ cells display strikingly more condensation formation than wild-type cells. In the brightfield images there are more nodular formations apparent in the FAK$^{-/-}$ cells. It is known from previous work with this culture system that nodular condensations are a hallmark of chondrogenic differentiation in micromass culture (Ahrens *et al.*, 1977). Therefore, these data show that the absence of *Fak* contributes to the progression of chondrogenesis.

3.8 Loss of *Fak* promotes expression of known chondrogenic genes, including *Ccn2*

To further assess chondrogenesis in *Fak* $^{-/-}$ cells, I examined mRNA expression of a number of known early chondrogenic genes, including *Ccn2* which was discussed above (Fig. 3.11 and 3.12 a-c). *Fak*$^{+/+}$ and *Fak*$^{-/-}$ MEFs were plated in micromass cultures for 6 days. RNA was harvested, and transcripts were analyzed by Real-Time RT-PCR. *Sox9* and *Sox6* expression did not change between cell types (Fig. 3.14 a, c). However, in the *Fak*$^{-/-}$ cells, *L-Sox5* and *Col2a1* mRNA expression were significantly higher than in the wild-type controls (Fig. 3.14b, d). *Agc* and *Hapln* mRNAs were not detected, likely

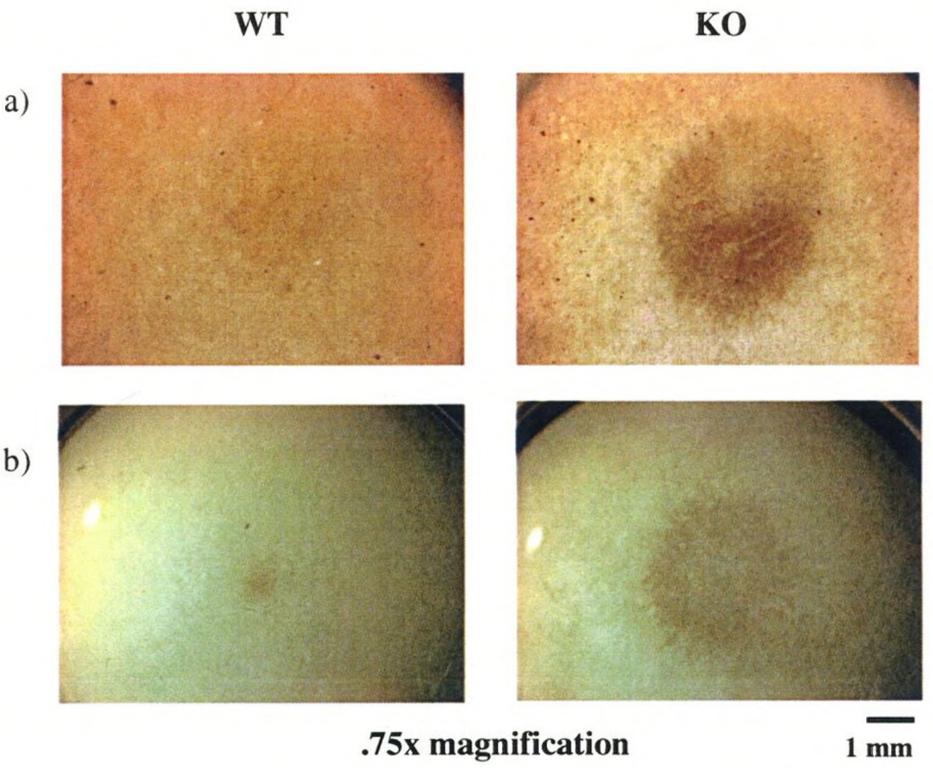


Figure 3.13 Loss of *Fak* increases chondrogenic differentiation. *Fak*$^{+/+}$ (WT) and $^{-/-}$ (KO) mouse embryonic fibroblasts (MEFs) 8.5 dpc were plated in micromass cultures for 6 days. On day 6 of culture images of the masses were taken. a) PNA stain and b) Bright field images display a striking difference between cell types. *Fak* $^{-/-}$ MEFs show markedly more condensation formations than *Fak* $^{+/+}$ MEFs in the PNA stained micromass cultures. Six different micromass cultures for each condition were examined. Representative micromass cultures are shown.

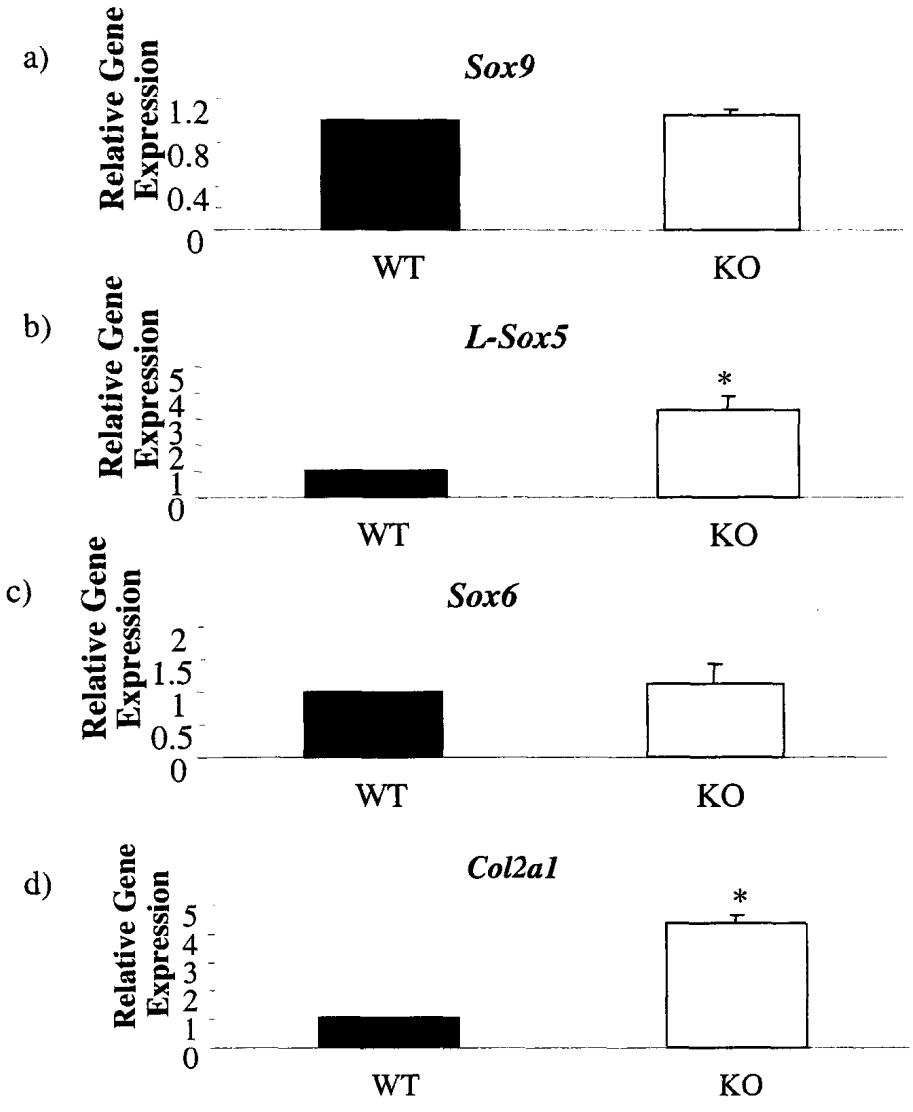


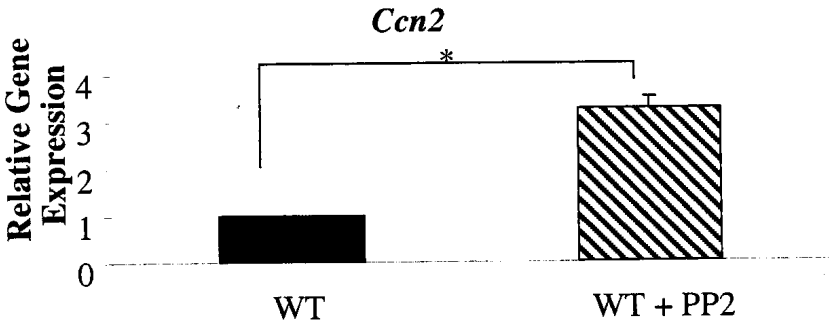
Figure 3.14 Loss of *Fak* increases mRNA expression of the chondrogenic markers *L-Sox5* and *Col2a1*. *Fak*$^{+/+}$ (WT) and $^{-/-}$ (KO) mouse embryonic fibroblasts (MEFs) 8.5 dpc were plated in micromass cultures for 6 days. RNA was harvested on day 6, and transcripts were analyzed by Real-Time RT-PCR. a) *Sox9* and c) *Sox6* do not change in mRNA expression. b) *L-Sox5* and d) *Col2a1* show significant elevation in mRNA expression levels in the *Fak* $^{-/-}$ MEFs. Data shown represent means relative to GAPDH ± SEM from three independent experiments and are normalized to control sample (each performed in triplicate) (* $p < 0.05$ using a Student's paired t-test). Note that *Agc* and *Hapln* transcripts were not detected in these samples.

because *Fak*$^{+/+}$ and $^{-/-}$ MEFs were generated from younger embryos that *Ccn2*$^{+/+}$ and $^{-/-}$ MEFs. These data indicate that the absence of *Fak* is sufficient to trigger expression of some known early chondrogenic markers in 8.5 dpc MEFs.

