THE ROLE OF *COL11A1* EXPRESSION DURING CARTILAGE DEVELOPMENT

by

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DEDICATION

To my wife, Michelle and daughter, Leelynn: thank you for understanding my goals and supporting them as if they were your own.

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ABSTRACT

It is currently a major scientific and medical goal to identify and characterize genetic defects and their impact in health and disease. For example, mutations in genes that encode collagen alpha chains can cause skeletal dysplasia and lead to premature degenerative joint disease. Collagen is the main structural protein in the ECM of connective tissues such as the cartilage, joints, ligaments and tendons. Therefore, the goal of this research is to define the impact of the alpha one chain of collagen type XI chain, encoded by the *COL11A1* gene in humans, on chondrocyte behavior during development of the cartilage. We hypothesize that altered expression of *COL11A1* dysregulates chondrocyte behavior during cartilage development by altering β-catenin dependent signaling pathways. To test this hypothesis, we inhibited the expression of the *COL11A1* homolog *col11a1a* in transgenic zebrafish expressing green fluorescence protein in neural crest derived cells and osteoblast. Then, the *col11a1a* deficient zebrafish were imaged by confocal microscopy to analyze the organization of cells contributing to craniofacial development. Inhibiting *col11a1a* expression reduced the size and shape of the developing Meckel's cartilage in zebrafish. These changes occurred because the cells failed to form the hallmark columns that normally promote longitudinal growth. In addition, premature and ectopic mineralization was found to occur in the cartilage tissue of the Meckel's cartilage. Additional investigation using the well-established embryonic mouse cell line ATDC5 led to the identification of changes in post translational modification of proteins which regulate cell behavior. Specifically, the AKT/GSK3β/β-

catenin proteins were modified to increase the activity of the transcription factor TCF/LEF. This activation was coupled to 1) changes in gene expression, 2) decreased cartilage growth, and 3) increased mineralization. In conclusion, the results confirm that loss of *COL11A1* homolog expression in zebrafish (*col11a1a*) and mouse (*Col11a1*) cells leads to severe chondrodysplasia that affects cells behavior and tissue shape. Treatment enhancing COL11A1 protein production in humans may be useful in inhibiting excessive mineralization. On the other hand, inhibition of COL11A1 protein production could be useful for tissue engineers and researchers developing treatments to accelerate the mineralization of cartilage during fracture healing in patients.

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LIST OF ABBREVIATIONS

Abbreviations of genes and proteins use the standard convention. Human gene names are presented italicized and in all caps. Human proteins are in all caps. Zebrafish genes are presented italicized and in all lower case. Zebrafish proteins are not italicized with the first letter capitalized and the remaining letters in lower case. Mouse genes are presented italicized with the first letter capitalized. Mouse proteins are presented in all caps.

μ-CT	Micro-Computed Tomography
ADAM	A Disintegrin and Metalloprotease
AKT	Protein Kinase B
AMO	Antisense Morpholino Oligionucleotide
APC	Adenomatous Polyposis Coli
ATDC5	Cell line derived from the mouse 129 teratocarcinoma AT805
β-TrCP	Beta-transducin repeat containing protein
CAM	Cell Adhesion Molecules
CDH2	Cadherin 2 also known as N-cadherin
Cho	Chondrodystrophic
CK1α	Casein Kinase 1α
COL	Collagen
COL2A1	Collagen type 2 alpha 1 chain
COL11A1	Collagen type 11 alpha 1 chain

COL10A1	Collagen type 10 alpha 1 chain
CNCC	Cranial Neural Crest Cells
DMEM	Dulbecco's Modified Eagle Medium
DPF	Days Post Fertilization
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGFP	Enhanced Green Fluorescent Protein
FGF	Fibroblast Growth Factor
FLI1a	Friend Leukemia Integration Factor 1a
GAG	Glycosaminoglycan
GSK3β	Glycogen Synthase Kinase 3-beta
HA	Hyaluronan
IACUC	Institutional Animal Care and Use Committee
IGF-1	Insulin-Like Growth Factor-1
IHH	Indian Hedgehog
ITS	Insulin, Transferrin, and Selenium
LEF	Lymphoid Enhancer-Binding Factor
LRP	Low Density Lipoprotein Related Proteins
mAb	Monoclonal Antibody
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
Neg	Negative

NCAM	Neural cell adhesion molecule
PCR	Polymerase Chain Reaction
RT-PCR	Real-Time Polymerase Chain Reaction
PI3K	Phosphoinositide 3-Kinase
PKA	Cyclic AMP-Dependent Protein Kinase
PKC	Protein Kinase C
RGD	Arginine Glycine Aspartic Acid sequence
RNAi	Ribonucleic Acid interference
RUNX2	Runt-Related Transcription Factor-2
siRNA	short interfering ribonucleic acid
SOX9	Sry-Related High-Mobilty Group Box-9
Sp7	Specificity protein 7/Osterix
TNC	Tenascin C
Tg	Transgenic
TGF	Transforming Growth Factor
TCF	T-cell specific transcription factor
VCAN	Versican
WNT	Wingless/integrated

CHAPTER ONE: INTRODUCTION

The Extracellular Matrix

Morphogenesis is the process of forming the tissue shape during development. Many factors influence the morphogenesis including autonomous cell behavior, cell-cell interactions, soluble growth factors and the extracellular matrix (ECM). Conceivably, some of the most fascinating influences come directly from the ECM. The ECM is a dynamic network of diverse molecules secreted during tissue development and morphogenesis (Rozario and DeSimone 2010). Molecules found outside the cell and that have formed an insoluble network through crosslinking interactions are typically considered the ECM. The molecules contributing to these molecular networks remain close to the site at which they were secreted by the cell, creating a conserved and predictable environment for the cell during the stages of development and morphogenesis.

Collagens, proteoglycans and glycosaminoglycans, and the non-collagenous glycoproteins are major molecules that contribute to the composition of the ECM (Hynes 2009). Collagens are the most abundant protein in the vertebrate body with at least 28 different collagen types identified (Shoulders and Raines 2009). The collagen alpha chains form trimeric molecules that assemble and form triple helical regions. Collagens are primarily considered structural molecules, providing strength and support for the tissue (Gordon and Hahn 2010). Glycosaminoglycans (GAGs) are linear and unbranched polymers of the disaccharides hexosamine and uronic acid. GAGs are classified by the

composition of their carboxyl, hydroxy and sulfate groups (i.e. chondroitin, dermatan, keratan and heparan sulfates). GAGs, excluding hyaluronan (HA), are covalently linked to core proteins to assemble the proteoglycans. On the other hand, HA links proteoglycans into an essential network critical for establishing the pericellular matrix (Evanko et al. 2007). HA can induce cell behavior through interactions with the cell surface receptor CD44 and provides one example of ECM induced cell signaling (Toole 2004). The non-collagenous glycoproteins include fibronectin, tenascin, and laminin all of which share common structural motifs such as Arg-Gly-Asp (RGD) sequences. The RGD sequences are essential for integrin mediated binding, and therefore provide a means for cell-matrix interactions (Shattil et al. 2010)

Both the individual molecules as well as the architecture of the ECM mediate cell behavior in diverse and complex ways. Cell surface receptors interact with individual structural motifs found within large extracellular molecules such as the collagens (Discher, Mooney, and Zandstra 2009). On the other hand, the matrix environment can both limit or facilitate the interaction of smaller morphogens with the appropriate receptors and co-receptors (Fuerer, Habib, and Nusse 2010). Of course, these interactions never occur in isolation, and the cell must interpret multiple matrix-cell as well as cell-cell interactions at a given time and respond favorably to generate and maintain a normal tissue. Additionally, the cell response itself can cue changes in the ECM that the cell must also respond to, creating a dynamic environment.

Although the molecules of the matrix can be very stable and long lived, changes to the ECM composition occur to facilitate tissue development and morphogenesis (Mouw, Ou, and Weaver 2014). These changes are mediated through the synthesis and

secretion of additional molecules and through the active degradation and replacement of the existing molecules. For example, the secretion of proteases such as matrix metalloproteinase (MMP) and a disintegrin and metalloprotease (ADAM) degrade and disrupt the stable molecular networks (Lu et al. 2011). Changes in gene expression altering protein translation and secretion, contribute to an evolving ECM and cellular behavior.

Cell migration is a fundamental process of development that requires ECM interactions. Focal adhesions are integrin based complexes that connect the cell cytoskeleton with extracellular substrates such as collagens and fibronectin, to generate traction forces and create cell locomotion (Cukierman et al. 2001). Differential compositions of integrin subunits and binding motifs provide directional cues for cellular migration. Additionally, diffusible factors such as morphogens that bind surface receptors can direct cell migration. Once cells reach the site of the future tissue, adhesion and differentiation processes can proceed.

Loosely associated vertebrate cells can form tight associations with each other and the extracellular matrix through cell adhesion molecules (CAMs). Cadherins are calcium dependent cell-cell adhesion molecules that hold cells together during vertebrate development, although they are also expressed in invertebrates. Cadherins are typically single-pass transmembrane glycoproteins that form dimers and connect the actin cytoskeleton of neighboring cells. The expression of cadherins are dynamic and can be considered cyclic during embryonic development to facilitate changes in cell location, providing another morphogenic mechanism (Takeichi 1991). For example, Neural cadherin (N-cadherin or Cdh2) expression can be detected in the developing neural tube

before it is down regulated as neural crest cell migration begins (Taneyhill and Schiffmacher 2017). Then, at new sites of tissue development, where cell adhesion is required to facilitate differentiation and patterning, such as in the developing brain or cartilage, N-cadherin expression reemerges.

Cell fate decisions and differentiation during development is also influenced by the composition of the ECM, although ultimately controlled by gene expression patterns driven by transcription factors. The contribution of the ECM is complicated by the multitude of molecules presented to the cell and apparent compensatory mechanisms that may mask the direct contribution of individual molecules. For example, studies disrupting the expression of matrix molecules through loss of function experiments often cause multiple defects or a spectrum of severity that are difficult to interpret as a direct effect. Additionally, disruption of the ECM can have no effect on cell differentiation, but still cause severe disorganization of the cells within the tissue. The cellular disorganization itself may expose cells to cues that alter cell fate and differentiation pathways disrupting the overall tissue pattern.

Tissue patterning during development is mediated through well conserved secreted signaling molecules such as the transforming growth factor (TGF), fibroblast growth factor (FGF), Wingless/integrated (Wnt) and hedgehog (Hh) families, in combination with the direct cell-cell signaling induced through Notch (Clevers 2006, Goldring, Tsuchimochi, and Ijiri 2006, Swartz et al. 2012, Melrose et al. 2016). The ECM can indirectly influence the availability of these molecules to the required cell surface receptor. For example, matrix molecules may bind and sequester soluble signaling molecules, preventing the ligand-receptor interactions from occurring or

attenuating the interaction. Alternatively, the receptors themselves may be masked from the ligand through direct or indirect ECM interactions. Additionally, the matrix composition alters the charge density and volume of the fluid which mediate concentration gradients and rates of diffusion. These mechanisms indirectly influence cell behavior through modulating the availability of the soluble morphogen but should be considered attributes of the ECM.

Endochondral Ossification

Endochondral ossification is a tightly regulated, multistep process of skeletal formation. Most of the bones in the vertebrate skeleton, such as the vertebrae, ribs and long bones, form through the process of endochondral ossification. Unlike intramembranous ossification, endochondral ossification must be preceded by the formation of cartilage anlagen that serve as templates. The steps involved in endochondral ossification are mesenchymal condensation, chondrogenic differentiation of mesenchymal cells and cartilage formation, primary ossification and secondary ossification (Figure 1) (Kozhemyakina, Lassar, and Zelzer 2015).

Initially, mesenchymal cells migrate to the site of the future bone and commit to the chondrogenic lineage in preparation of chondrogenesis, the formation of cartilage. The cells contributing to the vertebrate skeleton arise from multiple lineages, depending on the skeletal element. For example, cranial neural crest cells arise from the anterior-dorsal aspect of the closing neural tube, delaminate and migrate in a distinct stream to populate the ventral pharyngeal arches, which give rise to the craniofacial skeleton. On the other hand, cells contributing to the skull, middle ear, ribs and vertebrae of the axial skeleton arise from somites, or paraxial mesoderm, while the cells of the appendicular

skeleton arise from lateral plate mesoderm (Chai et al. 2000, Kague et al. 2012, Berendsen and Olsen 2015). Regardless of the initial source of the cells, they must migrate to the future site of the bone to initiate mesenchymal condensation and subsequently, chondrogenesis.

After successful migration, mesenchymal cells form high density cellular condensations and upregulate the expression of versican, tenascin, syndecan, N-CAM and N-cadherin. Cells surrounding the condensation form the perichondrium and establish the cartilage border. The perichondrium prevents additional mesenchymal cells from being recruited to the condensation. Proliferative cells in the center of the condensation will differentiate into cartilage forming chondrocytes (Hall and Miyake 2000).

Chondrocytes secrete a cartilage matrix that contains collagens, proteoglycans, hyaluronan and noncollagenous glycoproteins. Collagens type II, IX, and XI are highly expressed in the developing cartilage, as are sulfated proteoglycans. As chondrogenesis proceeds, chondrocytes mature and develop spatially recognizable zones with phenotypically unique characteristics that define the growth plate (Goldring et al. 2006, Berendsen and Olsen 2015) (Figure 2). Chondrocyte hypertrophy and matrix mineralization occurs as cells cease proliferation, increase in cell volume and differentiate into hypertrophic chondrocytes. These cells express collagen type X followed by increased matrix metalloproteinase-13 (MMP13) and alkaline phosphatase (Kozhemyakina et al. 2015). Neighboring perichondrial mesenchymal cells differentiate and form osteoblasts of the bone collar. Vascular invasion and ossification occurs in the final stage as hypertrophic chondrocytes undergo apoptosis. The vascularization is

necessary to recruit chondroclast that resorb the ECM around the hypertrophic cells and osteoblasts that secrete new bone and thus creating the primary ossification center of the bone.

The growth plate is a specialized tissue that enables the longitudinal elongation of the long bones and persists until the end of adolescence (Melrose et al. 2016). Chondrocytes in the growth plate undergo spatially organized differentiation and maturation processes. The differentiation stage is organized by the transcriptional activity regulated by tissue specific growth factors. Chondrocytes in the resting zone are dispersed within the extracellular matrix and are isolated from the ossification front. These cells are a pool of cells that contribute to the proliferative zone chondrocytes. Chondrocytes in the proliferative zone of the growth plate are flat and orientated with the long axis perpendicular to the plane of longitudinal growth. Clonal expansion of the cells generates a columnar structure with a stack like appearance (Figure 2). The cartilage producing cells in the upper proliferative zone synthesize and secrete abundant collagen type II and aggrecan. Chondrocytes in the prehypertrophic zone begin to increase in size and express Indian hedgehog (Ihh) and parathyroid hormone-related peptide receptors (Vortkamp et al. 1996). Cells in the hypertrophic zone are non-proliferative and secrete collagen type X instead of collagen type II. Interestingly, the fate of the hypertrophic chondrocyte is divided by at least potential outlooks. The first is that hypertrophic cells die by apoptosis and are subsequently replaced by osteoblast, and the second suggesting hypertrophic chondrocytes transdifferentiate directly into osteoblast (Hill et al. 2005, Yang et al. 2014).