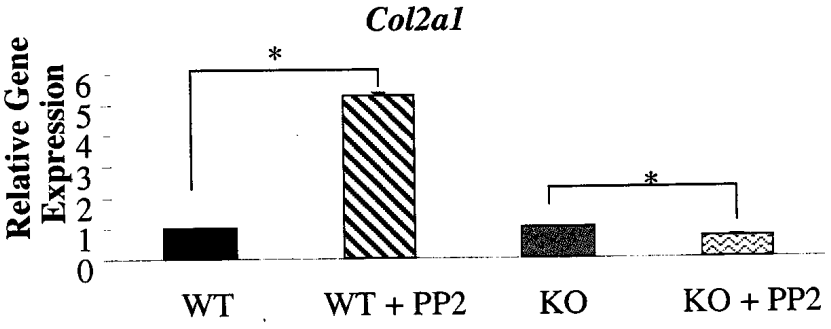
3.9 Inhibition of FAK/Src signaling increases *Ccn2* expression as well as chondrogenic matrix associated gene expression

Lastly, it was important to assess if the chondrogenic effects of FAK deficiency require up-regulation of CCN2. Since I did not have access to double-knockout MEFs, I employed the pharmacological FAK/Src inhibitor PP2, in conjunction with *Ccn2*$^{+/+}$ and *Ccn2*$^{-/-}$ MEFs. Cells were plated in micromass cultures and grown in the presence of DMSO (control) or 10 μM PP2 for six days. mRNA levels of *Ccn2, Col2a1, Agc, Sox6, Sox9 and L-Sox5* were significantly increased in wild-type cells treated with PP2, compared to control cultures (Fig. 3.15a-f). In contrast, expression levels of *Agc* and *Col2a1* were significantly decreased in *Ccn2* $^{-/-}$ cells treated with PP2 (Fig. 3.15 b, c). There was no difference seen in mRNA expression of *Sox6*, *Sox9* and *L-Sox5* in *Ccn2* $^{-/-}$ cells upon treatment with PP2 (Fig. 3.15 d-f). These data indicate that CCN2 is required for the induction of chondrogenesis by PP2 and likely by FAK deficiency. It is also interesting to note (data not shown) that there is no difference in *Fak* gene expression on day 6 of the *Ccn2* $^{+/+}$ and $^{-/-}$ micromass culture, which further supports the idea that CCN2 is in fact downstream of FAK.

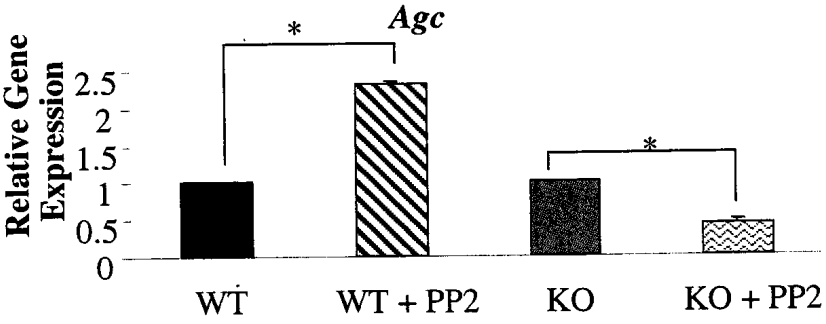
a)


Ccn2
Relative Gene Expression
0
1
2
3
4
*
WT
WT + PP2

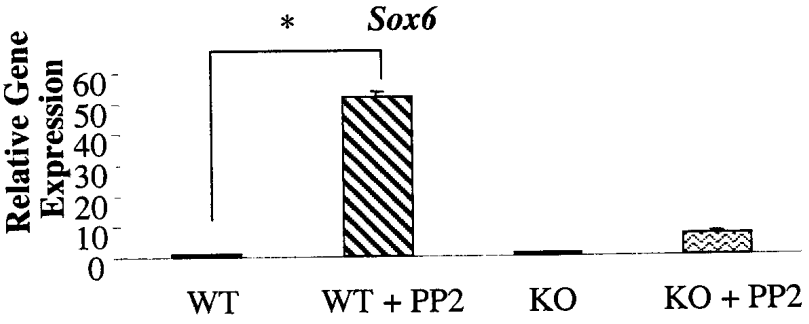
b)


Col2a1
Relative Gene Expression
0
1
2
3
4
5
6
*
*
WT
WT + PP2
KO
KO + PP2

c)


Agc
Relative Gene Expression
0
0.5
1
1.5
2
2.5
*
*
WT
WT + PP2
KO
KO + PP2

d)


Sox6
Relative Gene Expression
0
10
20
30
40
50
60
*
WT
WT + PP2
KO
KO + PP2

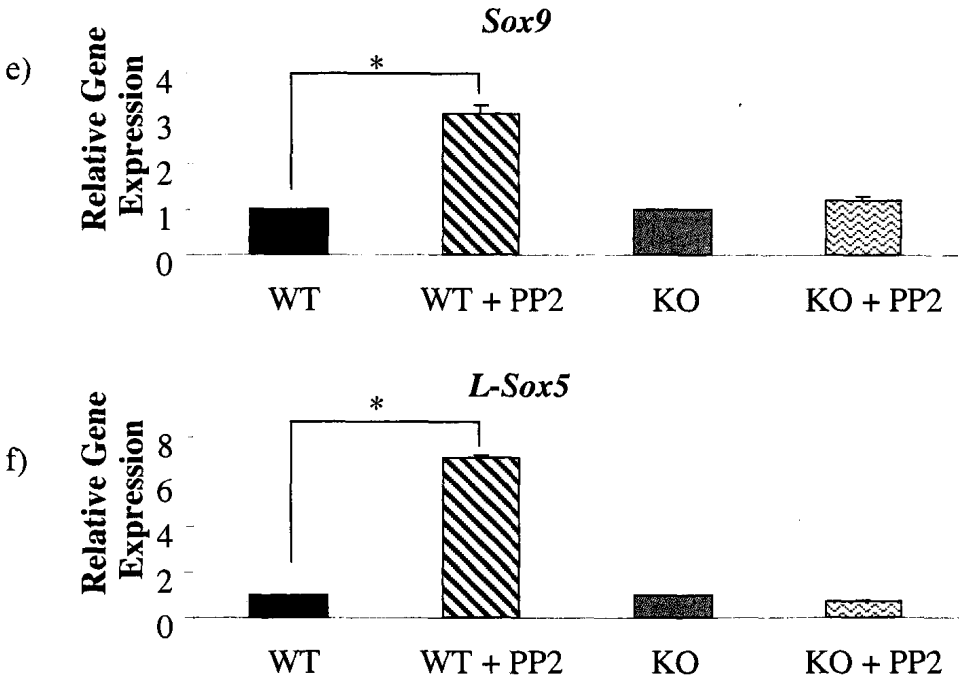


Figure 3.15 Inhibition of FAK/Src signaling affects mRNA expression of *Ccn2* and chondrogenic matrix associated genes. *Ccn2* $^{+/+}$ (WT) and $^{-/-}$ (KO) mouse embryonic fibroblasts (MEFs) were plated in micromass cultures and grown in DMSO or 10 μM of the FAK/Src inhibitor PP2 for a period of six days, RNA was harvested on day 6. mRNA levels of a) *Ccn2*, b) *Col2a1*, c) *Agc*, d) *Sox6*, e) *Sox9* and f) *L-Sox5* were significantly increased compared to control cultures in *Ccn2* $^{+/+}$ MEFs treated with PP2, as determined by Real-Time RT-PCR. In *Ccn2*$^{-/-}$ MEFs treated with PP2 there was a significant decrease in mRNA gene expression of b) *Col2a1* and c) *Agc*, and no change in d) *Sox6*, e) *Sox9* and f) *L-Sox5*. Data shown are relative to GAPDH and represent means ± SEM from three independent experiments and are normalized to day 6 WT control sample and 6 KO control sample (each performed in triplicate) (* $p < 0.05$ using a two-way ANOVA).

4.0 Discussion

4.1 Summary of results

Although the phenotype of *Ccn2*-deficient mice clearly demonstrates a requirement for this protein in endochondral ossification, the cellular and molecular basis of its role in chondrocytes is largely unknown. Previous microarray and functional data using $Ccn2^{-/-}$ MEFs suggested a role for CCN2 in early chondrogenesis, and in particular the differentiation from pluripotent mesenchymal precursor cells to chondrocytes (Chen *et al.*, 2004; Kennedy *et al.*, 2007). Thus, I hypothesized that CCN2 is required for ECM production in early chondrogenesis. My data demonstrate that CCN2 is important for early chondrogenic differentiation and is responsible for regulating the expression of chondrogenic ECM components.

Absence of *Ccn2* in MEFs plated in monolayer revealed a decrease in early chondrogenic markers, such as *Agc* and *Sox6* at the transcript and protein levels, and also demonstrated a significant decrease in the transcript levels for two other CCN family members, *Ccn1* and *Ccn5*. Moreover, mRNA expression of ECM associated proteins *Dcn* and *Hapln* as well as the cell adhesion protein *N-cad* were reduced in $Ccn2^{-/-}$ cells. However, my studies demonstrated that loss of *Ccn2* significantly increased *L-Sox5* and did not change *Sox9*, *Col2a1* or *Col10a1* mRNA expression. These results indicated that CCN2 is required for the expression of a specific subset of early chondrogenic markers. Although it is widely believed that the transcription of chondrocyte-specific genes is co-ordinately regulated by sox trio of transcription factors (Lefebvre *et al.*, 2001; Okazaki and Sandell, 2004), my results revealed a clear divergence between *Sox6* and proteoglycan transcription versus *Sox5*, *Sox9* and *Col2a1* in terms of their requirements for *Ccn2*. These data show a clear and novel connection between CCN2 expression and

the expression of known chondrogenic markers sox6 and aggrecan. Further investigation is required to establish the molecular mechanism underlying the selective requirement of CCN2 for the expression of chondrocyte-specific markers.

I then extended my results by examining the requirement for CCN2 in chondrocyte-specific gene expression by using the micromass culture model of chondrogenesis (Ahrens *et al.*, 1977; Lengner *et al.*, 2004). Chondrogenesis was induced by culturing *Ccn2*$^{+/+}$ and *Ccn2*$^{-/-}$ MEFs in micromass for 6 days, and gene expression was examined. *Ccn2* was expressed consistently throughout the 6 day time course, and, as expected, was absent in the *Ccn2*$^{-/-}$ cells. Consistent with the monolayer data, *Ccn5* was found to be significantly down regulated on all six days in culture, suggesting that expression of this gene depended on *Ccn2*. *Ccn1* mRNA expression was down regulated on days 1 and 3, but expression in *Ccn2*$^{-/-}$ cells returned to control levels on day 6 indicating that, whereas during early chondrogenesis *Ccn1* expression depended on CCN2, CCN2-independent mechanisms were operant during later stages of chondrogenic differentiation.

The mRNA expression of the sox trio of transcription factors, *Sox9*, *L-Sox5* and *Sox6* in the micromass culture system demonstrate a divergence between *Sox6* expression and *Sox9* and *L-Sox5* in *Ccn2*$^{-/-}$ cells. Expression of *Sox6* throughout the 6 days in culture was significantly down regulated in *Ccn2*$^{-/-}$ cells. Conversely, *Sox9* and *L-Sox5* did not show any differences between *Ccn2*$^{+/+}$ and *Ccn2*$^{-/-}$ cells on days 1 and 3 except on day 6, where expression of these genes increased significantly in *Ccn2*$^{-/-}$ cells. Furthermore, gene expression of the proteoglycans *Agc*, *Dcn* and the ECM protein, *Hapln* were significantly and consistently decreased in *Ccn2*$^{-/-}$ cells. Conversely, expression of

Col2a1 was increased in the *Ccn2*$^{-/-}$ MEF micromass cultures throughout the time course. Collectively, my data establish that CCN2 is required for early chondrogenic events and that sox6 and aggrecan are reliant on CCN2 for their expression, whereas type II collagen expression occurs via a CCN2-independent mechanism.

To uncover a mechanistic context for the role of CCN2 in chondrogenesis, I sought to establish how CCN2 itself was regulated in early chondrogenesis. CCN2 is a known regulator of adhesive signaling (Chen *et al.*, 2004; Gau and Brigstock, 2004) and adhesive signaling is an important aspect of the cell-cell contacts during chondrogenesis (DeLise et al., 2000a; Oberlender and Tuan, 1994). Therefore, I decided to investigate other known regulators of adhesive signaling. FAK is necessary for focal adhesion turnover (Furuta *et al.*, 1995; Ilic *et al.*, 1995), which occurs during adhesion events such as those during chondrogenesis (Bang *et al.*, 2000) and has been shown to act with CCN2 in adhesion events in other cell types (Chen *et al.*, 2001a; Chen *et al.*, 2004). Therefore, I examined the relationship between FAK and CCN2 expression. *Fak*$^{-/-}$ MEFs were isolated from 8.5 dpc embryos, a stage prior to the onset of chondrogenesis (Hall and Miyake, 2000), and plated in micromass culture. Micromass culture of *Fak*$^{+/+}$ cells was insufficient to induce expression of a chondrogenic program, consistent with the fact that 8.5 dpc embryos have not yet begun chondrogenesis (Hall and Miyake, 2000). To my surprise, loss of *Fak* in 8.5 dpc MEFs resulted in a significant increase in CCN2 gene and protein expression by day 6 of culture. Based on these results I anticipated other known chondrogenic markers may be up-regulated as well. Both *Col2a1* and *L-Sox5* were significantly increased in the *Fak*$^{-/-}$ MEFs, but *Sox9* and *Sox6* expression did not change. Conversely *Agc* and *Hapln* were not expressed either in the presence or absence of *Fak*,

indicating that loss of *Fak* was sufficient to recapitulate only a portion of the chondrogenic program. $Fak^{-/-}$ micromass cultures displayed strikingly more nodular formations, than the $Fak^{+/+}$ cultures, a feature which is characteristic of chondrogenesis in micromass culture (Ahrens *et al.*, 1977). The increase in condensation formation in the $Fak^{-/-}$ micromass cultures was observed by staining with PNA. Overall, these data suggest that FAK inhibits chondrogenesis, a novel finding.

Because loss of *Fak* increases the expression of chondrogenic markers including CCN2, I decided to investigate whether CCN2 operated downstream of the loss of FAK to promote chondrogenesis. To address this issue, I returned to using 13.5 dpc $Ccn2^{+/+}$ MEFs which are capable of chondrogenic differentiation in micromass. Inhibiting FAK/Src signaling using PP2 lead to a significant increase in *Ccn2, Col2a1, Agc, Sox6, Sox9,* and *L-Sox5* mRNAs in $Ccn2^{+/+}$ MEFs. Conversely, PP2 treatment decreased *Agc* and *Col2a1* mRNA expression in $Ccn2^{-/-}$ MEFs significantly. These data support the idea that FAK suppresses chondrogenesis by inhibiting CCN2 expression.

Therefore, my data have shown that chondrogenesis is regulated by both CCN2-dependent and independent pathways (Fig. 4.1). In particular, induction of *Sox6* and proteoglycan gene expression is dependent on CCN2, while *Sox9*, *L-Sox5* and *Col2a1* are induced normally or even higher in the absence of CCN2.

4.2 Contributions to the current state of knowledge of connective tissue growth factor and chondrogenesis.

Chondrogenesis is the first step in the development of the endochondral skeleton and is orchestrated by interplay between a number of molecules. A role for CCN2 in later

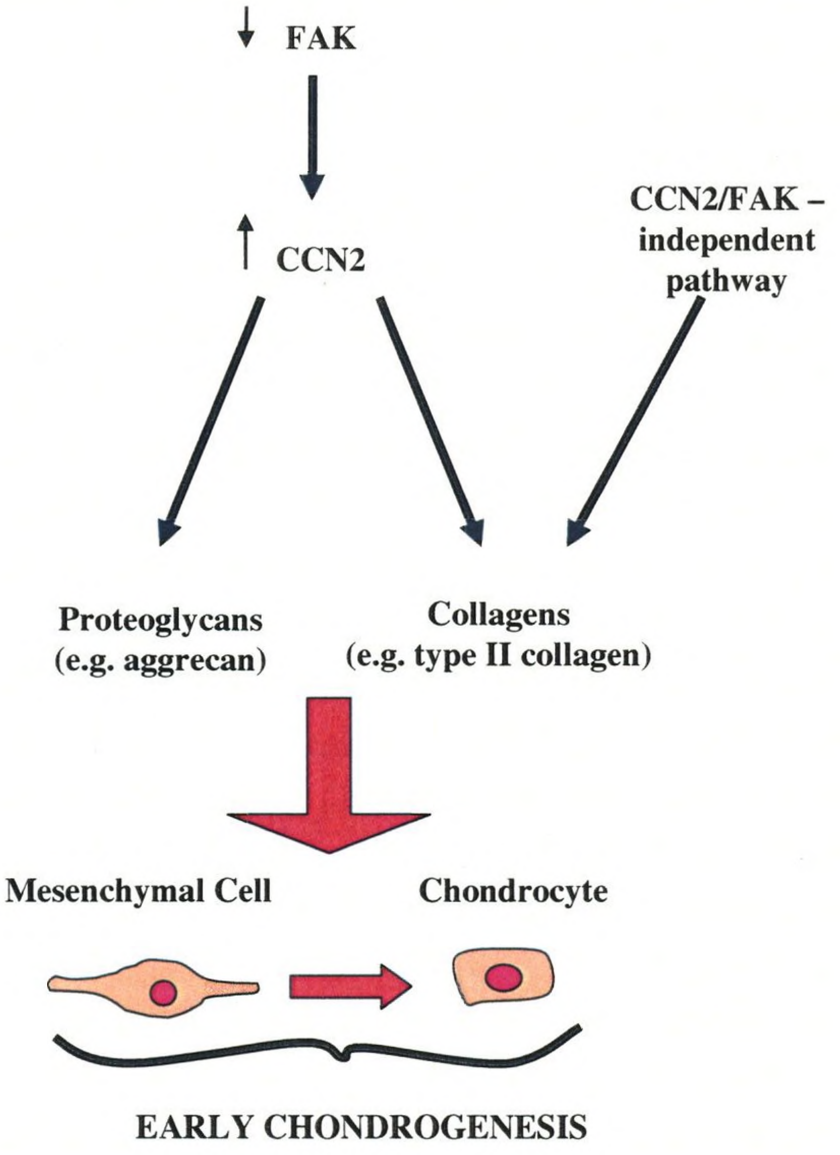


Figure 4.1 Summary of CCN2 function during early chondrogenesis. CCN2 is exerting its effects on chondrocyte development during the early stages of chondrogenesis. FAK is upstream of CCN2 and acts to suppress chondrogenesis. CCN2 acts to promote early chondrogenic events, and regulates the expression of extracellular matrix genes such as the proteoglycans, whereas collagen deposition seems to be regulated by a CCN2/FAK-independent pathway.

stages of chondrocyte maturation has been described (Nakanishi *et al.*, 1997; Takigawa *et al.*, 2003). I have demonstrated an essential role of CCN2 in the early stages of chondrogenesis.

In my first set of experiments, one interesting observation was that the *Ccn2*$^{-/-}$ cells also showed differential expression of other CCN family members. The CCN family is known to have a high degree of sequence homology among its members and is predicted to be similar in their modular structure (Bork, 1993; Leask and Abraham, 2006; Perbal, 2004). In the micromass cultures, *Ccn5* gene expression is consistently decreased in *Ccn2*$^{-/-}$ cells indicating that the expression of this gene may be reliant on the expression of CCN2, implicating a potential redundant function of CCN5 and CCN2. There has not been previous work connecting the expression of CCN2 and CCN5 in chondrogenesis. However, previous work has shown similarity in function and expression of CCN1 and CCN2 (Chen et al., 2001a; Kireeva et al., 1997). CCN1 and CCN2 also share similar binding characteristics since they bind to the same HSPGs; syndecan-4 and perlecan, through their fourth domain (Chen et al., 2004; Nishida et al., 2003; Todorovicc et al., 2005). They are also known to interact with similar integrins in a number of different cell types, as discussed above (Chen et al., 2007; Lau and Lam, 1999). CCN1 is also known to be expressed in chondrocytes and involved in the synthesis of collagen and other ECM components *in vitro* (Wong *et al.*, 1997). Both the CCN2 (Ivkovic *et al.*, 2003) and CCN1 (Mo *et al.*, 2002) loss of function animal models are lethal, however, despite the similarities in function and expression observed between CCN1 and CCN2, the animals exhibit different phenotypes. The *Ccn2*$^{-/-}$ mice display a generalized chondrodysplasia while the *Ccn1*$^{-/-}$ mice display abnormalities in their vessel integrity and placental

vasculature. Although the *Ccn2*$^{-/-}$ animals do not show the same vascular defects, they do exhibit a defect in angiogenesis of the developing bone (Ivkovic *et al.*, 2003).