Regulation of Chondrogenesis

The Sry-related high-mobilty group box (Sox) transcription factor, SOX9, has been identified as the master regulator for chondrogenic commitment and differentiation of mesenchymal cells (de Crombrugghe, Lefebvre, and Nakashima 2001, Lefebvre et al. 2007). Sox9 transcripts are expressed in both chondroprogenitor cells and differentiated chondrocytes and declines in hypertrophic chondrocytes (Lefebvre and Dvir-Ginzberg 2017). Sox9 binds the *Col2a1*, *Col11a2* and aggrecan promoters, making it essential for cartilage formation (Bell et al. 1997, Deng, Huang, and Yuan 2016). Interestingly, loss of SOX9 before mesenchymal condensation prevented the aggregation of mesenchymal cells and the loss of subsequent skeletal formation. On the other hand, deletion of SOX9 after mesenchymal condensation caused severe chondrodysplasia with decreased proliferation and maturation. In addition to the effects on proliferation, loss of SOX9 causes disorganization of cells in the growth plate. The expression of SOX9 is required to prevent proliferating chondrocytes from transitioning into hypertrophic chondrocytes (Akiyama et al. 2002). Furthermore, overexpression of SOX9 in chondrocytes decreased cell proliferation through interactions with the canonical Wnt signaling pathway (Akiyama 2004, Topol et al. 2009).

The Wnt signaling pathway plays conserved roles in regulating fundamental cell behavior during tissue development and morphogenesis in multiple tissue, including skeletal tissues (Clevers 2006). During skeletal development, Wnt influences the cell fate decisions of mesenchymal stem cells by directing them toward either the chondrocyte or osteocyte lineage (Day et al. 2005, Hill et al. 2005, Reinhold et al. 2006) High β-catenin

levels inhibit Sox9 expression and activity while promoting runt-related transcription factor-2 (Runx2) expression, therefore promoting differentiation toward the osteoblast lineage. Additionally, activation of T-cell specific transcription factor/lymphoid enhancer-binding factor (TCF/LEF) in chondrocytes accelerates hypertrophic differentiation and stimulates ectopic mineralization (Kitagaki et al. 2003). On the other hand, low Wnt/β-catenin signaling enhances *Sox9* expression and promotes the chondrocyte fate. Therefore, the balance of Wnt/β-catenin is an important molecular switch between chondrocyte and osteoblast fate choice in mesenchymal cells contributing to skeletal development.

Wnt molecules can act through at least three unique signaling pathways leading to distinct cellular responses: canonical Wnt signaling, noncanonical planar cell polarity, and Wnt/calcium dependent pathways. Canonical pathways refer to the involvement of β-catenin in the signal transduction. In the canonical Wnt signaling pathway, the absence of Wnt binding to the seven pass transmembrane surface receptor frizzled, promotes β-catenin phosphorylation at the amino terminal domain by glycogen synthase kinase (GSK) 3β and casein kinase 1α (CK1α) in a complex with the scaffolding protein axin and adenomatous polyposis coli (APC). This phosphorylation event on β-catenin targets it for subsequent ubiquitination and proteasome mediated degradation (Aberle et al. 1997). On the other hand, binding of Wnt to the frizzled receptor and co-receptor LDL related proteins (LRP) 5/6, leads to phosphorylation of GSK3β and thereby uniquely inhibiting GSK3β's kinase activity, and stabilizing β-catenin levels in the cytoplasm. Stable β-catenin accumulates and translocates to the nucleus where it interacts with T-cell

specific transcription factor/lymphoid enhancer-binding factor (TCF/LEF) to drive Wnt target gene transcription.

GSK3β is a serine/threonine kinase with multiple substrates including β-catenin (Doble and Woodgett 2003, Saegusa et al. 2009, Umschweif et al. 2013). Uniquely, GSK3β is an active kinase under normal conditions and is inhibited when phosphorylated at serine-9. Phosphorylation is mediated by several upstream kinases including phosphoinositide 3-kinase (PI3K)/AKT, cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC). As mentioned above, GSK3β mediated phosphorylation of β-catenin leads to the ubiquitination and proteasome-mediated degradation, therefore decreasing the activity of TCF/LEF transcription factors (Clevers 2006). Therefore, GSK3β is an important mediator of chondrocyte behavior and cell fate decisions through the regulation of β-catenin degradation and stabilization.

Collagen α1(XI)

Collagen type XI is a heterotrimeric protein containing three unique chains, α1(XI), α2(XI) and α3(XI) (Mendler et al. 1989, Fernandes et al. 2007). The collagen α1(XI) chain is translated as a procollagen alpha chain that will undergo future modifications through proteolytic cleavage of the amino and carboxyl terminal ends. The rate of proteolytic cleavage at the amino terminal domain is influenced by the variable region located between the amino terminal domain and the major triple helix (Medeck et al. 2003, Oxford et al. 2004).

The *COL11A1* gene contains 67 exons in which exons 6 through 9 (also referred to as exons 6a through 8) are subject to alternative splicing (Davies et al. 1998). Alternative splicing of *COL11A1* mRNA leads to complex combinations of exons 6A,

6B, 7 and 8 which give rise to variants of the variable region of the collagen α1(XI) protein. Exons 2-5 are expressed in all isoforms and code for the conserved amino propeptide. The variable region is included in the amino terminal domain but is not part of the proteolytically cleaved amino propeptide. Therefore, the variable region is retained in the mature collagen fibril and contributes to diverse isoforms of the collagen type α1(XI) chain. The expression of the isoforms is regulated during embryonic development and are tissue specific (Davies et al. 1998, Morris et al. 2000). Furthermore, the tissue specific expression of collagen type α1(XI) chain isoforms is temporally and spatially controlled by cis-acting elements (Chen et al. 2001).

Collagen type XI is a minor fibrillar collagen expressed in multiple tissues including brain, placenta, skeletal muscle, heart, lung, the vitreous bone and cartilage (Yoshioka et al. 1995). Collagen type α1(XI) chain is first detected during embryonic development in the developing limb and craniofacial cartilage. Additionally accumulating evidence has asserted that collagen type α1(XI) chain is a cancer biomarker (Bowen et al. 2008, García-Pravia et al. 2013, Wu et al. 2015). The tissue distribution has been investigated and confirmed in mouse, rat, chick and most recently, zebrafish models (Li 1995, Yoshioka et al. 1995, Fang et al. 2010).

The amino terminal domain of collagen α1(XI) chain remains exposed on the surface of the fibril for an extended amount of time (Morris and Bachinger 1987). The rate at which the amino propeptide is cleaved is influenced or potentially controlled, by the isoform present, which is dictated by the variable region primary sequence (Medeck et al. 2003, Oxford et al. 2004). The diameter of mature collagen fibrils in cartilage has been linked to collagen type XI expression. The amino terminal domain acts to sterically

hinder the additional collagen fibrils in the cartilage because the amino terminal domain remains extended at the collagen surface (Gregory et al. 2000, Holmes and Kadler 2006). The surface location of collagen α1 (XI) amino terminal domain enables the possibility for multiple interactions. Proteomic analysis has identified multiple molecules that show an affinity for the amino terminal domain. Several collagens (II, IX, XI, XII and XIV), proteoglycans biglycan, perlecan, chondroadherin, fibromodulin, epiphycan, thrombospondin 1 and 5, and matrilin 1 and 3, were found to interact with the amino terminal domain either directly or indirectly (Oxford et al. 2004).

Mutations in human *COL11A1* gene are the cause of Fibrochondrogenesis a neonatally lethal chondrodysplasia (Lazzaroni-Fossati 1978, Li 1995, Tompson et al. 2010). Heterozygous mutations in *COL11A1* cause milder forms of chondrodysplasia, Stickler's syndrome type II and Marshall's syndrome (Vijzelaar et al. 2013, Acke et al. 2014). *COL11A1* associated chondrodysplasia present with long bone and rib shortening with flared metaphysis or cupping, vertebral malformation, flattened midface, reduced jaw protrusion and sensorineural defects affecting hearing and vision (Whitley et al. 1984, Akawi, Al-Gazali, and Ali 2012, Hufnagel et al. 2014).

The chondrodystrophic mouse (cho) demonstrates the importance of *Col11a1* expression in development. The autosomal recessive chondrodysplasia is caused by a mutation generating a reading frame shift in the *Col11a1* gene that produces a premature stop codon (Li 1995). The collagen diameter in the cho mouse is abnormally thick, highlighting the ultrastructural influence of collagen type XI on fibril diameter (Fernandes et al. 2007). Recent analysis of the cho mouse demonstrated the importance of *Col11a1* in regulating mineralization of the bone collar and trabecular bone (Hafez et

al. 2015). These developmental defects irrefutably demonstrate the necessity of *COL11A1* expression in skeletal development.

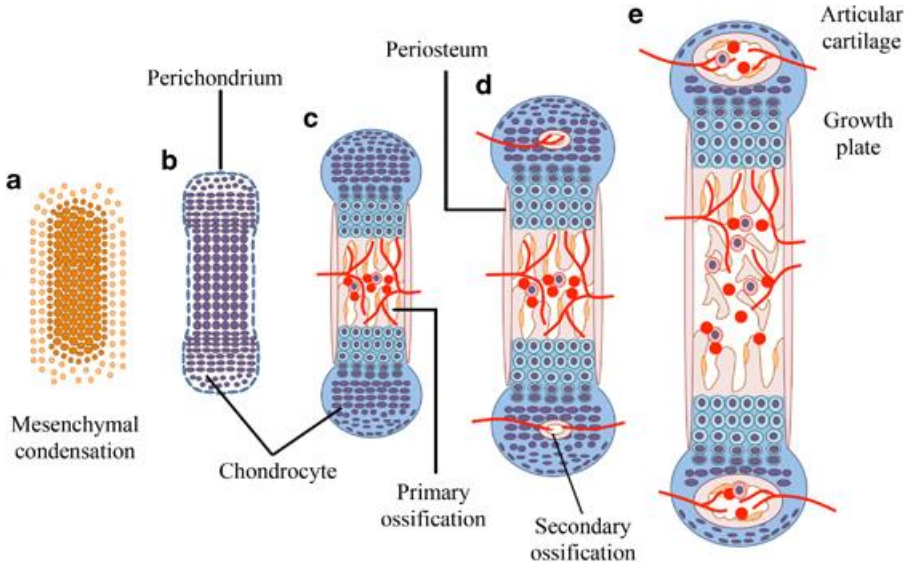

Figure 1.1. Schematic of cartilage development and endochondral ossification. Source: (Usami et al. 2016) The process of endochondral ossification requires multiple steps to successfully develop the cartilage and bone. (a) Mesenchymal cells first form a condensation at the future site of the cartilage. The cells of the condensation contribute to cells of the chondrocyte and osteoblast lineage. Chondrocyte differentiation and matrix production occurs in the mesenchymal condensation. (b) The cells of the condensation continue to grow, differentiation and organize the cartilage template, creating the growth plate. Perichondral cells border the cartilage template and prevent the addition of new mesenchymal cells. (c) Cells in the center undergo hypertrophy and create the primary ossification center that is occupied by microvessels. (d) Vessels invade at the epiphyses, and generate a secondary ossification center that contains osteoblast. (e) The growth plate continues to advance and promote longitudinal growth. The articular cartilage is formed at the superficial surface providing resilience in load-bearing situations and a smooth surface for the joint.

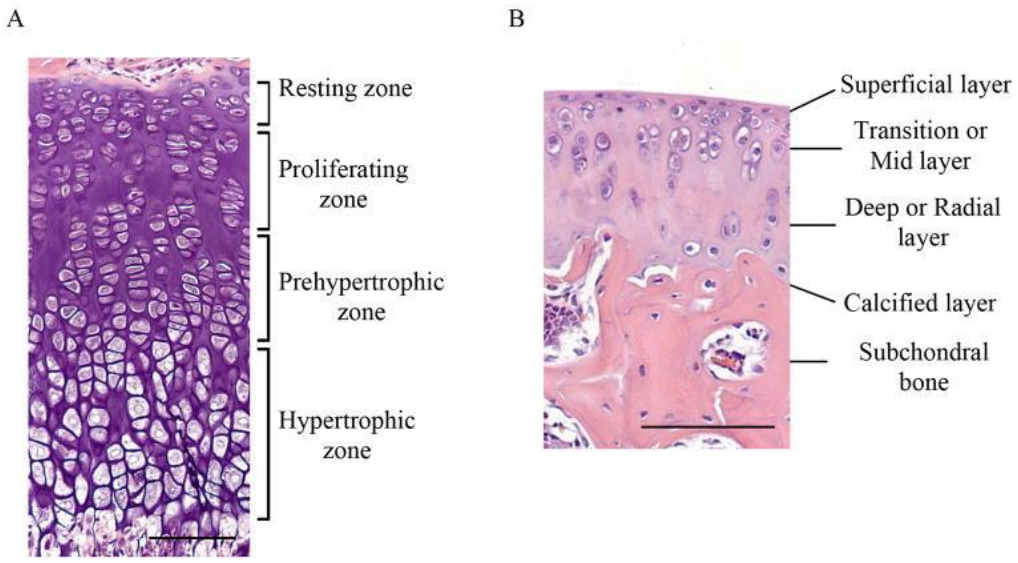

Figure 1.2. Organization of the growth plate and articular cartilage Source:(Usami et al. 2016). (A) The growth plate contains distinct zones with morphological differences in each: The resting zone, proliferating zone, prehypertrophic zone, and hypertrophic zone. (B) The articular cartilage also contains distinct zones: Superficial layer, mid layer, deep layer, calcified layer.

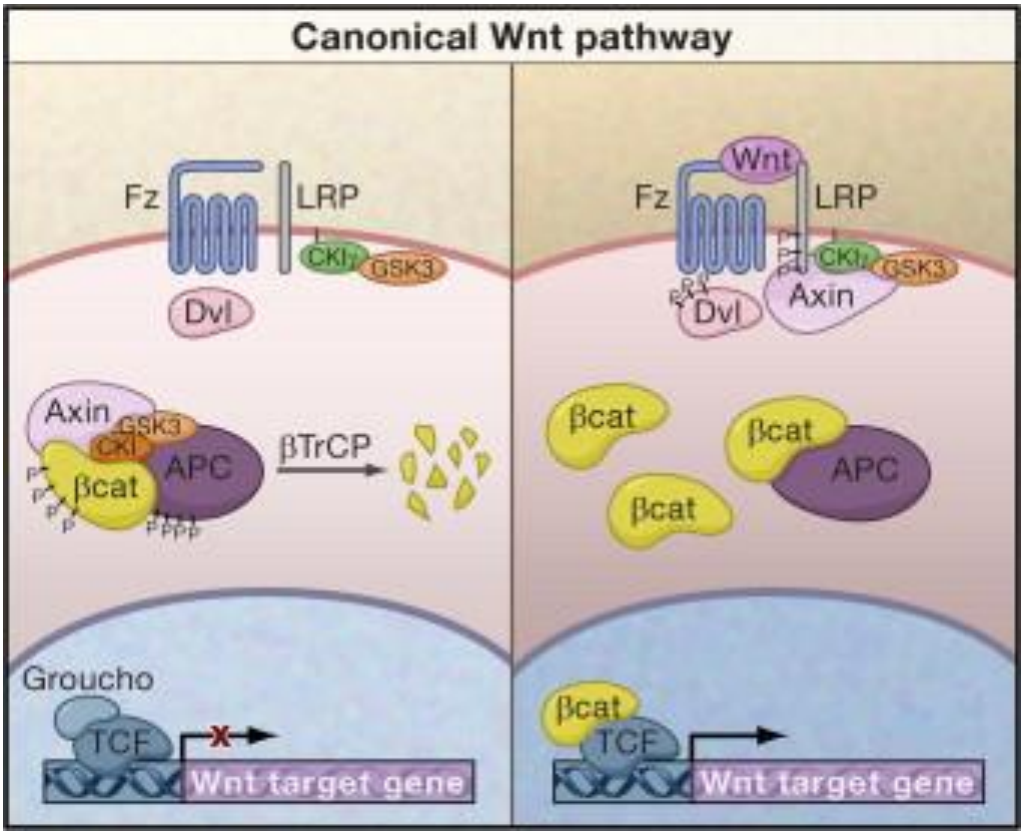

Figure 1.3. Canonical Wnt signaling pathway. Source: (Clevers 2006). In the absence of the Wnt ligand, CK1 and GSK3 phosphorylate β-catenin. Phosphorylated β-catenin is recognized by β-TrCP of the E3 ubiquitin ligase complex, ubiquitinated, and degraded by the proteasome. Therefore, in the absence of Wnt ligand, cytoplasmic β-catenin does not accumulate or enter the nucleus to activate Wnt target genes. In the presence of Wnt ligand, the Frizzled/ LRP complex activates the canonical signaling pathway. Axin is recruited to the membrane and β-catenin escapes phosphorylation and subsequent ubiquitination and degradation. Therefore, β-catenin accumulates in the cytoplasm and translocates to the nucleus. In the nucleus, β-catenin interacts with TCF/LEF and promotes the expression of Wnt target genes.