A potential reason why the skeletal malformations seen in the *Ccn2*$^{-/-}$ animals are not more severe could be the functional redundancy with other CCN family members, such as CCN1. Furthermore, the fact that *Ccn1*$^{-/-}$ mice do not reveal any skeletal irregularities can be attributed to the fact that the majority of *Ccn1*$^{-/-}$ mice die between 11.5 dpc and 14.5 dpc. If the *Ccn1*$^{-/-}$ mice could survive past midgestation I would expect to see chondrogenic defects, potentially similar to those seen in the *Ccn2*$^{-/-}$ mice, since *in vitro* studies of CCN1 expression indicate that it is involved in chondrogenesis and shows a similar pattern of expression to CCN2 (Chen et al., 2001a; Kireeva et al., 1997; O'Brien and Lau, 1992). Perhaps since CCN2 and CCN1 share many similarities it is a possibility that some CCN2-independent mechanisms during chondrogenesis may involve CCN1, such as the induction of type II collagen expression, or the up-regulation of l-sox5 and sox9.

Several previous studies have examined the relationship between the sox trio and their function, and from these studies it is widely believed that these three transcription factors act cooperatively during the initiation of chondrogenesis (Akiyama et al., 2002a; Lefebvre et al., 2001; Lefebvre et al., 1998). It is also believed that l-sox5 and sox6 are necessary for maximal activity of sox9 (Akiyama et al., 2002a; Lefebvre et al., 1998; Smits, 2001). Many studies have focused attention primarily on sox9 (Akiyama et al., 2002a; Lefevbre et al., 1997). For example in a recent study looking at CCN2 in chondrogenesis it was found that when CCN2 expression levels are high, sox9 expression is low, but l-sox5 and sox6 were not even examined, and the chondrogenic period

analysed was only the first 48 hours (Song *et al.*, 2007). My findings on the dependency of *Sox6* but not *Sox9* and *L-Sox5* on CCN2 expression are novel. Other work from our lab has demonstrated that these genes are not all necessarily expressed in parallel, and may be under differential regulation. It was found that *L-Sox5* and *Sox6*, but not *Sox9*, are down-regulated upon depolymerization of the actin cytoskeleton (Woods and Beier, 2006). Furthermore, it was shown that only *L-Sox5*, *Sox9* and *Col2a1*, but not *Sox6* and *Agc*, expression is induced upon cdc42 overexpression in ATDC5 cells (Woods *et al.*, 2007 submitted). Therefore, my data support a novel concept demonstrating differential regulation (and potentially function) of different sox genes during chondrogenesis.

I also demonstrated a novel finding involving the transcriptional targets of the sox trio; aggrecan and type II collagen. It is well established that the sox trio is responsible for the expression of aggrecan and type II collagen, but the precise mechanism of how they interact is poorly understood (Lefebvre *et al.*, 2001; Okazaki and Sandell, 2004). Current knowledge is heavily focused on the interaction of sox9 with an enhancer element in the first intron of the *Col2a1* gene (Bell, 1997; Bi *et al.*, 1999). Despite the fact that aggrecan is evolutionarily well conserved, the regulatory regions of this gene have not been completely described yet, but it is expected that it is regulated in a similar manner as type II collagen, since both ECM molecules are co-expressed in the early ECM of chondrocytes (Karsenty and Wagner, 2002). However, I have observed that the proteoglycans *Agc, Dcn* and the ECM protein, *Hapln* are all nearly absent in the knockout cells on all 6 days in culture. Although it is known that CCN2 is a major inducer of type II and X collagen *in vitro* and regulates the expression of type I collagen in fibrotic responses (Leask and Abraham, 2003; Nakanishi et al., 2000), my data show that the

expression of type II collagen is CCN2-independent in early chondrogenesis and even increased in the knockout micromass cultures. Therefore, my data demonstrate that CCN2 regulates the ratio of proteoglycans to collagen within the chondrogenic ECM. Recent studies in our lab have found a similar discrepancy in the regulation of type II collagen and aggrecan by cdc42. Cdc42 overexpression in ATDC5 cells results in the up-regulation of *L-Sox5*, *Sox9* and *Col2a1*, but not *Sox6* and *Agc* (Woods et al., 2007 submitted), which supports my data that there is a divergence in the regulation of type II collagen and aggrecan at least in part by the sox trio.

My studies analyzing the regulation of CCN2 by FAK, using both the $Fak^{+/+}$ and $^{-/-}$ MEFs and $Ccn2^{+/+}$ and $^{-/-}$ MEFs with the FAK/Src inhibitor PP2, revealed an interesting up-regulation of CCN2 and some markers of chondrogenesis upon loss of *Fak*. A possible explanation for these effects is that the absence of FAK promotes a shift from an environment that favours cell-ECM interactions to one that heavily favours cell-cell interactions. It is believed that the process of chondrogenesis is a sequence of events, where adhesion molecules are involved in mediating cell-cell interactions initially during cell condensations and commitment of mesenchymal cells to a chondrogenic lineage, followed by ECM mediation of cell-matrix interactions during the differentiation of cartilage (Kawai *et al.*, 1999). Another potential explanation for the increase in chondrogenic capability of the $Fak^{-/-}$ cells is that FAK is known to be required for migration (Gilmore and Romer, 1996; Ilic et al., 1995; Mitra et al., 2005), but cells that are undergoing chondrogenesis *in vivo* and the cells that are placed in the chondrogenic micromass system *in vitro* are not motile. The micromass system is designed to achieve heightened cell-cell contacts in a non-mobile environment.

Furthermore, the promotion of chondrogenesis by *Fak*$^{-/-}$ cells can be explained by a number of cellular possibilities, such as that the absence of FAK may be promoting an increase in the number of mesenchymal condensations or an increase in the differentiation of existing cells. These explanations could be the result of the increased amount of focal adhesion formation in the *Fak*$^{-/-}$ masses due to the decrease in focal adhesion turnover. Also interesting to note is the fact that the *Fak*$^{-/-}$ cells have a rounded cell morphology (Ilic et al., 1995; Sieg et al., 1999; Sieg et al., 1998), which is quite uncharacteristic of fibroblasts. It is widely accepted that pharmacological inhibition of actin polymerization, which promotes a rounded cell morphology, can drive dedifferentiated cells to re-express chondrogenic matrix molecules (Benya, 1988; Benya and Padilla, 1993; Brown and Benya, 1988) and can promote the differentiation of mesenchymal cells to chondrocytes (Loty et al., 1995; Zanetti and Solursh, 1984). Our lab showed that pharmacological inhibition of ROCK, an effector of RhoA, caused rounded cell morphology which is correlated to increased chondrogenic differentiation (Woods *et al.*, 2005). Therefore, a potential explanation for the absence of FAK promoting chondrogenesis could be the morphological changes that occur as a result of loss of FAK, consistent with the idea that rounded cell morphology promotes chondrogenesis.

Preliminary data from our lab have demonstrated that the rac1 signaling pathway which regulates cortical actin organization and promotes chondrogenesis also increases mRNA levels of *Ccn2* (Woods *et al.*, 2007 submitted). Overexpression of rac1 in the ATDC5 cell line results in increased levels of *Ccn2* mRNA compared to control cultures while genetic ablation of *Rac1* in micromass cultures inhibits chondrogenesis and decreases the expression levels of *Ccn2*.

In addition, the regulation of CCN2 expression in chondrogenesis has also been connected to the transcription factors, c-Maf and Lc-Maf. Overexpression of c-maf in the cell line C3H10T1/2 strongly induced *Ccn2* mRNA (Omoteyama *et al.*, 2006). Furthermore, *c-Maf* and *Ccn2* null mice share similarities in their phenotypes in that they both have increased hypertrophic zones in growth plates and decreased ossification in the center of the long bones (Ivkovic *et al.*, 2003; MacLean *et al.*, 2003). However, these connections have been described primarily for later stages of chondrogenesis involving hypertrophic chondrocytes, and it is not known if there is a connection between c-maf and CCN2 in early chondrogenesis.

The three major types of mitogen activated protein kinases (MAPK)s, ERK1/2, c-jun N-terminal kinase (JNK) and p38 MAPK, are implicated in playing a role in CCN2 signaling. It is believed that the proliferative effects of CCN2 are mediated by ERK and the effects on differentiation are mediated by p38 MAPK in chondrocytes (Yosimichi *et al.*, 2001). In a recent study by the same group using HCS-2/8 chondrosarcoma cells, CCN2 was also found to activate JNK expression which promotes the proliferation of chondrocytes (Yosimichi *et al.*, 2006). In the same study, it was found that CCN2 promoted activation of protein kinase C (PKC) which is known to act upstream of Akt (Yosimichi *et al.*, 2006). However this study mainly characterized the effect of Akt at the later stages of chondrogenesis, affecting primarily hypertrophic chondrocytes. The involvement of Akt and CCN2 is interesting, since it is known that CCN2 increases activation of Akt in human mesenchymal cells (Crean *et al.*, 2002), which might suggest an involvement in the progression of early chondrogenesis. The active role of Phosphoinositide 3 kinase (PI3K), another upstream mediator or Akt signaling, is still yet

to be fully understood in the initial development of chondrocytes and might potentially be connected to the activities of CCN2 in early chondrogenesis.