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CHAPTER TWO: ZEBRAFISH *COL11A1A* REGULATES CRANIOFACIAL MORPHOGENESIS DURING DEVELOPMENT

Abstract

Expression of the *col11a1a* gene is essential for normal skeletal development in both cartilage and bone. Although *col11a1a* loss of function mutations cause abnormal growth plate organization, the effect on craniofacial development has not been well studied. To further understand the effect of *col11a1a* gene function in craniofacial development, we analyzed the developing craniofacial skeleton in zebrafish using gene knockdown by injection of antisense morpholino oligonucleotide (AMO). We demonstrate that *col11a1a* knockdown impairs organization of the craniofacial cartilage and bone forming cells in and adjacent to the Meckel's cartilage. These results are consistent with reported cell behavior in the growth plate and suggest a conserved role for *col11a1a* expression in growth plate and jaw.

Introduction

The craniofacial skeleton is derived from cranial neural crest cells (CNCC) that migrate into the pharyngeal arches and establish a cartilage anlagen (Kimmel et al. 1998, Kague et al. 2012, Berendsen and Olsen 2015). The cranial neural crest cells of the seven pharyngeal arches occupy the appropriate segmented arch through predetermined migratory streams during craniofacial development (Schilling and Kimmel 1994). The first of seven pharyngeal arches give rise to the Meckel's cartilage which functions as the cartilage template of the mandible, or lower jaw (Mork and Crump 2015). Ossification of

the mandible occurs through a combination of intramembranous ossification at the mediolateral dentary and endochondral ossification at the distal-most cartilage (Eames et al. 2013).

During development, most of the skeleton is composed of cartilage, a firm yet flexible connective tissue composed primarily of chondrocytes embedded in a collagen and proteoglycan rich matrix. Collagens are a large family of extracellular molecules of at least 28 members found mostly in connective tissues (Carter and Raggio 2009, Ricard-Blum 2011). The functions of collagens are diverse and have been linked to cell adhesion, migration, structure and cell signaling during skeletal morphogenesis (Kadler et al. 2007).

The collagen type XI alpha 1 gene, *COL11A1*, is expressed by chondrocytes in the developing fetal cartilage, the growth plate, and articular cartilage. The translated protein is incorporated into collagen type XI fibrils and primarily localized to thin fibrils of the pericellular matrix of chondrocytes. Collagen type XI heterotrimeric molecules are composed of two unique alpha chains, α1(XI), α2(XI), as well as the α3(XI) chain which is identical to collagen α1(II)B (Poole, Flint, and Beaumont 1987, Eyre 2002). Autosomal recessive null mutations in *COL11A1* cause the lethal chondrodysplasia, Fibrochondrogenesis in humans (Tompson et al. 2010). Mutations that reduce or modify *COL11A1* gene expression cause milder forms of chondrodysplasia in Stickler's and Marshall's syndrome (Vijzelaar et al. 2013, Acke et al. 2014). Fibrochondrogenesis is clinically characterized by long bone and rib shortening with flared metaphysis or cupping, vertebral malformation, flattened midface, reduced jaw protrusion and

sensorineural defects affecting hearing and vision (Whitley et al. 1984, Akawi, Al-Gazali, and Ali 2012, Hufnagel et al. 2014).

Ultrastructural and biochemical analysis of collagen type XI alpha one chain molecules have provided valuable information regarding structure, location and binding interactions (Warner et al. 2006, McDougal et al. 2011, Brown et al. 2011, Ryan et al. 2015). In addition, clinical samples and animal models have indicated a clear link between chondrodysplasia, chain misassembly and *COL11A1* gene defects (Fernandes et al. 2007). This study seeks to elucidate the impact that zebrafish *col11a1a* gene defects have on tissue morphogenesis and cell organization during skeletal development. Evidence that *COL11A1* gene defects disrupt the growth plate and long bone development in human patients led us to hypothesize that zebrafish *col11a1a* is essential for craniofacial development. Based on this hypothesis, we predict that *col11a1a* gene defects will disrupt chondrocyte organization and subsequent mineralization in craniofacial development.

Methods

Fish Care and Transgenic Lines

Vertebrate animal use was approved by the Institutional Animal Care and Use Committee (IACUC Protocol 006-AC15-011)). Zebrafish embryos were obtained from ZIRC (Eugene, OR). The following lines were used in these studies; AB, Tg(sp7:EGFP) (DeLaurier et al. 2010) and Tg(Fli1a:EGFP) (Lawson and Weinstein 2002).

Morpholino Injections

Gene-specific and standard control antisense morpholino oligonucleotides (AMO) were purchased from Genetools, LLC (Philomath, OR). The *col11a1a* morpholino

targeted the translational start site with the following sequence: 5'-GGGACCACCTTGGCCTCTCCATGGT-3'. Morpholinos were diluted in water and 0.05% phenol red. The morpholinos were injected at a volume of 2 nL and at a concentration of 3 ng/nL.

Cartilage and Bone Staining

Five days post fertilization (dpf) zebrafish embryos were fixed overnight with 4% paraformaldehyde in phosphate buffered saline (PBS). The samples were washed with PBS with 0.1% Tween-20 (PBT) then dehydrated through a 30:50:90% series of ethanol followed by overnight incubation in Alcian blue solution of 0.1mg/mL in 75:25% ethanol:acetic acid overnight with rocking. Samples were rehydrated through 70:50:30% ethanol series and bleached in 1% hydrogen peroxide and monitored until the eyes were clear (2-3 hours).

For vital imaging of calcification, live zebrafish were incubated in 30 mL of the zebrafish housing system water with 200 µL of 0.5% Alizarin Red (final concentration 0.003%) for 3 hour. Fish were subsequently rinsed in zebrafish housing system water prior to imaging.

Imaging

Transgenic zebrafish were anesthetized in 0.016% tricaine methanesulfonate (MS-222) in system water prior to mounting in 0.6% (w/v) low melting point agarose containing MS-222. Confocal imaging was performed using a Zeiss LSM 510 Meta inverted laser scanning microscope. Alizarin Red vital stained images were collected by excitation between 530-560 nm and by monitoring emission at 580 nm. GFP transgene-expressing zebrafish images were generated by excitation at 488 and monitoring emission

at 509 nm. Wide field fluorescence microscopy was also performed using the EVOS Fl imaging system (ThermoFisher Scientific). Alcian blue stained zebrafish were mounted in glycerol and imaged using the Olympus BX53 light microscope.

Results

Knockdown of *Col11a1a* Leads To Skeletal Deformities In Zebrafish

Fibrochondrogenesis is clinically distinguished by wide long-bone metaphyses, abnormally pear-shaped vertebral bodies, flattened facial appearance, micrognathia and abnormal curvature of the spine. In addition, ultrastructural changes in the growth plate cartilage and chondrocyte dysplasia are observed at the microscopic level (Lazzaroni-Fossati 1978, Whitley et al. 1984, Tompson et al. 2010). We compared morphant and wild type zebrafish at 5 dpf. By this time most of the cartilage elements have developed and can be stained with Alcian blue to detect the cartilaginous ventral pharyngeal skeleton. *Col11a1a* antisense morpholino oligonucleotide (AMO) induced a chondrodysplasia that recapitulates Fibrochondrogenesis and morphants were distinguishable from stage-matched controls (Figure 1). The body axis of the morphants were curved ventrally, the midface had a flattened appearance and the jaw size was reduced. In addition to the skeletal defects, heart edema was observed (Figure 1B). Otoliths were abnormal and fused when compared to the controls (Figure 1D). Staining GAGs of the cartilage with Alcian blue showed the knockdown of *col11a1a* caused the development of cartilage derived from the pharyngeal arches to be deformed (Figure 1E). Specifically, the Meckel's cartilage of the control zebrafish was completely formed and extended to enable lower jaw articulation, indicating appropriate development had occurred. In contrast, Alcian blue staining of the morphants revealed that the Meckel's

cartilage was smaller compared to zebrafish injected with the control AMO and compared at stage matched times. Further examination of the Meckel's cartilage showed that not only does it fail to protrude, but the *overall* shape is altered. The lower jaw normally extends anteriorly and forms a single stack of cells by 5 dpf in normal zebrafish development (Piotrowski et al. 1996, Schilling et al. 1996). Inhibiting *col11a1a* expression by injection of AMO disrupted morphogenesis of the developing craniofacial skeleton, causing the eyes to appear that they are bulging. Also, the zebrafish otoliths became abnormally positioned in the otic vesicle as a condition of the severe dysplasia. Additionally, the 2nd arch failed to form anteriorly pointing segments at the midline of the zebrafish. The remaining posterior arches, ceratobranchial 1-5, were weakly stained and lacked the proper structure.

Cranial Neural Crest Derived Cells Form Segmented Pharyngeal Arches with Abnormal Shape

The cells of the pharyngeal arches were visualized by laser scanning confocal microscopy using the transgenic Fli1a:EGFP reporter (Figure 2). Fli1a is expressed in cranial neural crest derived mesenchymal cells of the developing cartilage as well as the aortic arches and endothelial cells of the vasculature. Enhanced green fluorescence protein (EGFP) expression in the neural crest derived cells of the developing pharyngeal skeletal can be detected until at least 7dpf (Lawson and Weinstein 2002). The EGFP was imaged 72-hours post fertilization to determine if the cells of the pharyngeal arches could organize in a manner sufficient to establish the cartilage template of the future craniofacial skeleton. We identified the neural crest derived cell pattern in the morphants and compared it to the control embryos (Figure 2). We predicted that the cells of the

pharyngeal arches would form condensations but not migrate and organize into ordered columns representing the normal cellular organization of the developing cartilage. Consistent with this prediction, the neural crest derived cells formed abnormally shaped pharyngeal arches in the morphants. We determined that the CNCCs occupied segmented pharyngeal arches, but subsequent patterning consistent with cartilage development failed to occur. Notably, the 1st and 2nd arches were closer to each other and neither arch extended anteriorly as seen in normal development (Figure 2B). Each pharyngeal arch was present and segmented from each other, and cell populations that could potentially contribute to cartilage development were present. The overall pattern at this stage was not consistent with normal cartilage development and patterning.

The lateral view of the normal zebrafish was used to visualize the extension of the Meckel's cartilage at this stage of development. Meckel's cartilage supports lower jaw formation by forming a template for mineralization. The morphant jaw failed to extend anteriorly to the extent that the control jaw, and cells of the palatoquadrate did not organize into stacked cells (Figure 2B). The result of these two events resulted in both a smaller and wider Meckel's cartilage, and formed kinks in the palatoquadrate where cell alignment normally contributes to generating a protruding structure. A ventral view of the ceratobranchial arches showed that they were smaller and cellular organization was distinguishable from the control zebrafish. The ceratobranchial arches did not extend anteriorly, but instead pointed toward the midline (Figure 2D).

Cells in the Meckel's Cartilage Fail To Converge And Extend In Col11a1 Morphants

We analyzed the cell organization of the Meckel's cartilage in 5 dpf zebrafish to determine if the observed craniofacial defects in *col11a1a* chondrodystrophies is correlated with unaligned cells. Additionally, we analyzed the effect on mineralization of the cartilage template. At this stage in development, the anterior region of the jaw cartilage should be fully developed. We observed that the organization of the cells in the morphant was distinct. The cells of the Meckel's cartilage form a single file column under normal conditions (Figure 3A). We observed both cartilage forming cells, which form the single column, and perichondral bone forming cells lining the Meckel's cartilage. The cartilage lining cells produced a mineralized matrix that was detectable with Alizarin Red in controls (Figure 3B and C). Mineralization along the normal jaw is intramembranous and contributes to the zebrafish dentary and maxilla. In contrast, cells in the morphant failed to organize into a single stack and the two-bilateral rod-like arches did not extend anteriorly (Figure 3D). Additionally, mineralization of the dentary was not observed, but instead mineralization of the cartilage where the two rods of the Meckel's cartilage meet at the anterior-most region was detected (Figure 3 E and F). This is a site presumed to mineralize through endochondral ossification (Eames et al. 2012). We found that the cells contributing to the Meckel's cartilage failed to form single file columns and that mineralization pattern was altered.

Bone Forming Cells Form Condensations at the Morphant Meckel's Cartilage

Sp7/osterix expression in bone forming cells has been reported in mice and zebrafish models (Nakashima et al. 2002, DeLaurier et al. 2010). To determine if the

bone forming cells lining the Meckel's cartilage were displaced by the abnormal template shape, we tracked the location of the EGFP positive cells in the transgenic sp7:EGFP zebrafish. We predicted that the osteocytes would not be able to organize and form a detectable mineralized jaw. At 5 dpf, the Meckel's cartilage defect in the morphant jaw was detectable in the transgenic morphants while the control zebrafish had a protruding jaw and normal midface (Figure 4A and B). Sp7:EGFP positive cells were detected by fluorescence microscopy adjacent to the developing Meckel's cartilage. The sp7:EGFP positive cells in the control zebrafish were detected lining the cartilage and the mineralization pattern followed the expression pattern of sp7:EGFP positive cells (Figure 4A). In the developing morphant jaw, sp7:EGFP cells were located in the mediolateral cartilage rods, but the cells did not line the cartilage as did the perichondral cells of the control. Instead, the cells formed small condensations adjacent to the cartilage and did not produced detectable mineralization at this time point (Figure 4D). Although sp7:EGFP cells were not detected at the distal tip of the cartilage where the cartilage rods meet, mineralization was detectable, suggesting accelerated endochondral ossification, potentially by increased calcifying chondrocytes.

Discussion

Collagen expression is linked to a large and diverse group of skeletal developmental diseases (Jobling et al. 2014, Deng, Huang, and Yuan 2016). Endochondral bone formation begins when mesenchymal cell condensations form and initiate production of the cartilage template. The cartilage increases in size and develops shape and structure through cell maturation and extracellular matrix production (Kronenberg 2003). While *col11a1a* has been linked to chondrodystrophies, the influence

of Col11a1a protein on cell behavior and organization remains unclear. Determining how a Col11a1a-deficient matrix may impact cartilage and bone morphogenesis through the spatial organization and behavior of cells is an important step in understanding skeletal development as well as degenerative diseases.

In this study, we analyzed the spatial arrangement of cells in zebrafish during craniofacial development after *col11a1a* knockdown by AMO injection. Through fluorescence microscopy and tissue staining, we determined that *col11a1a* deficiency disrupts the morphogenesis of pharyngeal arch derived cartilage and bone by preventing the normal arrangement of cells.

Collagen is the major component of the cartilage extracellular matrix secreted by chondrocytes. Collagen type XI plays a structural role in the cartilage through many interaction including nucleating and limiting the diameter of collagen type II fibrils and interacting with non-collagenous molecules (Wu and Eyre 1995, Brown et al. 2011). A previous study demonstrated severe changes to the extracellular matrix and collagen network when *col11a1a* AMO were injected into zebrafish (Baas et al. 2009). *Col11a1* mutations in the chondrodysplasia (cho) mouse also showed disorder chondrocytes and collagen fibers in the growth plate (Li et al. 1995, Fernandes et al. 2007). Micro-computed tomography (μ-CT) analysis of the same mouse model revealed alterations in the mineralization of the developing long bones, ribs, vertebrae and skull (Hafez et al. 2015). These reports are consistent with our findings indicating *col11a1a* is required for normal endochondral ossification in zebrafish.

The first pharyngeal arch contributes to the Meckel's cartilage and the bones of the inner ear (Schilling and Kimmel 1994, Chai et al. 2000, Kague et al. 2012). In

col11a1a morphants, the lower jaw was affected as well as the otoliths of the developing ear. Although the otoliths are present the size and location of the otoliths are altered. Previous research has identified collagen gene expression, specifically *col2a1* and *col11a1a* in the developing zebrafish ear (Fang et al. 2010; Nemoto et al. 2008) This is consistent with reported craniofacial and hearing defects that occur in Fibrochondrogenesis as well as Stickler's syndrome patients (Acke et al. 2012, Akawi et al. 2012).

These data support our conclusion that the cells in the craniofacial skeleton are subject to similar mechanisms as the cells of the long bone growth plate and that the absence of *col11a1a* impacts the cellular arrangement and alters mineralization. Based on these findings, we propose; 1) the loss of Col11a1a protein prevents cell-matrix interactions that permit cell intercalation and; 2) loss of Col11a1a protein prematurely promotes cartilage matrix mineralization. Testing these hypotheses and determining the molecular mechanisms involved will be the subject of future studies.

This study was limited to analyzing the overall shape of tissues, arrangement of cells that contribute to skeletal morphogenesis and the accumulation of proteoglycans and presence of mineralization. No effort was made in the current study to determine the gene or protein expression in the cell populations. It is important to note that chondrodystrophies linked to *COL11A1* present as a spectrum of disorders, such as Stickler's syndrome (Stevenson et al. 2012). Variations in the morphant phenotype and lethality were observed and therefore make it challenging to generalize broadly about the effect of *COL11A1* deficiencies. Because zebrafish *col11a1a* is expressed in many tissues, embryonic lethality could be a possible outcome.

Future work will aim to determine the molecular phenotype of the cartilage and bone cells. Subsequently, should abnormalities in cell identity be found, the mechanisms regulating the cell differentiation and fate should be further investigated. For example, is chondrocyte differentiation affected by the absence of co11a1a? Are the perichondral, bone lining cells differentiating properly? Is hypertrophic differentiation accelerated? Does transdifferentiation occur from hypertrophic chondrocytes to osteoblast? In addition to the cell identity, future studies will investigate why the cells fail to organize properly. It is possible that planar cell polarity programs are disrupted in *col11a1a* chondrodystrophies. The role of Col11a1a protein in cell signaling pathways that mediate planar cell polarity will be investigated in future studies. Integrin-matrix interactions may also be inhibited, based on the reported findings of integrin β1 knockdown (Aszodi et al. 2003).

In conclusion, loss of *col11a1a* gene expression not only affects the extracellular matrix composition and organization, but also has profound consequences for the spatial organization of cells during development of the craniofacial skeleton. Mechanisms involving Col11a1a protein during development described here may be conserved in other examples of tissue development and degeneration, such as cancer tumor growth (Lee, Kim and Kim 2012, Wu et al. 2015) and osteoarthritis (Xu et al. 2003, Rodriguez et al. 2004) Therefore, understanding the impact of *col11a1a* gene expression on cell behavior may contribute to the development of therapies that can target fundamental cell mechanisms of growth, differentiation, and homeostasis.

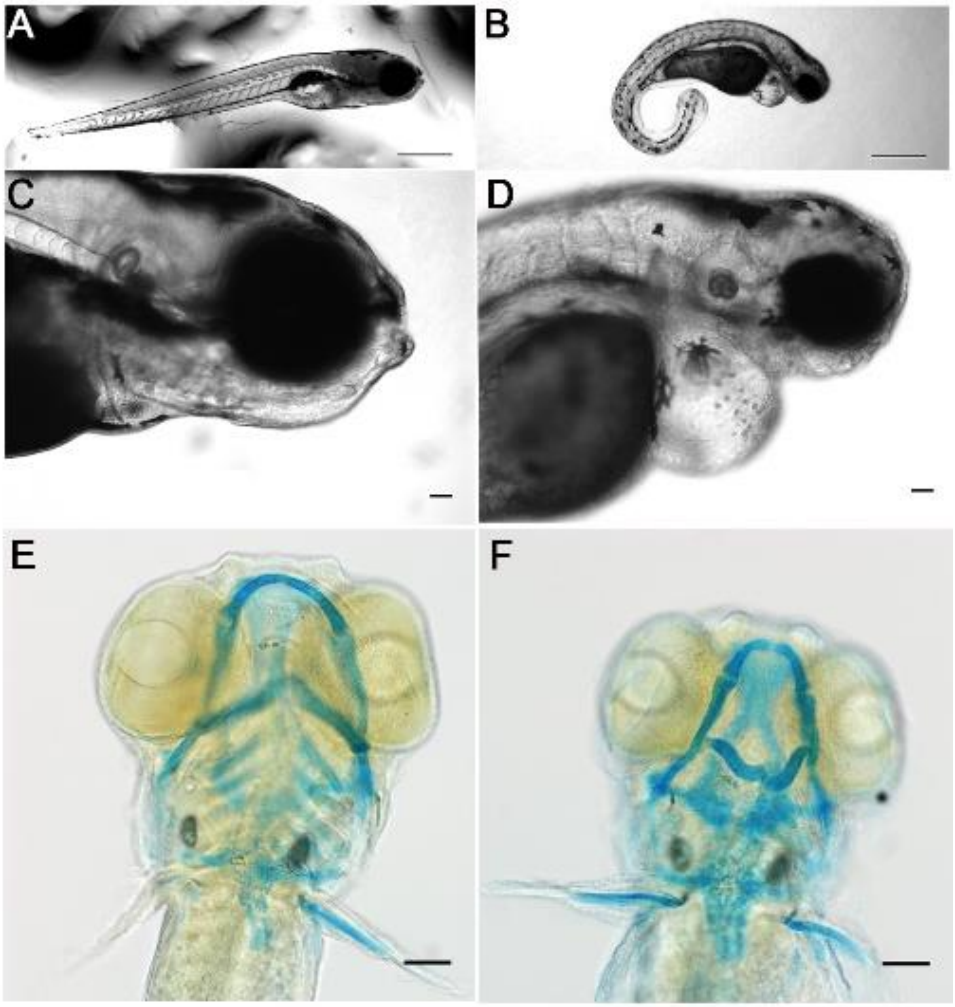

Figure 2.1. Col11a1a knockdown causes chondrodysplasia that recapitulates Fibrochondrogenesis in zebrafish. Injection of a col11a1a targeting AMO induces curvature of the body axis (B) while embryos injected with standard control AMO maintain a straight body axis (A). Two otoliths and normal face protrusion are present in the control zebrafish (C). In contrast, the morphant zebrafish have fused otoliths, heart edema, and an apparent reduction in the face and jaw protrusion (D). Alcian blue staining of the pharyngeal arch cartilage shows normal patterning and anteriorly facing cartilage (E). Alcian blue staining in the morphant cartilage shows perturbed pharyngeal cartilage patterning with a decreased protrusion of the 1st arch and posteriorly facing 2nd arch (F). Scale bar in A and B 0.5 mm. Scale bars in C-F 50 µm

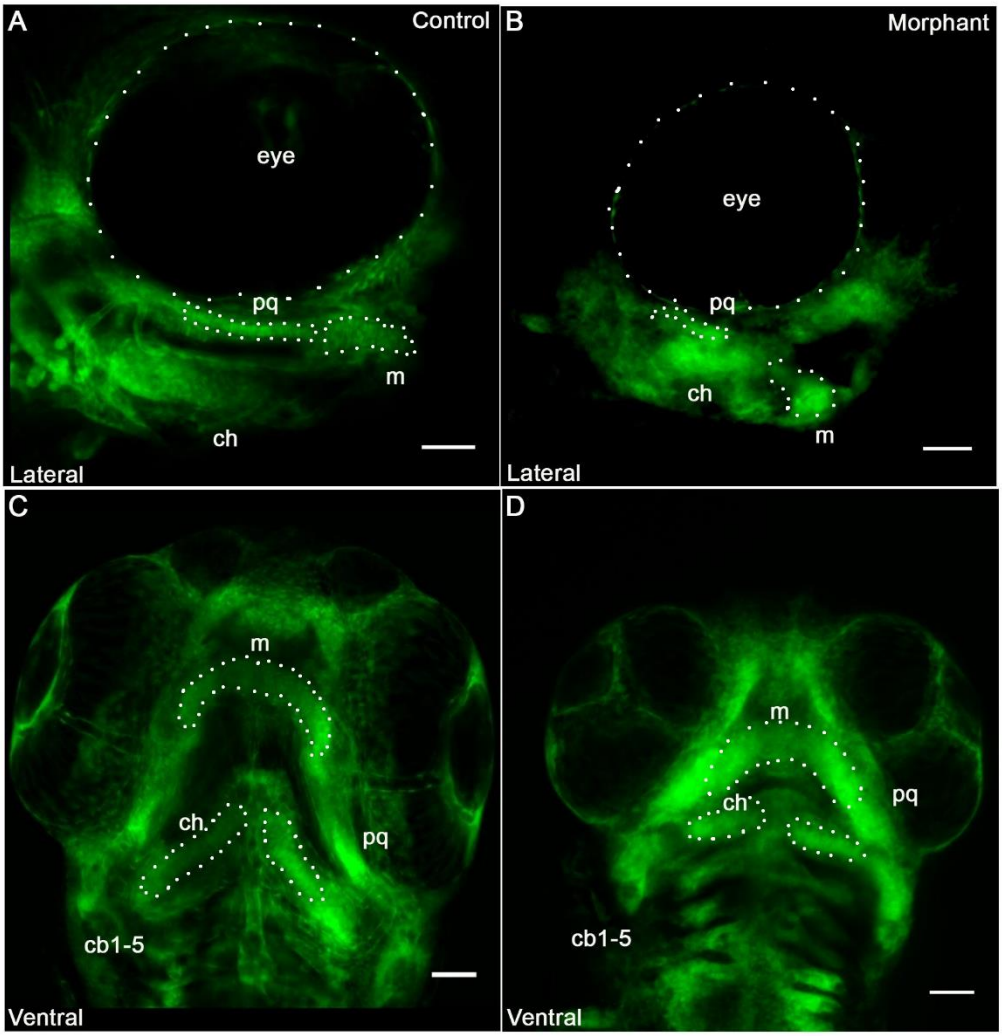

Figure 2.2. Cranial neural crest derived cells form segmented pharyngeal arches with abnormal shape in the absence of Col11a1a. Fli1a:EGFP positive cells indicated that the neural crest derived cells did occupy the pharyngeal arches. After occupying the pharyngeal arch however, cells failed to form organized cartilage structures. The cells aligned to form an elongated structure in the palatoquadrate (pq) that joins the Meckel's cartilage in the control morphant (A). The pq of the col11a1a morphant did not form a straight structure and the Meckel's did not extend anteriorly (B). The ventral view indicated segmentation of each of the developing pharyngeal arches in both the control (C) and the morphant (D). Scale bar 50 µm. (m; Meckel's cartilage, pq; palatoquadrate, ch; ceratohyal, cb; ceratobranchial)

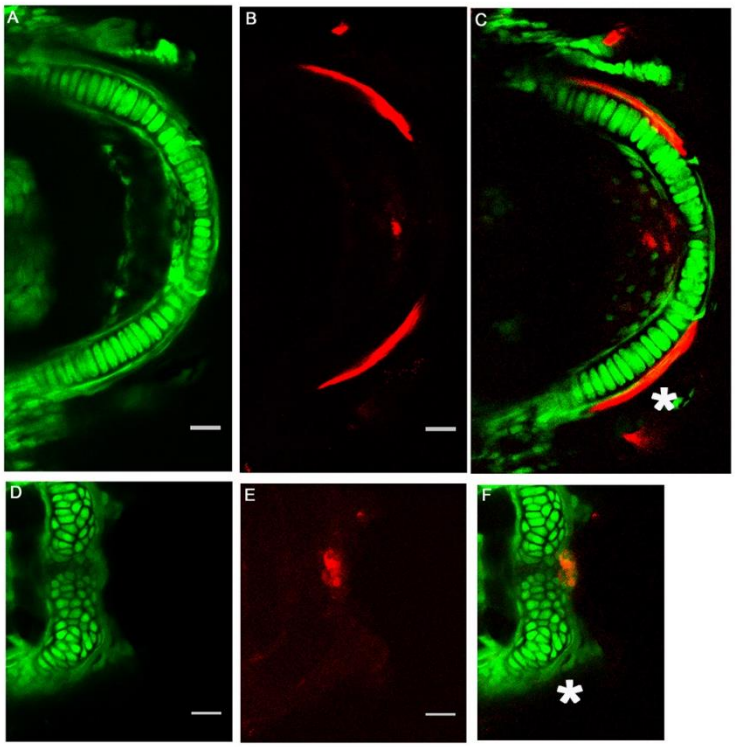

Figure 2.3. Col11a1a expression is required for Meckel's cartilage organization, elongation and normal mineralization. The Fli1a:EGFP expressing cells in the Meckel's cartilage and bone lining cells formed an organized stack of flat cells that extended the Meckel's cartilage anteriorly (A) Alizarin Red staining indicated that the bone lining cells produced a mineralized rod-like structure bordering the Meckel's cartilage (C, white star) and initiated mineralization at the distal tip (B and C) In contrast, the cells in the morphant were not flat and did not form a stack (D). Consequently, the Meckel's cartilage did not extend anteriorly. Mineralization of the bilateral rods in the morphant was not detected (F, white star), although mineralization was present at the distal tip (F). Scale bars 20 µm.