4.3 Limitations and suggestions for future research

My research was the first study to look at the role of CCN2 during early chondrogenesis using primary cells. My data have demonstrated a clear role for CCN2 in this process and has identified key targets of CCN2 such as sox6 and aggrecan and an upstream mediator of CCN2 expression, FAK. I have also been able to show that type II collagen expression is in a CCN2-independent pathway. I think my most interesting finding has been that in addition to being upstream of CCN2, FAK suppresses the progression of chondrogenesis. I believe my data are exciting for chondrocyte biology, but these findings also raise many more questions.

One major limitation of my research was that it was completely performed *in vitro*. In order to minimize this limitation I used primary MEFs from the *Ccn2*$^{+/+}$ and $^{-/-}$ mice as well as the *Fak*$^{+/+}$ and $^{-/-}$ mice. I also used the micromass culture system which is an excellent *in vitro* tool that best mimics an *in vivo* chondrogenic environment (Ahrens et al., 1977; James et al., 2005; Lengner et al., 2004; Woods and Beier, 2006; Woods et al., 2005). Nevertheless, inherent limitations existed due to the lethality of the genes that were mutated in these mice. The early stage embryonic lethality of the *Fak* $^{-/-}$ mice proved to be a limitation since it was not possible to use the *Fak* MEFs at a stage later than 8.5 dpc (Furuta *et al.*, 1995; Ilic *et al.*, 1995). Had the *Fak* mice lived to 13.5 dpc for example, the 13.5 MEFs would have been capable of completely undergoing chondrogenic differentiation. It would be most interesting to see an animal model with a double knockout for *Fak* and *Ccn2*; however, this would require tissue-specific gene

inactivation, for example through the Cre-Lox system. It would be exciting to see the expression patterns of the known chondrogenic markers in this scenario. It would also be interesting to look at conditional knockouts of *Fak* and *Ccn2* in different tissues in order to examine further potential connections between these two genes. A double null animal for both *Ccn1* and *Ccn2* would also be interesting, since these two genes are thought to have similar roles in development, yet very different phenotypes in their knockout animal models. I would anticipate very severe generalized skeletal and vascular abnormalities, since this double null animal would potentially inhibit any sort of compensatory mechanism exhibited by CCN1 for CCN2.

Although my research was focused on CCN2, I did briefly examine the effects of loss of CCN2 on two other CCN family members, CCN1 and CCN5, but I did not fully address the possibility of functional redundancy within this family. The CCN family was discovered only 15 years ago (Bradham *et al.*, 1991; Brigstock, 1999; Lau and Lam, 1999; Perbal, 2001), therefore much remains unknown about these matricellular proteins. In fact there are many inherent limitations within this field of research itself. With no commercial neutralizing antibodies available, no standardised recombinant CCN2 and no known specific signaling receptor, study of the CCNs proves to be challenging. With similar predicted structures (Bork, 1993; Brigstock, 2003; Perbal, 2004) and known patterns of similar expression (Kireeva et al., 1997; O'Brien and Lau, 1992; Perbal, 2004) it will be important to address in the future just how much these proteins depend on one another for their function.

An aspect of chondrogenesis I did not address in my study was the role of the matrix metalloproteinases (MMPs) in early chondrogenesis. Since my results indicate a

strong connection between CCN2 expression and regulation of components within the ECM, it would be interesting to examine if any of the MMPs are affected as well. For example, MMP9 is required for ECM remodeling, since it is known to degrade collagen and proteoglycans and is expressed in cartilage (D'Angelo *et al.*, 2001; Sternlicht and Werb, 2001). In the *Ccn2*$^{-/-}$ animal, low levels of MM9 were found in the growth plate (Ivkovic *et al.*, 2003). It would be useful to see if there is a loss in the expression of this gene during any stages of early chondrogenesis and see if that interaction is involved in the regulation of collagens and proteoglycans during this stage.

Another limitation was the use of the pharmacological inhibitor PP2 as a FAK/Src inhibitor. The concentration of this inhibitor was optimized in our labs to control for toxic effects, however, one cannot completely rule out whether inhibitors display nonspecific effects. Furthermore, the role of Src kinases in chondrogenesis is not known. It would be ideal to have an inhibitor that is specific for FAK, but this is not yet commercially available. Additionally, development of siRNAs to knockdown FAK would circumvent the issue of inhibitor specificity in order to study the effects on known chondrogenic markers' however, transfection efficiency is a serious challenge yet to be overcome for application of siRNA technology in chondrogenic cells.

Another interesting, albeit costly suggestion for future work would be to do a microarray for the *Fak*$^{+/+}$ and $^{-/-}$ MEF micromass cultures. A high throughput genome wide screen of all the genes that are being affected by the loss of FAK during chondrogenesis would be most interesting. I would suspect that an increase in many known chondrogenic markers would be seen in the knockout. This study could

potentially open the door to many other questions and provide insight into other genes found in the FAK signaling cascade.

A large gap remains in the literature about the regulation of expression of the two main early chondrogenic ECM components, type II collagen and aggrecan. It would be most useful to undertake a study that delineates regulatory sequences within the *Agc* gene. Such a study would potentially allow for the understanding of to what degree these two genes are co-regulated. So far, most research has focused on type II collagen (Bell, 1997; Bi *et al.*, 1999), and aggrecan has not received much attention. Another avenue to achieve a better understanding of aggrecan and type II collagen expression would be through examination of the individual and specific functions of the sox trio. Much like the dilemma for type II collagen and aggrecan, where the attention has only been focused on one of them, the attention has been primarily focused on sox9 (Akiyama et al., 2002a; Lefevbre et al., 1997; Sekiya et al., 2000) and not l-sox5 or sox6. In fact, it is still widely accepted that l-sox5 or sox6 serve overlapping functions. My data, which shows that the expression of sox6 and early chondrogenic proteoglycans are dependent on CCN2, and other work in our lab, as stated above (Woods and Beier, 2006; Woods et al., 2007 submitted) have demonstrated a clear divergence between the expression of the sox trio and the regulation of type II collagen and aggrecan. Further investigation is needed to gain a more comprehensive understanding of the mechanism behind this novel divergence.

4.4 Significance

In order to best understand treatment options for skeletal diseases we must first understand the onset, development and progression of pathologies. This can be best

initiated with an understanding of the molecular mechanisms at play in physiological circumstances and their pathological derivations. My work has identified a new role for CCN2 in chondrocyte biology. My data has proven that CCN2 is required for early chondrogenesis and is responsible for regulating the components of the ECM and has identified FAK as an upstream mediator of CCN2. Furthermore, I have identified that type II collagen deposition during chondrogenesis occurs in a CCN2-independent fashion.

Discovering that the presence of CCN2 but the absence of FAK promotes chondrogenesis can have potential implications in the treatment of skeletal diseases that involve loss of cartilage such as osteoarthritis, or improper development of cartilage such as chondrodysplasias. Novel therapeutic interventions in the future could include using our knowledge about these genes to promote the progression of chondrogenesis *in vivo* and generate new cartilage. My research has contributed to a more comprehensive understanding of embryonic cartilage development and will hopefully one day contribute to new therapeutic treatment options for people afflicted with skeletal disorders.

5.0 References

5.0 References

Ahrens, P., Solursh, M., and Reiter, R. (1977). Stage-related capacity for limb chondrogenesis in cell culture. *Developmental Biology* **60,** 69-82.

Akiyama, H., Chaboissier, M. C., Martin, J. F., Schedl, A., and de Crombrugghe, B. (2002). The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes and Development* **16,** 2813-2828.

Babic, A., Chen, C., and Lau, L. (1999). Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin alphavbeta3, promotes endothelial cell survival, and induces angiogenesis in vivo. *Molecular Cell Biology* **19,** 2958-2966.

Bang, O.-S., Kim, E.-J., Chung, J. G., Lee, S.-R., Park, T. K., and Kang, S.-S. (2000). Association of Focal Adhesion Kinase with Fibronectin and Paxillin is required for precartilage condensation of chick mesenchymal cells. *Biochemical and Biophysical Research Communications* **278,** 522-529.

Bell, D. M. (1997). SOX9 directly regulates the type-II collagen gene. *Nature Genetics* **16,** 174-178.

Benya, P. D. (1988). Modulation and re-expression of the chondrocyte phenotype; Mediation by cell shape and microfilament modification. *Pathology and Immunopathology Research* **7,** 51-54.

Benya, P. D., and Padilla, S. R. (1993). Dihydrocytochalasin B enhances transforming growth factor beta-induced re-expression of the differentiated chondrocyte

phenotype without stimulation of collagen synthesis. *Experimental Cell Research* **204,** 268-277.

Bi, W. (2001). Haploinsufficiency of Sox9 results in defective cartilage primordia and premature skeletal mineralization. *Proceedings of the National Academy of Science of the United States of America* **98,** 6698-6703.

Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R., and de Crombrugghe, B. (1999). Sox9 is required for cartilage formation. *Nature Genetics* **22,** 85-89.

Blom, I. E., van Dijk, A. J., Wieten, L., Duran, K., Ito, Y., Kleij, L., deNichilo, M., Rabelink, T. J., Weening, J. J., Aten, J., and Goldschmeding, R. (2001). In vitro evidence for differential involvement of CTGF, TGF{beta}, and PDGF-BB in mesangial response to injury. *Nephrology Dialysis Transplantation* **16,** 1139-1148.

Bork, P. (1993). The modular architecture of a new family of growth regulators related to connective tissue growth factor. *Federation of European Biological Societies Letters* **327,** 125 - 130.

Bradham, D., Igarashi, A., Potter, R., and Grotendorst, G. (1991). Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *Journal of Cell Bioliolgy* **114,** 1285-1294.

Brigstock, D. (2003). The CCN family: a new stimulus package. *Journal of Endocrinology* **178,** 169 - 175.