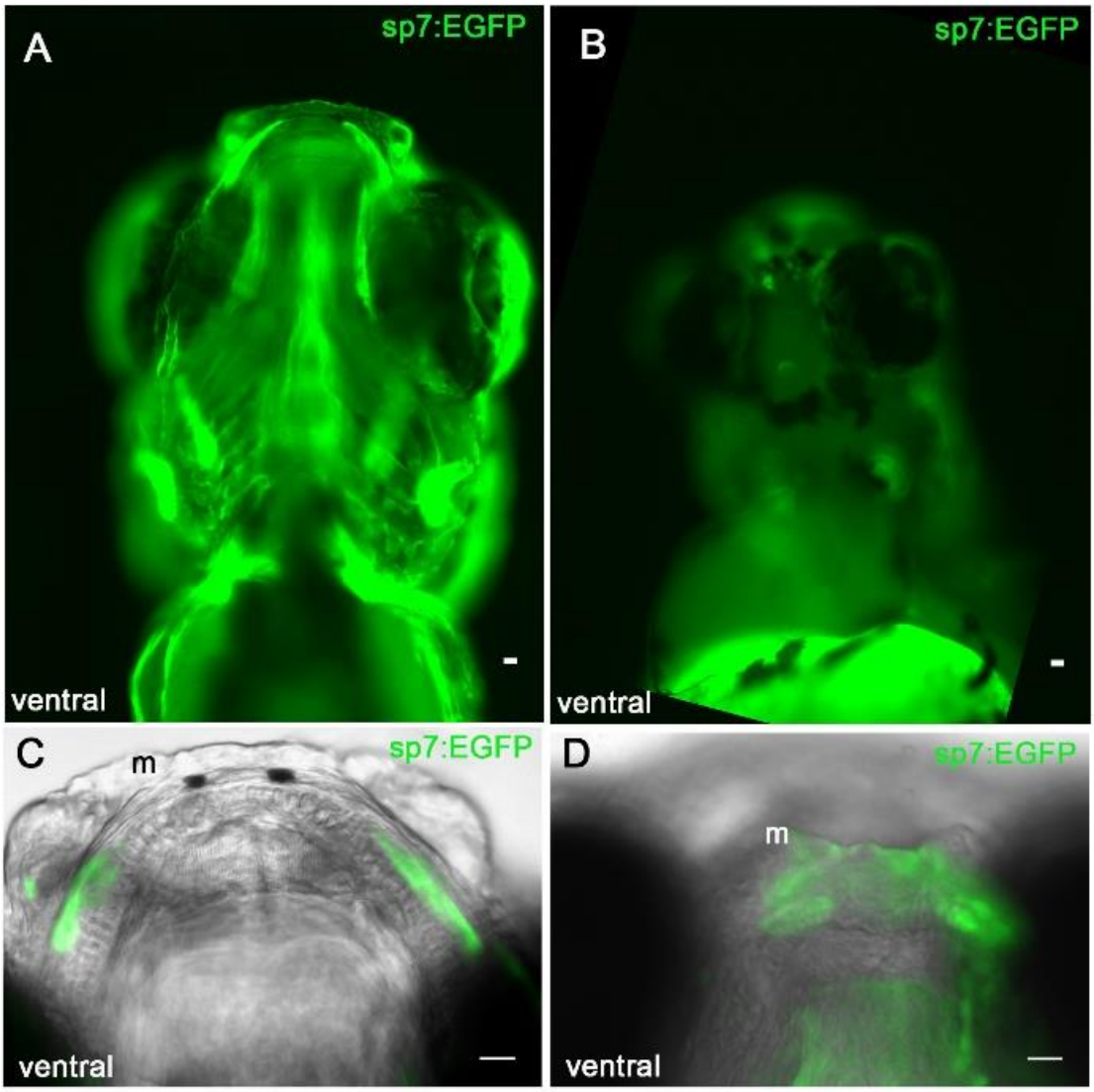

Figure 2.4. Col11a1a knockdown disrupts the organization of bone forming cells lining the Meckel's cartilage. Ventral view of sp7:EGFP positive cells imaged in 5 dpf control zebrafish (A and C) and morphant (B and D). sp7:EGFP expressing cells line the cartilage template in control zebrafish (C). sp7:EGFP expressing cells form small condensations at the mediolateral cartilage but fail to expand in morphants (D). Scale bar 20 µm.

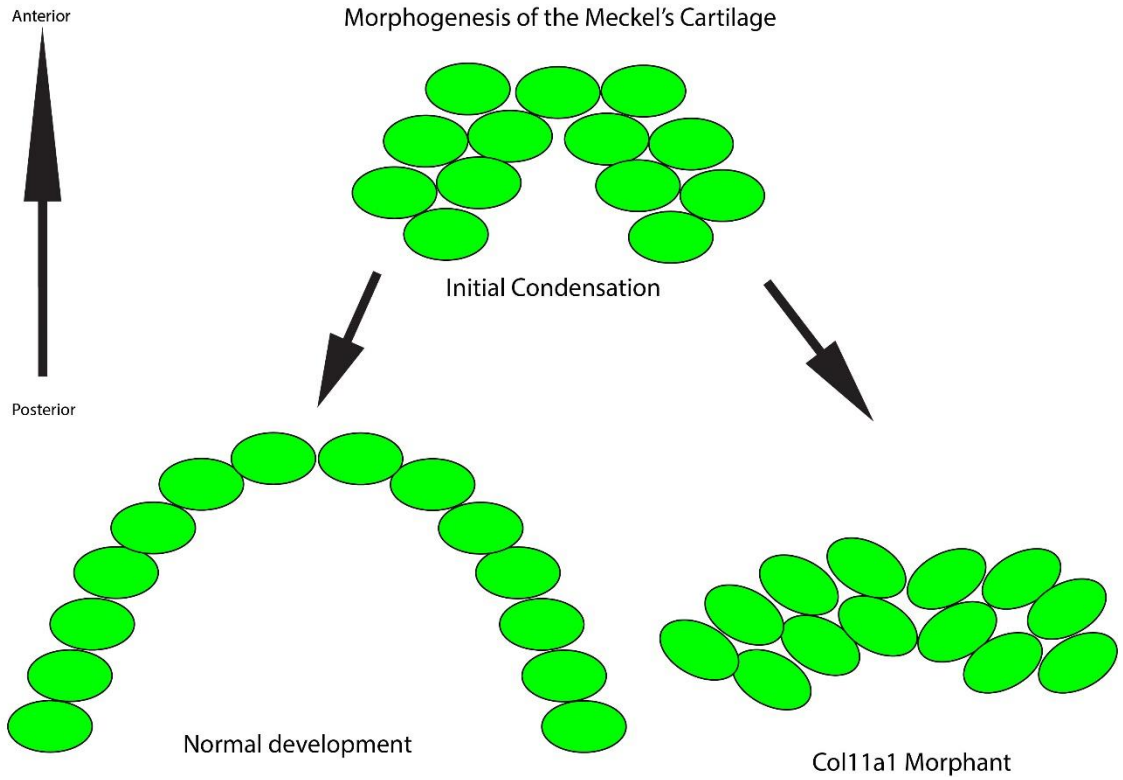

Figure 2.5. Model of Meckel's cartilage development and the cellular organization in normal development and in the col11a1a morphant. Cranial neural crest cells form initial condensations. During normal development, the cells reorganize and form a single file stack of cells that promotes extension anteriorly. In contrast, inhibition of col11a1a expression prevents reorganization into stacked cells. Therefore, the Meckel's cartilage does not extend anteriorly.

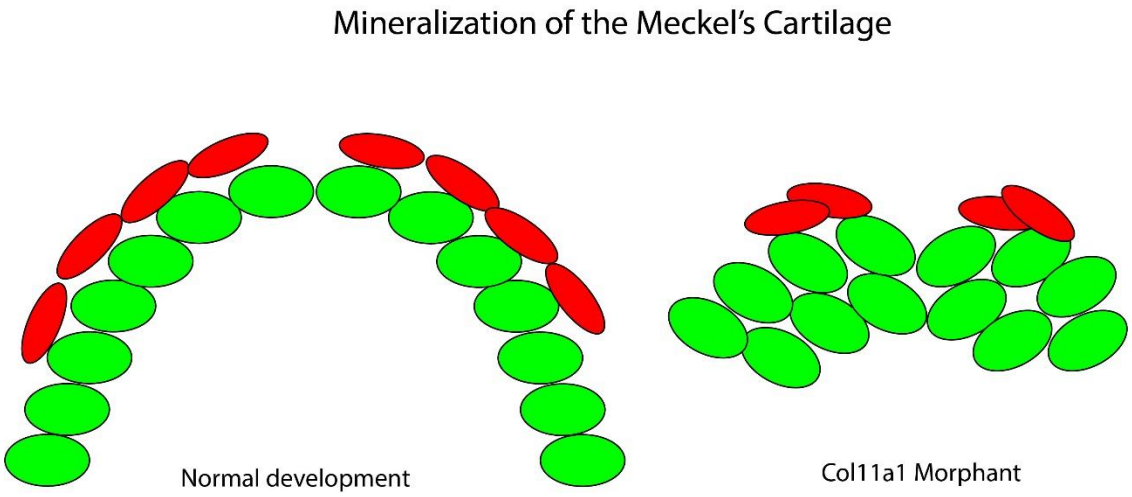

Figure 2.6. Model of Meckel's cartilage mineralization in the col11a1a morphant. The organized chondrocytes (green) serve as a template for bone forming cells (red) to generate a calcified matrix. These cells extend along the template and form two bilateral mineralized rods. Bone forming cells in the morphant have less template to serve as guide for the bone lining cells. Therefore, these cells cluster and produce abnormal mineralization at the cartilage template.

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CHAPTER THREE: Col11a1 expression regulates the chondrocyte phenotype during chondrogenesis in ATDC5 cells

Abstract

Cartilage development is a tightly regulated process that involves multiple molecules. Therefore, the loss of expression of a single gene can alter cell behavior and disrupt the development of the cartilage. *Col11a1* encodes the alpha one chain of the minor fibrillar collagen type XI that is essential in skeletal development. We used an RNAi mediated knockdown approach to investigate the role of *Col11a1* expression during chondrogenesis in ATDC5 cells. We hypothesized that *Col11a1* expression promotes the transition of mesenchymal cells to the chondrogenic phenotype, and that reduction of *Col11a1* expression will delay or inhibit this transition. The results are consistent with a role for COL11A1 protein in the regulation of cell shape, matrix production, mineralization and gene expression through a mechanism that involves AKT/GSK3β/β-catenin and increases TCF/LEF activity during chondrogenesis. These data indicate an underappreciated role for COL11A1 protein in the regulation of cell behavior.

Introduction

Endochondral ossification requires that a cartilage template is formed prior to initiating ossification (Hall and Miyake 2000). The process of endochondral ossification is the primary bone forming mechanism during vertebrate development (Kronenberg 2003). Therefore, successful vertebrate development requires the formation of a cartilage

template through chondrogenesis. To successfully form cartilage analgen, the cells must proceed through a series of maturation steps that begins with prechondrogenic mesenchymal cells forming a condensation and initiating the expression of genes involved in chondrocyte differentiation and matrix formation. The chondrocytes continue to mature and further differentiate into hypertrophic chondrocytes that contribute to matrix remodeling and ossification. The cells involved in this initial cartilage template remain in the growth plate and are responsible for longitudinal growth of the bone (Kronenberg 2003, Berendsen and Olsen 2015). Errors occurring during the formation of the cartilage can cause skeletal dysplasia or increase the susceptibility to the early onset of connective tissue diseases such as osteoarthritis.

COL11A1 gene expression is essential for normal skeletal development, evident by diseases in humans and by the observation that mutations in the *Col11a1* gene result in severe tissue disorganization in mice (Li et al. 1995, Hafez et al. 2015). Fibrochondrogenesis and Stickler's syndrome type II are human skeletal dysplasias associated with mutations in the *COL11A1* gene (Tompson et al. 2010a, Hufnagel et al. 2014). Additionally, genetic alteration in *COL11A1* contributes to intervertebral disc disease and degradation of the articular cartilage (Noponen-Hietala et al. 2003, Rodriguez et al. 2004, Raine et al. 2013).

Collagen type XI is composed of three alpha chains, α1(XI), α2(XI), and α3(XI) (Morris and Bächinger 1987, Fernandes et al. 2007). Collagen type XI both nucleates fibril formation and regulates the fibril diameter (Blaschke et al. 2000, Gregory et al. 2000, Holmes and Kadler 2006). The amino terminal domain of the α1(XI) chain is a unique domain to clade B fibrillar collagens that interacts with multiple extracellular

molecules, making it essential in providing integrity to the matrix network (Bernard et al. 1988, Oxford et al. 2004, Warner et al. 2006, Brown et al. 2011). Despite our knowledge of the structural role of collagen type XI in the extracellular matrix of cartilage, the biological impact of *COL11A1* gene mutations on cell behavior and molecular phenotype remain unknown.

Previous studies have established the importance of COL11A1 protein in skeletal development, maintenance and health, but they have not investigated how decreased *COL11A1* expression affects the chondroprogenitor cell behavior during chondrogenesis. The chondroprogenitors are derived from mesenchymal stem cells and their maturation into overt chondrocytes is a tightly regulated, sequential process (Goldring, Tsuchimochi, and Ijiri 2006). The chondrocytes undergo proliferation and upregulate chondrogenic gene expression of *COL2A1, ACAN,* and *SOX9*. These cells further change their expression to include upregulation of the hypertrophic chondrocyte markers *COL10A1*, *RUNX2,* and *MMP13*. Deviation from the normal expression pattern may alter the extracellular matrix environment permanently, causing chondrodysplasia and susceptibility to degenerative cartilage disease. Furthermore, cells interact with and react to the extracellular environment; therefore, dysplastic tissue has the potential to alter downstream cellular behaviors such as differentiation. In addition to nucleation and collagen fibril diameter, we propose the hypothesis that *COL11A1* expression is required to promote chondroprogenitor cell maturation during endochondral ossification and chondrogenesis. Based on this hypothesis, we predict that skeletal dysplasia resulting from loss of *COL11A1* expression alters chondrocyte phenotype and disrupts cell signaling events during chondrogenesis.

To test this hypothesis, we utilized the mouse ATDC5 chondrogenic cell line and induced differentiation using media containing insulin, transferrin, selenium (ITS), and ascorbic acid. ATDC5 cells are a well-established murine cell line used for studying the process of chondrogenesis and the transcriptome has been well documented (Chen et al. 2005, Altaf et al. 2006, Yao and Wang 2013). To investigate the early role of *Col11a1* in chondroprogenitor cells, we first identified the expression pattern of *Col11a1* and then used an RNAi approach to inhibit the upregulation of *Col11a1* during chondrocyte maturation. We analyzed the expression of chondrogenic markers in the knockdown cell culture as well as the activity of the β-catenin signaling pathway. We found that in the absence of *Col11a1* expression, cellular morphology differed from control cultures. We also identified changes in the phosphorylation of AKT, GSK3β and β-catenin. The pattern of phosphorylation was consistent with the increased transcriptional activity of TCF/LEF and downstream gene expression. These results suggest that *Col11a1* expression mediates changes in cell phenotype by attenuating the β-catenin signaling pathway during chondrogenesis and endochondral ossification.

Methods

Cell Culture

Mouse chondrogenic cell line ATDC5 cells were cultured in DMEM/F12 containing 5% fetal bovine serum and 100 I.U./mL penicillin and 100 µg/mL streptomycin. Cells were passaged at 80% confluence by dissociating with 0.25% trypsin/EDTA for 3-5 mins at 37°C. For differentiation experiments, the culture media was replaced with differentiation media after the cells reached 100% confluency. Differentiation media was DMEM:F12 supplemented with Insulin (10 µg/mL),

Transferrin (0.5 µg/mL) Selenium (0.0067 µg/mL) (ITS, Gibco) and 50 µg/mL ascorbic acid 2-phosphate (A2P) (Wako Chemicals, USA). The differentiation media was replaced every other day for the duration of the experiment.

Col11a1 Knockdown by Sirna Transfection

ATDC5 cells were seeded in 6-well plates with 2.5 x 10^6 cells per well in culture media and allowed to reach 80-90% confluency prior to transfection. siRNA targeting *Col11a1* was designed and purchased from Invitrogen. The 21-base pair double stranded siRNA was generated from interrogating RefSeq NM_007729.2 and targeting exon 2 at nucleotide location 590. Lipofectamine RNAiMAX was used for transfections following standard protocols. Transfections were performed in triplicate with a siRNA-lipid complex master mix prepared in Opti-MEM media. The final master mix solution contained 1 mL Opti-MEM, 10 µL of 10 µM siRNA stock and18 µL of Lipofectamine RNAiMAX. Control wells were transfected with negative control siRNA and performed in triplicate with each experiment under the same conditions as the experimental siRNA. BLOCK-iT Alexa Fluor Red Fluorescent Oligo was used to determine and optimize the transfection efficiency. A transfection efficiency of 90-100% was achieved when determined by monitoring uptake of the fluorescent oligonucleotide. Cell cultures recovered for 24 hours before changing the media to differentiation medium.