Brigstock, D. R. (1999). The connective tissue growth factor/Cysteine- rich 61/Nephroblastoma overexpressed (CCN) family. *Endocrinology Review* **20,** 189-206.

Brigstock, D. R. (2002). Regulation of angiogenesis and endothelial cell function by connective tissue growth factor (CTGF) and cysteine-rich 61 (CYR61). *Angiogenesis* **5,** 153 - 165.

Brown, P. D., and Benya, P. D. (1988). Alterations in chondrocyte cytoskeletal architecture during phenotypic modulation by retinoic acid and dihydrocytochalasin B-induced re-expression. *Journal of Cell Biology* **106,** 171-179.

Chan, D., Cole, W. G., Chow, C. W., Mundlos, S., and Bateman, J. F. (1995). A Col2a1 mutation in achondrogenesis type II results in the replacement of type II collagen by type I and III collagens in cartilage. *Journal of Biological Chemistry* **270,** 1747-1753.

Chen, C., Chen, N., and Lau, L. (2001a). The angiogenic factors Cyr61 and connective tissue growth factor induce adhesive signaling in primary human skin fibroblasts. *Journal of Biological Chemistry* **276,** 10443 - 10452.

Chen, C. C., Young, J., Monzon, R. I., Chen, N., Todorovicc, V., and Lau, L. F. (2007). Cytotoxicity of TNFalpha is regulated by integrin mediated matrix signaling. *The EMBO Journal* **26,** 1257-67.

Chen, H., Liu, C. T., and Yang, S. S. (1981). Achondrogenesis: A review with special consideration of achondrogenesis type II (Langer-Saldino). *American Journal of Medical Genetics* **10,** 379-394.

Chen, Q., Johnson, D. M., Haudenschild, D. R., and Goetinck, P. F. (1995). Progression and recapitulation of the chondrocyte differentiation program: cartilage matrix protein is a marker for cartilage maturation. *Developmental Biology* **172,** 293-306.

Chen, Y., Abraham, D. J., Shi-wen, X., Pearson, J. D., Black, C. M., Lyons, K. M., and Leask, A. (2004). CCN2 (connective tissue growth factor) promotes fibroblast adhesion to Fibronectin. *Molecular Biology of the Cell* **15,** 5635-5646.

Chen, Y., Blom, I. E., Sa, S., Goldschmeding, R., Abraham, D. J., and Leask, A. (2002). CTGF expression in mesangial cells: Involvement of SMADs, MAP kinase, and PKC. *Kidney International* **62,** 1149-1159.

Chen, Y., Segarini, P., Raoufi, F., Bradham, D., and Leask, A. (2001b). Connective tissue growth factor is secreted through the Golgi and is degraded in the endosome. *Experimental Cell Research* **271,** 109-117.

Cohn, M. J., Tickle, C. (1996). Limbs: A model for pattern formation within the vertebrate body plan. *Trends in Genetics* **12,** 253-257.

Crean, J. K., Finaly, D., Murphy, M., Moss, C., Godson, C., Martin, F., and Brady, H. R. (2002). The role of p42/44 MAPK and protein kinase B in connective tissue growth factor induced extracellular matrix protein production, cell migration, and actin cytoskeletal rearrangement in human mesangial cells. *Journal of Biological Chemistry* **277,** 44187-94.

D'Angelo, M., Billings, P., Pacifici, M., Leboy, P., and Kirsch, T. (2001). Authentic matrix vesicles contain active metalloproteinases (MMP). *Journal of Biological Chemistry* **276,** 11347-11353.

de Crombrugghe, B., Lefebvre, V., Behringer, R. R., Bi, W., Murakami, S., and Huang, W. (2000). Transcriptional mechanisms of chondrocyte differentiation. *Matrix Biology* **19,** 389-394.

DeLise, A. M., Fischer, L., and Tuan, R. S. (2000a). Cellular interactions and signaling in cartilage development. *Osteoarthritis and Cartilage* **8,** 309-334.

DeLise, A. M., Stringa, E., Woodward, W. A., Mello, M. A., and Tuan, R. S. (2000b). Embryonic limb mesenchyme micromass culture as an *in vitro* model for chondrogenesis and cartilage maturation. *Methods in Molecular Biology* **137,** 359-375.

Dessau, W. J., von der Mark, K., von der Mark, H., and Fischer, S. (1980). Changes in the patterns of collagens and fibronectin during limb bud chondrogenesis. *Journal of Embryology and Experimental Morphology* **57,** 51-60.

Eyre, D. R., Upton, M. P., Shapiro, F. D., Wilkinson, R. H., and Vawter, G. F. (1986). Nonexpression of cartilage type II collagen in a case of Langer-Saldino achondrogenesis. *American Journal of Human Genetics* **39,** 52-67.

Fell, H. B. (1925). The histogenesis of cartilage and bone in the long bones of the embryonic fowl. *Journal of Morphology* **40,** 417-459.

Foster, J. W. (1994). Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature* **372,** 525-530.

Friedrichsen, S., Heuer, H., Christ, S., Winckler, M., Brauer, D., Bauer, K., and Raivich, G. (2003). CTGF expression during mouse embryonic development. *Cell Tissue Research* **312,** 175 - 188.

Furuta, Y., D. , Ilic, S., Kanazawa, N., Takeda, T., Yamamoto, S., and Aizawa, S. (1995). Mesodermal defect in late-phase of gastrulation by a targeted mutation of focal adhesion kinase, FAK. *Oncogene* **11,** 1989.

Gau, R., and Brigstock, D. R. (2004). Connective Tissue Growth Factor (CCN2) induces adhesion of rat activated hepatic stellate cells by binding of its C-terminal domain to integrin alpha(v)beta(3) and heparan sulfate proteoglycan. *J Biol Chem* **279,** 8848-55.

Gilmore, A., and Romer, L. (1996). Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decreases cell motility and proliferation. *Molecular Biology of the Cell* **7,** 1209-1224.

Gleghorn, L., Ramesar, R., Beighton, P., and Wallis, G. (2005). A mutation in the variable repeat region of the aggrecan gene (AGC1) causes a form of spondyloepiphyseal dysplasia associated with severe, premature osteoarthritis. *American Journal of Human Genetics* **77,** 484-490.

Grotendorst, G. R. (1997). Connective tissue growth factor: a mediator of TGF-[beta] action on fibroblasts. *Cytokine & Growth Factor Reviews* **8,** 171-179.

Gruneberg, H. (1963). The pathology of development. a study of inherited skeletal disorders in animals. *Oxford. Blackwells Scientifc Publications.*

Hacker, U., Nybakken, K., and Perrimon, N. (2005). Heparin sulphate proteoglycans: The sweet side of development. *Nature Reviews Molecular Cell Biology* **6,** 530-541.

Hall, B. K. (1988). The embryonic development of bone. *American Scientist* **76,** 174-181.

Hall, B. K., and Miyake, T. (2000). All for one and one for all: condensations and the initiation of skeletal development. *BioEsssays* **22,** 138-147.

Hardingham, T., and Bayliss, M. (1990). Proteoglycans of articular cartilage:changes in aging and in joint disease. *Seminars in Arthritis and Rheumatism* **20,** 12-33.

Hardingham, T. E., and Fosang, A. J. (1992). Proteoglycans: many forms and many functions. *Federation of American Societies for Experimental Biology Journal* **6,** 861-870.

Hascall, V. C., and Heinegard, D. (1974). Aggregation of cartilage proteoglycans. I. The role of Hyaluronic acid. *Journal of Biological Chemistry* **249,** 4232-4241.

Hishikawa, K., Oemar, B. S., Tanner, F. C., Nakaki, T., Luscher, T. F., and Fujii, T. (1999). Connective Tissue Growth Factor induces apoptosis in human breast cancer cell line MCF-7. *Journal of Biological Chemistry* **274,** 37461-37466.

Holmes, A., Abraham, D. J., Sa, S., Shiwen, X., Black, C. M., and Leask, A. (2001). CTGF and SMADs, maintenance of scleroderma phenotype is independent of SMAD signaling. *Journal of Biological Chemistry* **276,** 10594-10601.

Horton, W. A. (1993). Cartilage morphology. *In* " Extracellular matrix and heritable disorder of connective tissue" (P. M. R. a. B. Steinman, Ed.), pp. 73–84. Alan R. Liss, New York.

Igarashi, A., Okochi, H., Bradham, D. M., and Grotendorst, G. R. (1993). Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. *Molecular Biology of the Cell* **4,** 637–645.

Ikeda, T., Kawaguchi, H., Kamerkura, S., Ogata, N., Mori, Y., Nakamura, K., S., I., and Chung, U. (2005). Distinct roles of Sox5, Sox6, and Sox9 in different stages of chondrogenic differentiation. *Journal of Bone and Mineral Metabolism* **23,** 337-40.

Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* **377,** 539-44.

Inoki, I., Shiomi, T., Hashimoto, G., Enomoto, H., Nakamura, H., Makino, K.-I., Ikeda, E., Takata, S., Kobayashi, K.-I., and Okada, Y. (2002). Connective tissue growth factor binds vascular endothelial growth factor (VEGF) and inhibits VEGF-induced angiogenesis. *Federation of American Societies for Experimental Biology Journal* **16,** 219-221.

Ivkovic, S., Yoon, B. S., Popoff, S. N., Safadi, F. F., Libuda, D. E., Stephenson, R. C., Daluiski, A., and Lyons, K. M. (2003). Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. *Development* **130,** 2779-2791.

James, C. G., Appleton, C. T., Ulici, V., Underhill, T. M., and Beier, F. (2005). Microarray analyses of gene expression during chondrocyte differentiation identifies novel regulators of hypertrophy. *Molecular Biology of the Cell* **16,** 5316-5333.

Karsenty, G. (1999). The genetic transformations of bone biology. *Genes and Development* **13,** 3037-3051.

Karsenty, G. (2001). Chondrogenesis just ain't what it used to be. *Journal of Clinical Investigation* **107,** 405-407.

Karsenty, G. (2003). The complexities of skeletal biology. *Nature* **423,** 316-318.

Karsenty, G., and Wagner, E. F. (2002). Reaching a Genetic and Molecular Understanding of Skeletal Development. *Developmental Cell* **2,** 389-406.

Kawai, J., Akiyama, H., Shinego, C., Ito, H., Konishi, J., and Nakamura, T. (1999). Effects of transforming growth factor beta signaling on chondrogenesis in mouse chondrogenic EC cells, ATDC5. *European Journal of Cell Biology* **78,** 707-714.

Kennedy, L., Liu, S., Shi-Wen, X., Chen, Y., Eastwood, M., Carter, D. E., Lyons, K. M., Black, C. M., Abraham, D. J., and Leask, A. (2007). CCN2 is necessary for the function of mouse embryonic fibroblasts. *Experimental Cell Research* **313,** 952-964.

Kireeva, M., Latinkic, B., Kolesnikova, T., Chen, C., Yang, G., Abler, A., and Lau, L. (1997). Cyr61 and Fisp12 are both ECM-associated signaling molecules: activities, metabolism, and localization during development. *Experimental Cell Research* **233,** 63-77.

Kornack, U., and Mundlos, S. (2003). Genetic disorders of the skeleton: A developmental approach. *American Journal of Human Genetics* **73,** 447-474.

Kronenberg, H. M. (2003). Development regulation of the growth plate. **423,** 332-336.

Krueger, R. C., Kurima, K., and Schwartz, N. B. (1999). Completion of the mouse Aggrecan structure and identification of the defect in the cmd-Bc as a near complete deletion of the murine aggrecan. *Mammalian Genome* **10,** 1119-1125.

Krueger, R. C. J., Fields, T. A., Hildreth, J., and Schwartz, N. B. (1990). Chick cartilage chondroitin sulfate proteoglycan core protein. I. Generation and characterization of peptides and specificity for glycosaminoglycan attachment. *Journal of Biological Chemistry* **265,** 12075-12087.

Lau, L. F., and Lam, S. C. T. (1999). The CCN family of angiogenic regulators: The integrin connection. *Experimental Cell Research* **248,** 44-57.

Leask, A., and Abraham, D. (2003). The role of connective tissue growth factor, a multifunctional matricellular protein, in fibroblast biology. *Biochemistry and Cell Biology* **81,** 355 - 363.

Leask, A., and Abraham, D. J. (2006). All in the CCN family: essential matricellular signaling modulators emerge from the bunker. *Journal of Cell Science* **119,** 4803-4810.

Leask, A., Denton, C. P., and Abraham, D. J. (2004). Insights into the molecular mechanism of chronic fibrosis: The role of connective tissue growth factor in scleroderma. *Journal of Investigative Dermatology* **122,** 1-6.

Lefebvre, V., Behringer, R. R., and de Crombrugghe, B. (2001). L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. *Osteoarthritis Cartilage* **9,** 69-75.

Lefebvre, V., Li, P., and de Crombrugghe, B. (1998). A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. *The EMBO Journal* **17,** 5718-5733.

Lefevbre, V., Huang, W., Harley, V. R., Goodfellow, P. M., and de Crombrugge, B. (1997). Sox9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha I (II) collagen gene. *Molecular Cell Biology* **17,** 2336-2346.

Lengner, C. J., Lepper, C., van Wijnen, A. J., Stein, J. L., Stein, G. S., and Lian, J. B. (2004). Primary mouse embryonic fibroblasts: A model of mesenchymal cartilage formation. *Journal of Cellular Physiology* **200,** 327-333.

Li, H., Schwartz, N. B., and Vertel, B. M. (1993). cDNA cloning of chick cartilage chondroitin sulfate (Aggrecan) core protein and identification of a stop codon in the aggrecan gene associated with the chondrodystrophy, nanomelia. *Journal of Biological Chemistry* **268,** 23504-23511.

Li, S. W., Prockop, D. J., Helminen, H., Fassler, R., Lapvetelainen, T., Kiraly, K., Peltarri, A., Arokoski, J., Lui, H., and Arita, M. (1995). Transgenic mice with targeted inactivation of the col2a1 gene for Collagen II develop a skeleton with membranous and periosteal bone but no endochondral bone. *Genes and Development* **9,** 2821-2830.

Lillis, A. P., Mikhailenko, I., and Strickland, D. K. (2005). Beyond endocytosis: LRP function in cell migration, proliferation and vascular permeability. *Journal of Thrombosis and Haemostasis* **3,** 1884-1893.

Loty, S., Forest, N., Boulekbache, H., and Sautier, J. M. (1995). Cytochalasin D induces changes in cell shape and promotes *in vitro* chondrogenesis: A morphological study. *Biology of the Cell* **83,** 149-161.

MacLean, H. E., Kim, J. I., Glimcher, M. J., Wang, J., Kronenberg, H. M., and Glimcher, L. H. (2003). Absence of transcription factor c-maf causes abnormal terminal differentiation of hypertrophic chondrocytes during endochondral bone development. *Developmental Biology* **262,** 51-63.

Mitra, S. K., Hanson, D. A., and Schlaepfer, D. D. (2005). Focal adhesion kinase: In command and control of cell motility. *Nature Reviews Molecular Cell Biology* **6,** 56-68.

Mo, F. E., Muntean, A. G., Chen, C. C., Stolz, D. B., Watkins, S. C., and Lau, L. F. (2002). CYR61 (CCN1) is essential for placental development and vascular integrity. *Molecular Cell Biology* **22,** 8709-8720.

Mori, T., Kawara, S., Shinozaki, M., Hayashi, N., Kakinuma, T., Igarashi, A., Takigawa, M., Nakanishi, T., and Takehara, K. (1999). Role and interaction of connective tissue growth factor with transforming growth factor-beta; in persistent fibrosis: A mouse fibrosis model. *Journal of Cellular Physiology* **181,** 153-159.

Moussad, E. E.-D. A., and Brigstock, D. R. (2000). Connective tissue growth factor: What's in a name? *Molecular Genetics and Metabolism* **71,** 276-292.

Nakanishi, T., Kimura, Y., Tamura, T., Ichikawa, H., Yamaai, Y.-i., Sugimoto, T., and Takigawa, M. (1997). Cloning of a mRNA preferentially expressed in chondrocytes by differential display-PCR from a human chondrocytic cell line that is identical with Connective Tissue Growth Factor (CTGF) mRNA,. *Biochemical and Biophysical Research Communications* **234,** 206-210.

Nakanishi, T., Nishida, T., Shimo, T., Kobayashi, K., Kubo, T., Tamatani, T., Tezuka, K., and Takigawa, M. (2000). Effects of CTGF/Hcs24, a product of a hypertrophic chondrocyte-specific gene, on the proliferation and differentiation of chondrocytes in culture. *Endocrinology* **141,** 264 - 273.

Nakanishi, T., Yamaai, T., Asano, M., Nawachi, K., Suzuki, M., Sugimoto, T., and Takigawa, M. (2001). Overexpression of connective tissue growth factor/hypertrophic chondrocyte-specific gene product 24 decreases bone density in adult mice and induces dwarfism. *Biochemical and Biophysical Research Communications* **281,** 678-681.

Nishida, T., Kubota, S., Fukunaga, T., Kondo, S., Yosimichi, G., Nakanishi, T., Takano-Yamamoto, T., and Takigawa, M. (2003). CTGF/Hcs24, hypertrophic chondrocyte-specific gene product, interacts with perlecan in regulating the proliferation and differentiation of chondrocytes. *Journal of Cell Physiology* **196,** 265-75.

Nishida, T., Nakanishi, T., Asano, M., Shimo, T., and Takigawa, M. (2000). Effects of CTGF/Hcs24, a hypertrophic chondrocyte-specific gene product, on the proliferation and differentiation of osteoblastic cells in vitro. *Journal of Cell Physiology* **184,** 197-206.

O'Brien, T., and Lau, L. (1992). Expression of the growth factor-inducible immediate early gene cyr61 correlates with chondrogenesis during mouse embryonic development. *Cell Growth and Differentiation* **3,** 645 - 654.

Oberlender, S. A., and Tuan, R. S. (1994). Expression and functional involvement of N-Cadherin in embryonic limb chondrogenesis. *Development* **120,** 177-187.

Oemar, B. S., and Luscher, T. F. (1997). Connective Tissue Growth Factor : Friend or Foe? *Arteriosclerosis, Thrombosis, and Vascular Biology* **17,** 1483-1489.

Okazaki, K., and Sandell, L. J. (2004). Extracellular Matrix Gene Regulation. *Clinical Orthopaedics and Related Research* **427,** S123-S128.

Olsen, B. R., Reginato, A. M., and Wang, W. (2000). Bone Development. *Annual Reviews Cellular and Developmental Biology* **16,** 191-220.

Omoteyama, K., Ikeda, H., Imaki, J., and Sakai, M. (2006). Activation of connective tissue growth factor gene by the c-Maf and Lc-Maf transcription factors. *Biochemical and Biophysical Research Communications* **339,** 1089-1097.

Ornitz, D. M., and Marie, P. J. (2002). FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. *Genes and Development* **16,** 1446-1465.

Parsons, J., Martin, K., Slack, J., Taylor, J., and Weed, S. (2000). Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. *Oncogene* **19,** 5606-5613.

Perbal, B. (2001). NOV (nephroblastoma overexpressed) and the CCN family of genes: structural and functional issues. *Molecular Pathology* **54,** 57 - 79.

Perbal, B. (2004). CCN proteins: multifunctional signalling regulators. *Lancet* **363,** 62 - 64.

Reichenberger, E. M., Aigner, T., von der Mark, K., Stoss, H. V., and Bertling, W. (1991). *In situ* hybridization studies on the expression of type X Collagen in fetal human cartilage. *Developmental Biology* **148,** 562-572.