3d Micromass Cultures

Micromass cultures were generated by dissociating cells with 0.25% trypsin/EDTA from the wells and resuspending to 2.0 x 10^7 cells/mL in differentiation medium containing ITS and 50 µg/mL ascorbate 2-phosphate. Ten microliters of the cell suspension was gently spotted in tissue culture wells. The cells were allowed to adhere

for 2-3 hours in a tissue culture incubator at 37°C with 5% CO_2 before adding additional medium. The medium was changed every other day for the duration of the experiment.

Quantitative Rt-Pcr (Qpcr) Analysis

RNA was extracted and purified using the RNAeasy minikit following manufacturer's instructions (Qiagen). Isolated RNA was analyzed by spectrophotometry for purity and quantity and used immediately for cDNA synthesis or alternatively, aliquoted and stored at -80°C. RNA was reverse transcribed into cDNA using the RT^2 first strand kit. The cDNA template was analyzed by qRT-PCR reaction using Sybr Green and specific primers or directly in RT^2 profiler PCR arrays in 96 well format. Reactions were carried using the Roche Lightcycler 96 target specific primers (Table 1). The relative amount of PCR product was normalized to the indicated reference genes and the change in threshold cycle (dCt) was compared using Student's t-test. The fold change was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008)

Luciferase Assays

Cignal 45-pathway reporter array was used to identify changes in transcription factor activity following manufactures instructions. Lipofectamine 2000 was used with Opti-Mem to simultaneously reverse transfect *Col11a1* siRNA and a mixture of transcription factor responsive firefly luciferase reporter and a constitutively expressing Renilla constructs. 5.0 $x10^3$ cells per well were added to each well of a 96-well plate containing 100 µL of transfection media containing 10 µM *Col11a1* siRNA and 0.8 µL Lipofectamine 2000 per well. The cells were incubated at 37°C with 5% CO_2 for 24 hours prior to changing the media to the indicated treatment. Dual luciferase reporter assay

system and Glomax multi detection system was used to measure the activity of the transcription factors following standard protocol.

Western Blot Analysis

Cell lysates were extracted using ice-cold radioimmunoprecipitation (RIPA) cell lysis buffer (ThermoFisher Scientific) supplemented with protease and phosphatase inhibitor cocktails added directly to the wells of six-well plates after washing with cold phosphate buffered saline (PBS). The cell lysates were collected and added to microcentrifuge tubes and the supernatant was cleared by centrifugation at 4°C, 14,000xg for 15 min and transferred to a new tube. Protein lysates were quantified using bicinchoninic (BCA) protein assay (Pierce). Proteins were separated by SDS PAGE and then transferred to nitrocellulose membranes for western blot analysis. SDS-PAGE gels were stained with coomassie blue to verified equal amounts of loading and transfer for each sample. Membranes were blocked using a 5% (w/v) non-fat milk solution in Tris-buffered saline with 0.1% Tween-20, pH 7.4 (TBST) buffer for 1 hour at room temperature prior to incubation with primary antibodies. Primary antibodies were diluted in 5% (w/v) bovine serum albumin (BSA) in TBST prior to overnight incubation of the membranes at 4°C with shaking. Immunoblots were washed and then incubated with horseradish peroxidase (HRP) conjugated secondary antibodies for 1 hour at room temperature. Signal was detected using Pierce enhanced chemiluminescence (ECL) western blotting substrate. Western blots were performed using the following rabbit primary antibodies from Cell Signaling Technology at a dilution of 1:1000 unless otherwise stated: phospho-β-catenin (Ser33/37/Thr41), β-catenin (6B3) mAb, AKT, phospho-AKT (1:2000) (Ser473) (D9E) XP mAb, Gsk-3beta, phospho-GSK3-β (Ser9)

(5B3) mAb, β-Actin (13E5) mAb. Collagen type XI α1 chain specific rabbit polyclonal antibodies were designed to recognize epitopes within the amino propeptide (7990) or variable region 2 (1703) (Davies et al. 1998).

Alcian Blue And Alizarin Red Staining

Cells were differentiated for the indicated times and then stained with 0.1% Alcian blue 8GX in 1N HCl at 4°C overnight. For detection of matrix calcification, Alizarin Red solution (pH 4.2) was added for 5 min at room temperature and then washed with deionized water three times before imaging.

Immunocytochemistry

Cells were seeded on sterilized coverslips in 6-well plates in differentiation medium. Cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and washed 3 times with cold PBS. When required, permeabilization was performed by incubating samples for 10 min in 0.1% Triton X-100. Non-specific binding was blocked using 1% (w/v) BSA in PBS + 0.1% Tween20 (PBST) for 30 min at room temperature. Samples were incubated with diluted antibodies in 1% (w/v) BSA/PBST in a covered chamber overnight. Cells were incubated with secondary antibodies for 1 hour at room temperature in the dark. Counter stain for nuclei was performed by using Prolong gold anti fade mounting media with DAPI.

Results

Characterization Of *Col11a1* And Chondrogenic Mrna Expression During Chondrogenesis In Atdc5 Cells

We characterized the expression profile of the *Col11a1* gene in the mouse chondrogenic ATDC5 cell line induced to differentiate with ITS and A2P (Figure 1). Day

0 of the experiment was designated as the time at which culture medium was exchanged for differentiation medium in confluent cultures. Using quantitative RT-PCR, we determined that *Col11a1* mRNA was increased 7.6-fold at day 7 relative to day 0, and 156-fold by day 14. We also demonstrated that *Col11a1* mRNA levels significantly decreased between day 14 and 21 to 20% of the levels observed on day 14 (Figure 1A). Western blot analysis using two separate rabbit polyclonal antibody directed to the collagen α1(XI) chain amino terminal epitope or variable region 2 (V2) confirmed expression at each of the investigated time points (Figure 1B). Protein levels were detectable at day 0 and day 7 and in agreement with the mRNA levels, collagen α1(XI) chain protein reached maximally detectable levels in cell lysates by day 14. The protein detected on day 21 remained high despite the significant decrease in mRNA levels, a result that is not surprising given the long half-life on collagen proteins (Verzijl et al. 2000, Toyama and Hetzer 2013).

In addition to *Col11a1*, we confirmed the expression of chondrogenic differentiation markers in culture (Figure 1C). The expression patterns that we observed were consistent with previously reported results in ATDC5 cells using ITS and A2P to induce chondrogenic differentiation (Chen et al. 2005, Altaf et al. 2006). *Col2a1* and *Acan* mRNA were increased at day 7 of differentiation. We did not observe significant changes in *Sox9* mRNA between day 0 and 7 (Figure 1C).

<u>*Col11a1* Knockdown Induces Fibroblast-Like Cytoskeletal Phenotype, Inhibits Cartilage Growth, And Increases Mineralization</u>

To investigate the requirement for *Col11a1* expression during the transition of prechondrogenic cells to differentiated chondrocytes, we transfected ATDC5 cells with

double stranded siRNA complexes targeting *Col11a1* mRNA. We optimized and verified the effectiveness of this approach by monitoring mRNA and protein levels in ATDC5 cells. RNAi transfection prevented the increase in *Col11a1* mRNA previously observed on day 7 (Figure 2). Expression on day 3 was decreased by 90% compared to negative control transfected cells and by more than 75% on day 7 (Figure 2A). Western blot analysis confirmed a decrease in total collagen α1(XI) chain protein levels in the cell lysates on day 7, although some protein was detectable (Figure 2B). Due to collagen's long half-life, it is likely that the protein detected was translated prior to transfection with siRNA. New protein translation was blocked through RNAi degradation complexes as we could not detect collagen α1(XI) chain by immunofluorescence in transfected cells (Figure 2C). We observed changes in the actin cytoskeletal organization 3 days after *Col11a1* siRNA transfection with cells appearing more elongated and fibroblast-like than control cells (Figure 2C).

The development of the cartilage extracellular matrix can be determined by the presence of sulfated glycosaminoglycans in developing cartilage nodules. Additionally, late stage chondrogenesis is marked by increased matrix calcification. Micromass cultures transfected with *Col11a1* siRNA and subsequently grown for 7 days produced less extracellular matrix than cells transfected with negative control siRNA as indicated by the decreased Alcian blue staining (Figure 3A and B). Additionally, increased mineralization was detectable in knockdown micromass cultures after 7 days in differentiation media (Figure 3C and D).

Inhibition Of *Col11a1* Does Not Affect Genes Involved in Mesenchymal Condensation but Does Affect Genes Involved in Matrix Production and Remodeling

The expression of mesenchymal condensation and stereotypical chondrogenic differentiation markers were analyzed by quantitative RT-PCR. Condensation of mesenchymal prechondrogenic cells is a prerequisite for chondrogenic differentiation and subsequent chondrocyte maturation. The expression of versican (*Vcan*), tenascin (*Tnc*), N-cadherin (*Cdh2*) and N-CAM (*Ncam*) are required for successful condensation to occur (Hall and Miyake 1995). The expression of *Vcan, Tnc, Cdh2,* and *Ncam* were unaffected by *Col11a1* knockdown in ATDC5 cells (Figure 4). However, in the absence of *Col11a1* expression, the mRNA levels of collagen α1(II)chain (*Col2a1*) and matrix metallopeptidase-13 (*Mmp13*) were increased, while the expression of *Sox9, Acan,* and *Col10a1* did not significantly change (Figure 5).

To further investigate the link of between *Col11a1* mRNA expression and cartilage development, we utilized a quantitative RT-PCR profiler array for extracellular matrix and adhesion genes. Consistent with our previous results, Mmp13 and Col2a1 were upregulated in the assay. Additionally, Hyaluronan and Proteoglycan Link Protein 1 (*Hapln1*), Connective tissue growth factor (*Ctgf*), Integrin beta 3 (*Itgb3*), Intercellular adhesion molecule 1 (*Icam1*), Laminin beta 3 (*Lamb3*), Laminin alpha 3 (*Lama3*) and collagen α3(IV)chain (*Col4a3*) were significantly increased by *Col11a1* knockdown (Figure 6).

β-Catenin Signaling Pathway Activity is Increased When *Col11a1* Expression is Inhibited During Chondrogenesis and is Independent of Wnt Induction

Wnt/β-catenin signaling and the regulation of TCF/LEF transcriptional activity is essential in the development of the cartilage (Tamamura et al. 2005, Dao et al. 2012). Unregulated activity in cells of the chondrocyte lineage negatively influence tissue morphogenesis and consequently leads to disease (Usami et al. 2016). GSK3β is a constitutively expressed, multifunction, protein kinase involved in a variety of cellular mechanisms, including homeostasis of the cartilage (Doble and Woodgett 2003, Miclea et al. 2011). Phosphorylation of GSK3β at serine 9 uniquely inhibits the molecules kinase activity (Doble and Woodgett 2003, Kawasaki et al. 2008). Active GSK3β can phosphorylate β-catenin when in complex with Axin2, leading to the degradation and a decrease in the cytoplasmic levels (Aberle et al. 1997, Delcommenne et al. 1998).

GSK3β is phosphorylated at Serine 9 in response to stimuli that include Wnt3a and insulin. Inhibition of *Col11a1* expression increased the phosphorylation of GSK3β at serine 9 and decreased amino terminal β-catenin phosphorylation (Figure 7A). Interestingly, we also identified increased AKT phosphorylation at serine 473 in the absence of insulin. In addition to changes in AKT, GSK3βand β-catenin phosphorylation, we observed increased TCF/LEF activity when measured by luciferase assay with the TOP:FLASH reporter construct (Figure 7B). β-Catenin levels and localization fluctuate with cell behavior, gene expression and activation of cell signaling pathways. To identify changes in cell shape and localization of β-catenin caused by *Col11a1* expression we used immunofluorescence microscopy. Actin fibers in control cells had a cortical actin pattern consistent with a chondrocyte phenotype. In contrast, inhibition of *Col11a1*

expression prevented cortical actin and favored cell spreading. Additionally, β-catenin was localized more perinuclear and β-catenin appeared throughout the cytoplasm of the cell (Figure 8). Stimulation of the canonical Wnt signaling pathway with recombinant Wnt3a protein was not inhibited by *Col11a1* knockdown as indicated by substantial GSK3β phosphorylation and decreased β-catenin phosphorylation in both the negative control and *Col11a1* siRNA samples.

Discussion

This is the first demonstration of a link between the extracellular matrix protein collagen α1(XI) chain and the activation of the AKT/GSK3β/β-catenin pathway during skeletal development. This cell culture model system provides a means by which to ask cellular questions relevant to chondrodysplasia *in vitro*. These results support the hypothesis that *Col11a1* expression is required for chondrocyte maturation during chondrogenesis and endochondral ossification. Mutations in the human *COL11A1* gene cause severe chondrodysplasia, affecting bones that develop by endochondral ossification, including the craniofacial skeleton, long bones, and the vertebrae (Li et al. 1995, Tompson et al. 2010a, Stevenson et al. 2012, Faletra et al. 2014, Hafez et al. 2015). Although the shape and structure of skeletal tissues impacted by *COL11A1* mutations have been described radiologically and at the ultrastructural level, the response of the cells to the altered matrix have not been fully characterized (Fernandes et al. 2007). Since the cells are ultimately responsible for both generating and maintaining tissues, understanding the consequences of the absence or reduction of *COL11A1* expression has important ramifications for skeletal development, disease progression and therapies. We

hypothesize that *COL11A1* expression is required for mesenchymal, prechondrogenic cells to transition to mature chondrocytes.

In this study, we investigated the relationship between chondrocyte phenotype and the expression of *Col11a1* using a mouse cell culture model system. We inhibited the expression of *Col11a1* during chondrogenesis by transfecting prechondrocytes with siRNA targeting *Col11a1* mRNA prior to inducing differentiation with ITS and ascorbate 2-phosphate (A2P). The siRNA prevented the normal upregulation of *Col11a1* expression, thus creating a cell culture model system in which to investigate the effects of COL11A1 protein deficiency on chondroprogenitor maturation during cartilage development. We found that cells maintain a mesenchymal morphology, reduce matrix production, alter gene expression and activate AKT/GSK3β/β-catenin pathway in the absence of *Col11a1* expression. Based on these results we propose that *Col11a1* expression attenuates β-catenin signaling in chondroprogenitor cells to regulate chondrocyte maturation and endochondral ossification.

Collagen type XI's role in skeletal development has previously been described as a structural constituent regulating collagen nucleation and fibril diameter. Additionally, *Col11a1* expression in cell culture models, including ATDC5 cells, has been used to identify cells as having a chondrocyte phenotype (Davies et al. 1998, Chen et al. 2005). This study clearly supports that *Col11a1* is expressed in chondrogenic cells during chondrogenesis and agrees with the hypothesis that *Col11a1* expression is essential for skeletal development (Li et al. 1995). In addition to being a chondrocyte marker, the results of this study are consistent with the idea that *Col11a1* is an early mediator of

chondrogenesis required to establish the overt chondrocyte phenotype from a mesenchymal prechondrocyte and provides a plausible and testable mechanism.

Mutations in the *COL11A1* gene cause the severe skeletal dysplasia, Fibrochondrogenesis. Skeletal development is severely affected in Fibrochondrogenesis, causing craniofacial defects such as micrognathia and long bone defects with flared metaphysis attributed to disorganization of the growth plate (Eteson et al. 1984, Whitley et al. 1984, Tompson et al. 2010). Additionally, the cartilage of the larynx is often weak and causes respiratory distress which is ultimately lethal. These morphological descriptions support COL11A1's role to promotes cell shape, behavior and cartilage growth. The results presented in this study suggest that the inability of cells to stabilize the cell shape and cortical actin may prevent the necessary cell behavior from occurring during chondrogenesis. For example, cell-cell interactions requiring N-cadherin and β-catenin promote cell shape, prechondrogenic cell condensations, and regulate Wnt signaling (Komori et al. 1997, Modarresi et al. 2005, Gao et al. 2010, Marie et al. 2014). Down regulation of these cell adhesions in favor of matrix-cell interactions is an essential step in chondrogenesis. Therefore, the inability of cells to establish stable cell-cell adhesions or transition to cell-matrix interactions may cause changes at the cellular level consistent with *Col11a1* associated chondrodysplasia in mouse.

The observed increases in mineralization may be due to accelerated hypertrophic differentiation or cartilage calcification in the absence of intermediate steps. Phosphorylation of GSK3β has previously been shown to promote hypertrophic differentiation (Kawasaki et al. 2008). Hypertrophic chondrocytes are a normal part of the endochondral ossification pathway and are required to initiate mineralization in the

growth plate under normal conditions (Ballock et al. 1993, Vega et al. 2004). On the other hand, premature initiation of the hypertrophic differentiation program has detrimental effects on the cartilage growth and integrity (van der Kraan and van den Berg 2012, Jayasuriya et al. 2014). Additionally, hypertrophic chondrocytes can potentially transdifferentiate into osteoblasts during normal endochondral ossification (Yang et al. 2014).

Interestingly, we found that increased expression of *Col2a1* and *Mmp13* occurs in the absence of the collagen α1(XI) chain. These results indicate that the expression of *Col2a1* and *Mmp13* is dependent on collagen α1(XI) through an unidentified genetic network. The upregulation of *Mmp13* was previously described in the articular cartilage of chondrodysplasia (cho) mouse with heterozygous null mutation in *Col11a1* (Xu et al. 2003, 2010). The researchers proposed that increased *Mmp13* expression degraded the pericellular matrix and exposed chondrocyte discoidin domain-2 receptor (DDR2) to interact with the surrounding matrix, mainly collagen type II. The changes in gene expression in this study agree with their hypothesis.

It is relevant to explain that the *Col2a1* mRNA transcripts detected in our assays were total *Col2a1* mRNA, and could not account for alternative splicing; therefore, it is possible that the increase in *Col2a1* mRNA is primarily *Col2a1* containing exon 2, which is highly expressed in mesenchymal cells. In contrast *Col2a1* transcripts lacking exon 2 are primarily found in differentiated chondrocytes (Hering et al. 2014; McAlinden et al. 2008; Sandell et al. 1991; Sandell, et al. 1994). Future assays will utilize this unique splicing pattern to further understand the cellular phenotype involved in *Col11a1* expression.

The genes *Hapln1, Ctgf,* and *Itgb3* each have essential roles in tissue morphogenesis and cartilage development and were upregulated by *Col11a1* knockdown. *Hapln1* is essential member of the cartilage matrix whose expression stabilizes aggrecan and hyaluronan links (Yamada and Watanabe 1999). *Hapln1* overexpression has recently been linked to mesenchymal stem cell phenotype associated with the expression of β-catenin (Mebarki et al. 2016). Ctgf is involved in both insulin mediated AKT/GSK3β signaling and hypertrophic chondrocyte differentiation (Nishida et al. 2003, Zhou et al. 2008, Huang, Brugger, and Lyons 2010, Oh et al. 2016). Additionally, the up regulation of *Itgb3*, which along with the ItgaV protein subunit, interacts with the IGF-1 receptors enhances AKT signaling (Saegusa et al. 2009). In agreement with the changes in gene expression, we observed that AKT and GSK3β phosphorylation was increased in the absence of COL11A1 protein in ATDC5 cells and that TCF/LEF activity was increased overall. A previous study found that GSK3β inhibition in the cartilage induces cartilage degradation and decreases glycosaminoglycan detection (Miclea et al. 2011). Interestingly, inhibition of GSK3β was recently shown to increase the volume of trabecular bone (Arioka et al. 2013, Tatsumoto et al. 2016). Additionally, GSK3β inhibitors enhanced fracture healing by altering the mineralization pattern and skipping the formation of a cartilage template (Sisask et al. 2013). Consistent with these findings, we have observed in previous studies, that COL11A1 protein deficiency alters mineralization patterns, increase bone collar thickness and leads to wider trabecular structures, yet this is the first time we have implicated chondroprogenitor cells in the process of calcification (Kahler et al. 2008, Hafez et al. 2015).

RNA silencing using siRNA knockdown has limitations in that it may not completely degrade all target mRNA and expression may recover over time. We confirmed the stability of the knockdown using quantitative RT-PCR, western blot analysis and immunofluorescence microscopy. Because of the transient nature of the mRNA silencing, this study focused on expression occurring within the first week of culture. Differentiation was accelerated by including ITS and ascorbate 2-phosphate in the medium. This *in vitro* model provides insight into the cellular response to altered cartilage matrix induced by genetic defects in chondrocytes, yet cannot provide valuable information concerning neighbor tissue interactions.

This work suggests that we may be able to rescue the COL11A1 protein deficiency by modulating AKT, GSK3β, or β-catenin activity in mouse. Future directions will improve upon the understanding of the relationship between human COL11A1 and AKT/GSK3β/β-catenin. Future studies will include activators and inhibitors of these cell signaling intermediates in cell culture and animal model systems. COL11A1 gene defects are also associated with intervertebral disc degeneration and osteoarthritis and may induce similar cellular responses. Additionally, questions regarding the roles of the other collagen type XI and V alpha chains as well as alternative spliced forms of collagen α1(XI) remain to be investigated. This information may lead to development of cell based and tissue regeneration therapies for surgical intervention or fracture healing.

The requirement for *Col11a1* gene expression to establish the chondrocyte phenotype during cartilage development is presented here. We show that although considered a structural protein, loss of *Col11a1* gene expression impacts cellular behavior and cell signaling pathways. Undoubtedly the mechanisms influencing the cell behavior

are diverse and complex and therefore we are unlikely to isolate a single factor responsible for any deviations in behavior and function. Yet, understanding the cell phenotype at a particular stage provides the opportunity for investigation of cell targeted therapies.

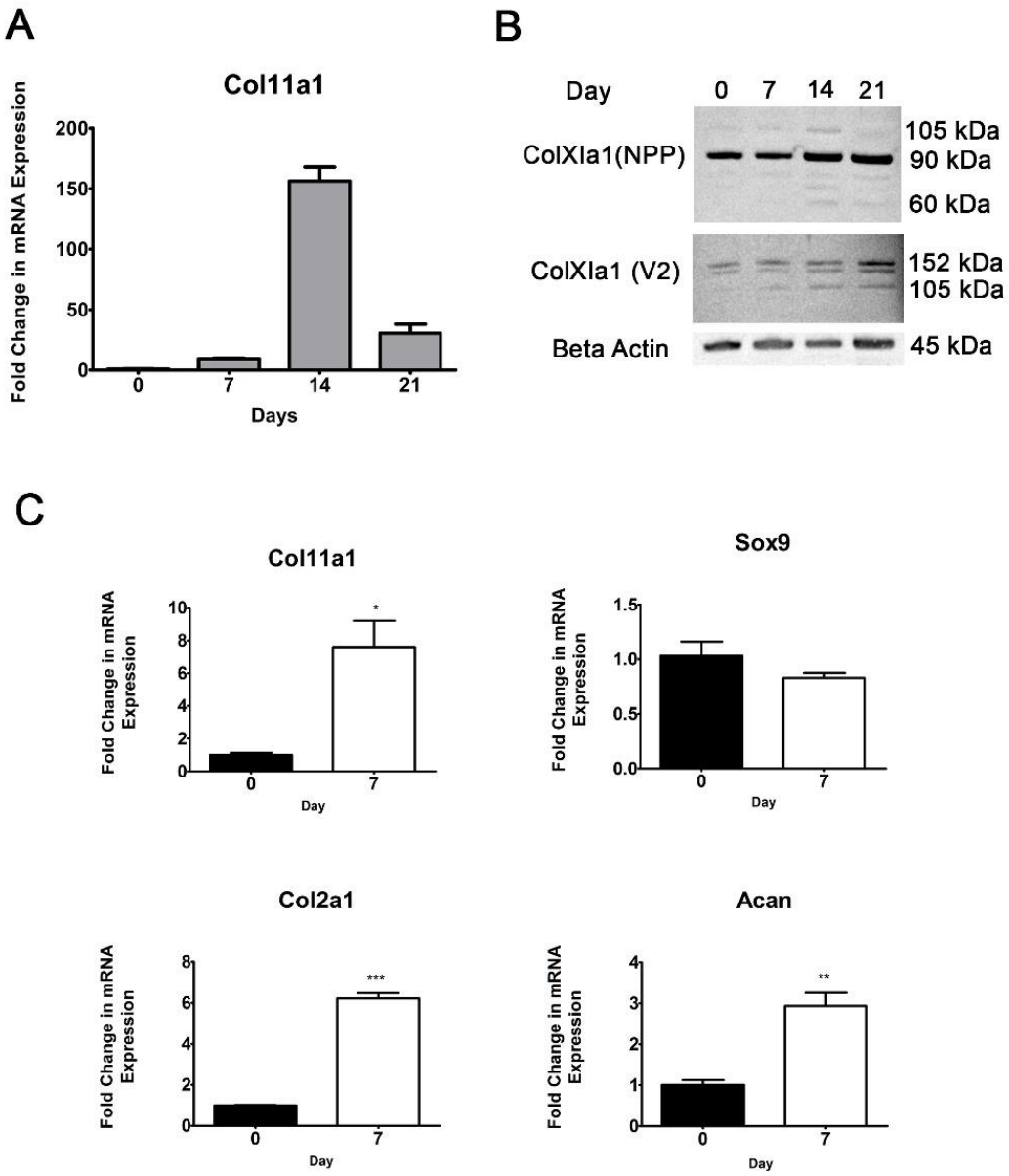

Figure 3 1. Col11a1 gene expression during chondrogenesis in ATDC5 cells. Differentiation media was added to confluent cell cultures at day 0. Quantitative RT-PCR was performed to detect Col11a1 mRNA in ATDC5 during chondrogenesis (A). Western blot analysis using two polyclonal antibodies that recognize unique epitopes were used to detect protein expression over the same time course (B). Col11a1 mRNA expression correlates with the expression of established chondrogenic markers Sox9, Col2a1, and Acan (C). Data is represented as mean and the standard deviation. Significance was determined using the t-test, n=3 *p < 0.05, **p<0.005, ***p < 0.0005.

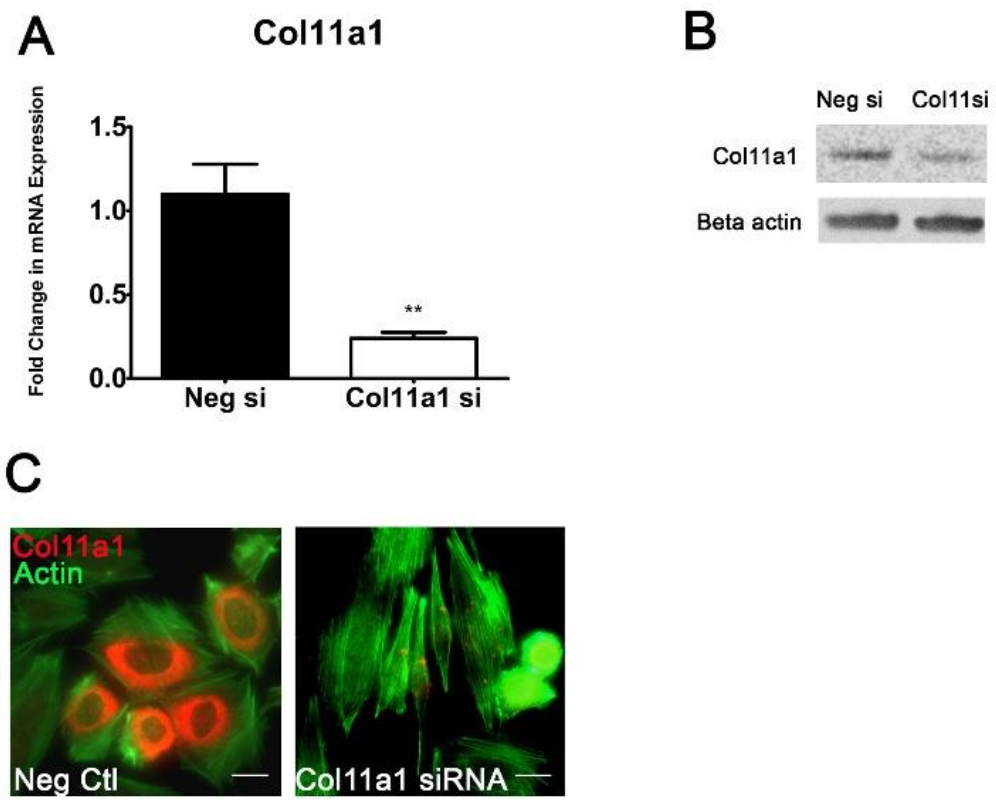

Figure 3 2. ***Col11a1*** **knockdown causes fibroblast-like actin organization. qRT-PCR was used to quantify the decrease of** ***Col11a1*** **mRNA and protein 7 days after siRNA transfection.** ***Col11a1*** **mRNA was decreased to 0.24 ± 0.03, N=3, relative to negative control siRNA transfections at day 7 (A). Protein levels detected using COL11A1 polyclonal antibody show a decrease in COL11A1 protein. Beta actin used to demonstrate equal loading per lane (B). Immunofluorescence detecting COL11A1 protein (red) and actin (green) verify the loss of detectable COL11A1 protein and changes in the actin cytoskeleton at 3-days post transfection. Scale bar in is 20 µm. Data is represented as mean and SD., data analyzed using the t-test, **p < 0.005.**

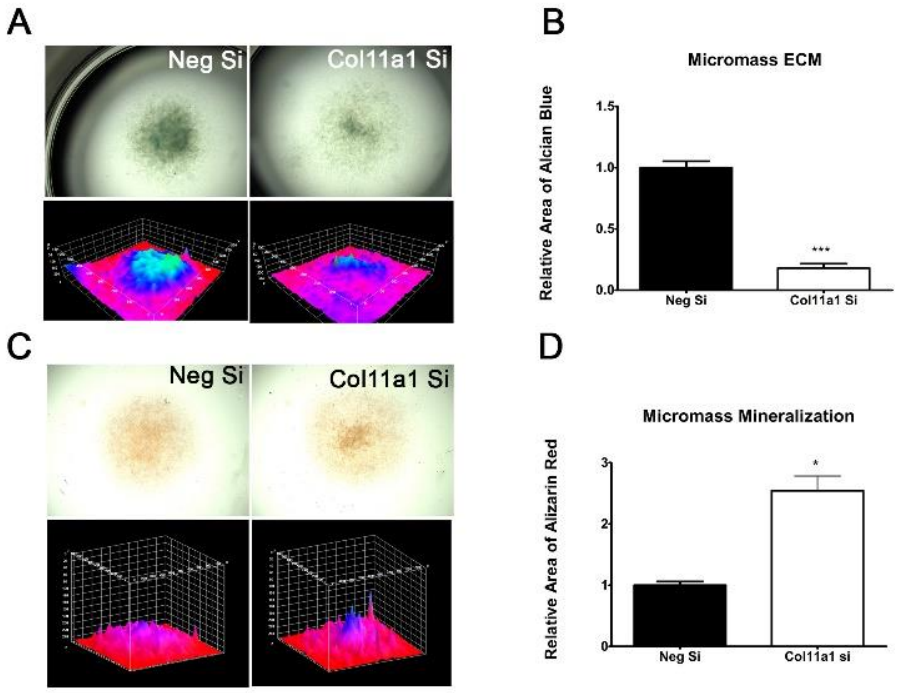

Figure 3 3. Knockdown of Col11a1 expression decreases the area of Alcian blue staining and increases Alizarin Red staining. Alcian blue staining was used to quantify the proteoglycan production in 7 day micromasses. Col11a1 siRNA decreases the relative area of proteoglycans detected (A and B). Surface area maps provide a visual representation of the staining intensity and shape. An opposite trend was observed when staining for calcium with Alizarin Red in micromass cultures. The relative area and intensity increased in the micromass. (C and D).