Ruoslahti, E. (1988). Structure and biology of proteoglycans. *Annual Review of Cell Biology* **4,** 229-255.

Schober, J., Chen, N., Grzeszkievicz, T., Jovanovic, I., Emeson, E., Ugarova, T., Ye, R., Lau, L., and Lam, S. (2002). Identification of integrin alphaMbeta2 as an adhesion receptor on peripheral blood monocytes for CYR61 (CCN1) and connective tissue growth factor (CCN2): immediate-early gene products expressed in atherosclerotic lesions. *Blood* **99,** 4457 - 4465.

Scott, J. E. (2003). Elasticity in extracellular matrix 'shape modules' of tendon, cartilage, etc. A sliding proteoglycan-filament model. *Journal of Physiology.*. **553,** 335-43.

Segarini, P. R., Nesbitt, J. E., Li, D., Hays, L. G., Yates, J. R., III, and Carmichael, D. F. (2001). The Low Density Lipoprotein Receptor-related Protein/alpha 2-Macroglobulin Receptor is a receptor for Connective Tissue Growth Factor. *Journal of Biological Chemistry* **276,** 40659-40667.

Sekiya, I., Tsuji, K., Koopman, P., Wantanabe, H., Yamada, Y., Shinomiya, K., Nifuji, A., and Noda, M. (2000). Sox9 enhances aggrecan promoter/enhancer activity and is upregulated by retinoic acid in a cartilage derived cell-line TC6. *The Journal of Biological Chemistry* **275,** 10738-10744.

Shi-Wen, X., Chen, Y., Denton, C. P., Eastwood, M., Renzoni, E. A., Bou-Gharios, G., Pearson, J. D., Dashwood, M., du Bois, R. M., Black, C. M., Leask, A., and Abraham, D. J. (2004). Endothelin-1 promotes myofibroblast induction through the ETA receptor via a rac/phosphoinositide 3-kinase/Akt-dependent pathway and is essential for the enhanced contractile phenotype of fibrotic fibroblasts. *Mol Biol Cell.* **15,** 2707-19.

Shi-Wen, X., Stanton, L., Kennedy, L., Pala, D., Chen, Y., Howat, S. L., Renzoni, E. A., Carter, D. E., Bou-Gharios, G., Stratton, R., Pearson, J. D., Beier, F., Lyons, K. M., Black, C. M., Abraham, D. J., and Leask, A. (2006). CCN2 is necessary for adhesive responses to TGFbeta 1 in embryonic fibroblasts. *Journal of Biological Chemistry* **281,** 10715-26.

Shiang, R. (1994). Mutations in the transmembrane domain of FGFR3 cause the most common genetic form of dwarfism, achondroplasia. *Cell* **78,** 335-342.

Shimo, T., Kanyama, M., Wu, C., Sugito, H., Billings, P. C., Abrams, W. R., Rosenbloom, J., Iwamoto, M., Pacifici, M., and Koyama, E. (2004). Expression

and roles of connective tissue growth factor in Meckel's cartilage development. *Developmental Dynamics* **231,** 136-147.

Sieg, D. J., Hauck, C. R., and Schlaepfer, D. D. (1999). Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *Journal of Cell Science* **112,** 2677.

Sieg, D. J., Ilic, D., Jones, K. C., Damsky, C. H., Hunter, T., and Schlaepfer, D. D. (1998). Pyk2 and Src-family protein-tyrosine kinases compensate for the loss of FAK in fibronectin-stimulated signaling events but Pyk2 does not fully function to enhance FAK cell migration. *The EMBO Journal* **17,** 5933.

Slee, R. B., Hillier, S. G., Largue, P., Harlow, C. R., Miele, G., and Clinton., M. (2001). Differentiation-dependent expression of connective tissue growth factor and lysyl oxidase messenger ribonucleic acids in rat granulosa cells. *Endocrinolgy* **142,** 1082-1090.

Smits, P. (2001). The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. *Developmental Cell* **1,** 277-290.

Song, J., Aswad, R., Kanaan, R. A., Rico, M. C., Owen, T. A., Barbe, M. F., Safadi, F. F., and Popoff, S. N. (2007). Connective tissue growth factor (CTGF) acts as a downstream mediator of TGF-beta1 to induce mesenchymal cell condensation. *Journal of Cell Physiology* **210,** 298-410.

Stanton, L.-A., Underhill, T. M., and Beier, F. (2003). MAP kinases in chondrocyte differentiation. *Developmental Biology* **263,** 165-175.

Stanton, L. A., Sabari, S., Sampaio, A. V., Underhil, T. M., and Beier, F. (2004). p38 MAP Kinase signalling is required for hypertrophic chondrocyte differentiation. *Biochemical Journal* **378,** 53-62.

Sternlicht, M., and Werb, Z. (2001). How matrix metalloproteinases regulate cell behavior. *Annual Reviews of Cell and Developmental Biology* **17,** 463-516.

Surveyor, G. A., Wilson, A. K., and Brigstock, D. R. (1998). Localization of connective tissue growth factor during the period of embryo implantation in the mouse. *Biology of Reproduction* **59,** 1207-1213.

Takigawa, M. (2003). CTGF/Hcs24 as a multifunctional growth factor for fibroblasts, chondrocytes and vascular endothelial cells. *Drug News and Perspective* **16,** 11-21.

Takigawa, M., Nakanishi, T., Kubota, S., and Nishida, T. (2003). Role of CTGF/Hcs24/ecogenin in skeletal growth control. *Journal of Cell Physiology* **194,** 256-266.

Tan, L., Peng, H., Osaki, M., Choy, B. K., Auron, P. E., Sandell, L. J., and Goldring, M. B. (2003). Egr-1 mediates transcriptional repression of colIIa1 promoter activity by Interleukin-1. *The Journal of Biological Chemistry* **278,** 17688-17700.

Todorovicc, V., Chen, C. C., Hay, N., and Lau, L. F. (2005). The matrix protein CCN1 (CYR61) induces apoptosis in fibroblasts. *Journal of Cell Biology* **171,** 559-568.

Tuan, R. S. (2004). Biology of development and regenerative skeletogenesis. *Clinical Orthopaedics & Related Research* **427,** S105-S117.

Van Beek, J. P., Kennedy, L., Rockel, J. S., Bernier, S. M., and Leask, A. (2006). The induction of CCN2 by TGFbeta1 involves Ets-1. *Arthritis Res Ther.* **8,** R36.

van der Eerden, B. C. J., Karperien, M., and Wit, J. M. (2003). Systemic and local regulation of the growth plate. *Endocrinology Review* **24,** 782-801.

von der Mark, K., Gauss, V., von der Mark, H., and Muller, P. (1977). Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. *Nature* **267,** 531-532.

Wagner, E. F., and Karsenty, G. (2001). Genetic control of skeletal development. *Current Opinion in Genetics & Development* **11,** 527-532.

Wagner, T. (1994). Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell* **79,** 1111-1120.

Wagner, T., Wirth, J., Meyer, J., Zabel, B., and Held, M. (1994). Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell* **79,** 1111-1120.

Watanabe, H., Kimata, K., Line, S., Strong, D., Gao., L., Kozak, C. A., and Yamada, Y. (1994). Mouse cartilage matrix deficiency (cmd) caused by a 7 bp deletion in the aggrecan gene. *Nature Genetics* **7,** 154-158.

Watanabe, H., Yamada, Y., and Kimata, K. (1998). Roles of Aggrecan, a large chondroitin sulfate proteoglycan, in cartilage structure and function. *Journal of Biochemistry* **124,** 687-693.

Wong, M., Kireeva, M., Kolesnikova, T., and Lau, L. (1997). Cyr61, product of a growth factor-inducible immediate-early gene, regulates chondrogenesis in mouse limb bud mesenchymal cells. *Developmental Biology* **192,** 492 - 508.

Woods, A., and Beier, F. (2006). RhoA/ROCK signaling regulates chondrogenesis in a context-dependent manner. *The Journal of Biological Chemistry* **281,** 13134-13140.

Woods, A., Wang, G., and Beier, F. (2005). RhoA/ROCK signaling regulates sox9 expression and actin organization during chondrogenesis. *Journal of Biological Chemistry* **280,** 11626-11634.

Woods, A., Wang, G., Dupuis, H., Shao, Z., and Beier, F. (2007 submitted). Rac1 signaling stimulates n-cadherin expression, mesenchymal condensation and chondrogenesis. *Journal of Cell Biology*.

Wozniak, M. A., Modzelewska, K., Kwong, L., and Keely, P. J. (2004). Focal adhesion regulation of cell behavior. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1692,** 103-119.

Wright, E., Hargrave, M. R., Christiansen, J., Cooper, L., and Kun, J. (1995). The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos. *Nature Genetics* **9,** 15-20.

Yang, F., Tuxhorn, J. A., Ressler, S. J., McAlhany, S. J., Dang, T. D., and Rowley, D. R. (2005). Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis. . *Cancer Res* **65,** 8887-8895.

Yosimichi, G., Kubota, S., Takashi, N., Kondo, S., Yanagita, T., Nakao, K., Takano-Yamamoto, T., and Takigawa, M. (2006). Roles of PKC, PI3K and JNK in multiple transduction of CCN2/CTGF signals in chondrocytes. *Bone* **38,** 853-863.

Yosimichi, G., Nakanishi, T., Nishida, T., Hattori, T., Takano-Yamamoto, T., and Takigawa, M. (2001). CTGF/Hcs24 induces chondrocyte differentiation through a

p38 mitogen-activated protein kinase (p38MAPK), and proliferation through a p44/42 MAPK/extracellular-signal regulated kinase (ERK). *European Journal of Biochemistry* **268,** 6058-6065.

Zanetti, N. C., and Solursh, M. (1984). Induction of chondrogenesis in limb mesenchymal cultures by disruption of the actin cytoskeleton. *Journal of Cellular Biology* **99,** 115-123.

Zelzer, E., and Olsen, B. R. (2003). The genetic basis for skeletal diseases. *Nature* **423,** 343-348.