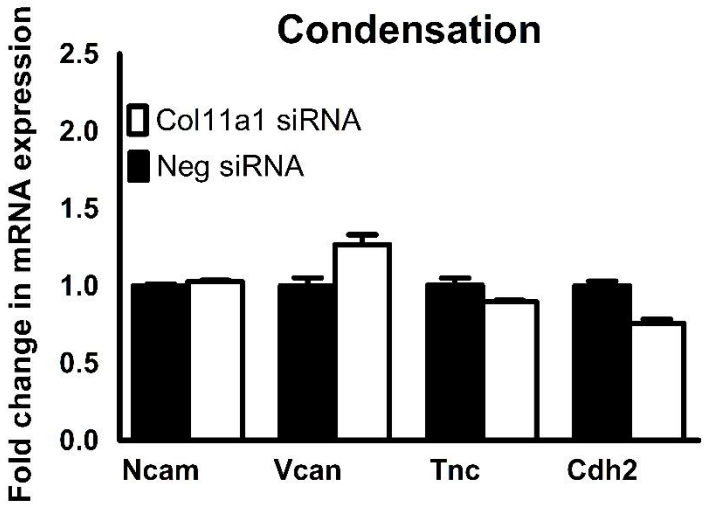

Figure 3 4. COL11A1 expression is not required for the expression of cellular condensation markers during chondrogenesis in ATDC5 cells. The mRNA expression of Ncam, Vcan, Tnc, and Cdh2 were not significantly altered in response to COL11A1 knockdown during chondrogenesis. Data were analyzed using an unpaired t-test and represented as the mean with the standard deviation (n=3).

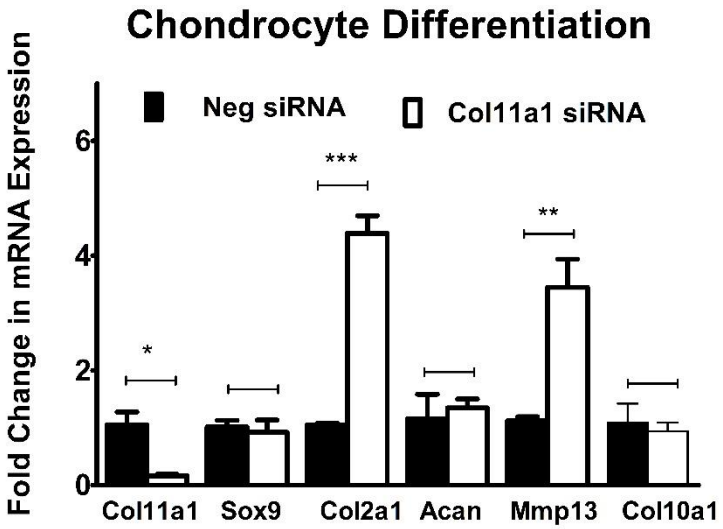

Figure 3 5. The expression of Col2a1 and Mmp13 are regulated by Col11a1 knockdown. ATDC5 cells were transfected with either Neg siRNA or Col11a1 siRNA prior to chondrogenic differentiation. Expression of Sox9, Acan, and Col10a1 mRNA was not significantly different following inhibition of Col11a1 expression. Col2a1 and Mmp13 mRNA expression was significantly increased by the inhibition of Col11a1 expression. Data were analyzed using the unpaired t-test and represented as the mean with the standard deviation (n=3). *=P-value <0.05, **<0.005, *<0.0005.**

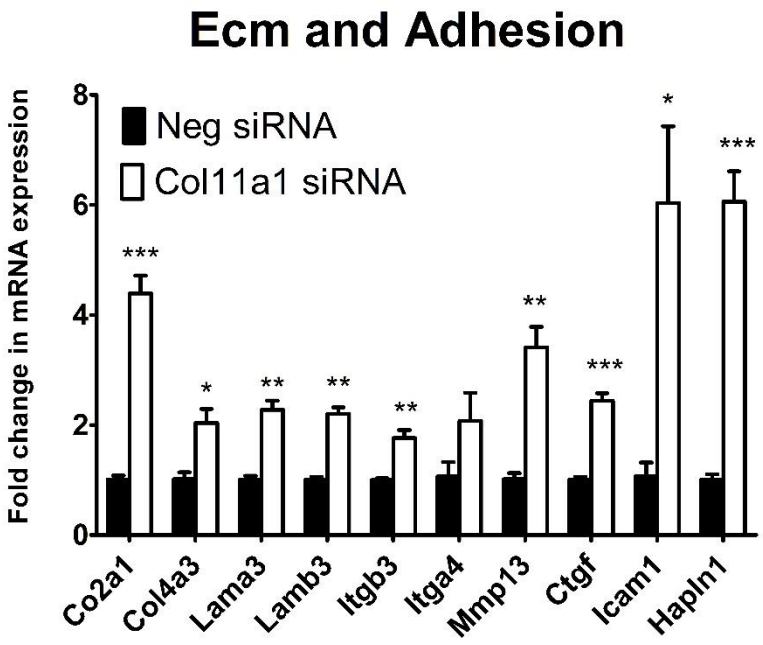

Figure 3 6. Extracellular matrix and adhesion gene transcription is regulated by Col11a1 expression levels. Changes in mRNA expression levels were determined by comparing the expression level of mRNA for each target gene relative to β-actin. The relative expression of the Col11a1 siRNA transfected cells was compared to the negative control siRNA transfected cells. The fold change was calculated using the 2-ΔΔCt method. The data were analyzed using unpaired t-test and represented as the mean and standard deviation (n=3). *=P-value <0.05, **<0.005, *<0.0005.**

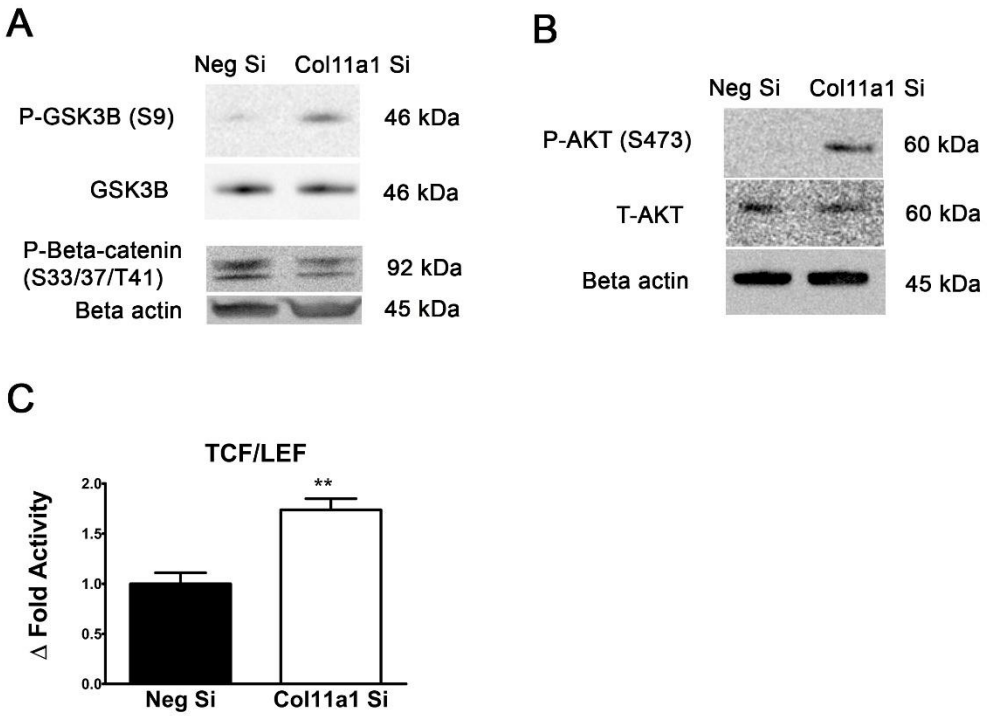

Figure 3 7. Col11a1 knockdown increases AKT/GSK3β/β-catenin signaling activity and increases TCF/LEF activity. Col11a1 siRNA treatment increased the phosphorylation of GSK3β while decreasing phosphorylation levels of β-catenin (A). Additionally, Col11a1 siRNA induced phosphorylation of AKT at serine 473 (B). The activity of the TCF/LEF transcription factor was significantly increased relative to negative control siRNA transfected cells. (C).

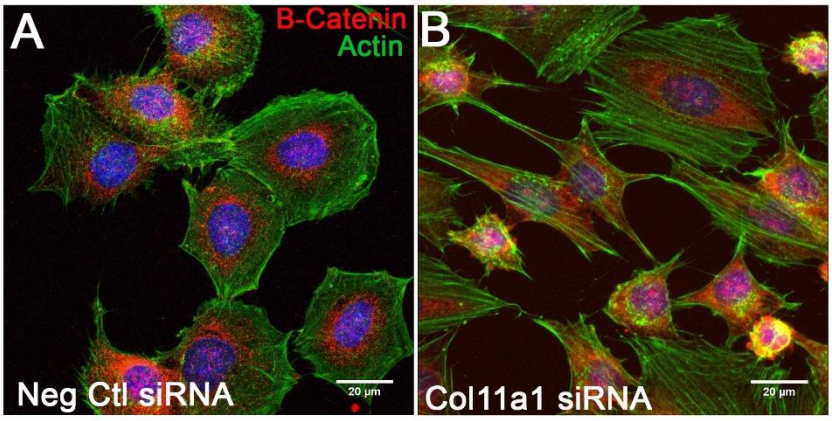

Figure 3 8. Immunofluorescence imaging of the actin cytoskeleton and β-catenin localization. Increased cell spreading and increased β-catenin nuclear localization was found following Col11a1 knockdown. Immunofluorescence of the actin cytoskeleton (green) shows cortical actin in Neg Ctl siRNA transfected cells (A) and an increase in actin stress fibers and increased cell spreading in cells transfected with Col11a1 siRNA (B). β-catenin (red) is primarily perinuclear in the Neg Ctl transfected cells (A) and is present throughout the cytoplasm and localized to the nucleus. (B). Scale bars are 20 µm.

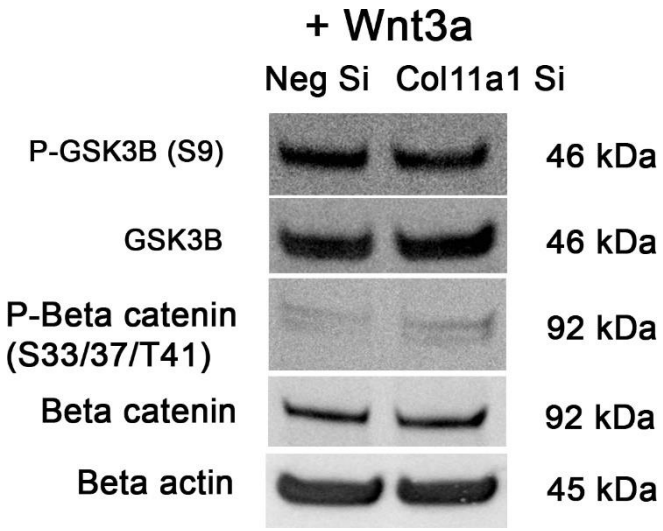

Figure 3 9 Inhibition of Col11a1 expression does not prevent Wnt3a induced phosphorylation of GSK3β or inhibition of β-catenin phosphorylation. The addition of 100 ng/mL of recombinant Wnt3a induced phosphorylation of GSK3B and inhibited β-catenin phosphorylation.

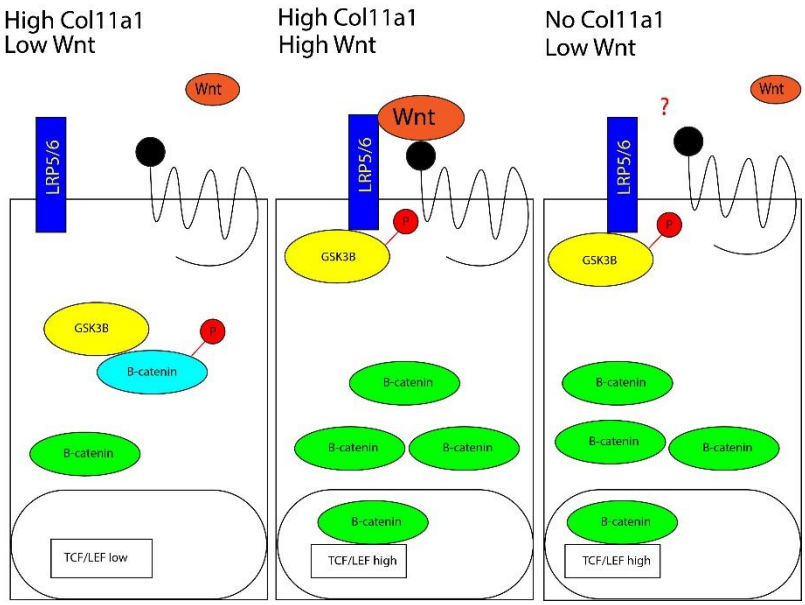

Figure 3 10. Model of β-catenin signaling in the absence of Col11a1 expression. In the absence of Wnt or under low Wnt conditions GSK3β phosphorylates β-catenin and leads degradation, keeping cytoplasmic levels low and preventing nuclear translocation. High levels of Wnt lead to receptor complexes that recruits GSK3β away from β-catenin and leads to GSK3β phosphorylation and subsequent accumulation of B-catenin and increased TCF/LEF activity. Inhibition of Col11a1 expression creates a high Wnt like situation where the cells responds to low Wnt levels with increased TCF/LEF activity, decreased β-catenin phosphorylation and increased GSK3β phosphorylation. Collagen α1(XI) chain may prevent Wnt receptor complexes from forming and therefore aberrantly activating β-catenin signaling pathways.

Table 3.1 Table of Primers used for RT-PCR

Target	Forward Sequence	Reverse Sequence
Mouse Col2a1	ACGAAGCGGTGGCAACCTCA	CCCTCGGCCCTCATCTCTACATCA
Mouse HPRT	CTGGTGAAAAGGACCTCTCGAA	CTGAAGTACTCATTATAGTCAAGGGCAT
Mouse PPIA	CGCGTCTCCTTCGAGCTGTTTG	TGTAAAGTCACCACCCTGGCACAT
Mouse Col10a1	TGCCCGTGTCTGCTTTTACTGTCA	TCAAATGGGATGGGGGCACCTACT
Mouse Sox9	GAGGCCACGGAACAGACTCA	CAGCGCCTTGAAGATAGCATT
Mouse Acan	CCTCGGGCAGAAGAAAGA	GTCTCATGCTCCGCTTCTGT
Mouse Mmp13	AGTTGACAGGCTCCGAGAAA	GGCACTCCACATCTTGGTTT
Mouse Col11a1	TGGAAACCCACACCGGAAA	TGCCTCTGTTTGTGCTACTGT

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