

EFFECTS OF BONE MORPHOGENETIC PROTEIN-2 AND PARATHYROID  
HORMONE-RELATED PEPTIDE ON COLLAGEN XI DURING SKELETAL  
DEVELOPMENT

by

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of the requirements for the degree of  
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## ABSTRACT

Long bones develop via endochondral ossification, a process in which cartilage precedes bone. During endochondral ossification prechondrogenic cells undergo proliferation and apoptose as cells of the periosteum differentiate into bone. The process of endochondral ossification is regulated by a group of endocrine as well as paracrine molecules that includes bone morphogenetic protein-2 (BMP-2) and parathyroid hormone-related peptide (PTHrP). Collagens are extracellular matrix molecules that are present in a spatiotemporal manner during endochondral ossification. Collagen XI alpha 1 (Col11a1) is a minor fibrillar collagen that is alternatively spliced during development and can result in up to eight different spliceforms. During endochondral ossification, Col11a1 spliceforms have a distinct expression profile in the growing long bone. The significance of the different spliceforms has not been characterized. Here, I studied the effects of BMP-2 and PTHrP on the expression of Col11a1 in pre-chondrogenic mouse ATDC5 and pluripotent mouse C2C12 cells. I also investigated the role of different Col11a1 spliceforms in BMP-2-mediated C2C12 osteoblast differentiation. My results suggest a role for BMP-2 in regulating Col11a1 splicing and expression that includes the induction of exons 6A, 7, and 8. Further I show that while PTHrP alone did not affect Col11a1 expression, when combined with BMP-2, PTHrP was able to diminish BMP-2-induced Col11a1 expression. In addition, Col11a1 NTD[p6b-7] reduced BMP-2 activity in C2C12 cells while Col11a1 NTD[p7] did not have any significant effects. Col11a1 NTD[p6b-7] also reduced the expression of alkaline phosphatase and collagen 1 alpha 1

and increased the expression of Runx2 and osteocalcin while NTD[p7-8] induced the expression of osteocalcin, Runx2, and collagen alpha 1. When Col11a1 was knocked down, the expression of BMP-2-induced markers alkaline phosphatase, Runx2, osteocalcin, and collagen 1 alpha 1 were reduced at 24h post induction with BMP-2, suggesting a role for collagen XI alpha 1 during early osteoblast differentiation. In ATDC5 cells, I determined a role for PTHrP in the induction of collagen XI alpha 1 exons 6A, 7, and 8 during early chondrocyte differentiation and exon 6B during late chondrocyte differentiation. Overall the results of this project show a novel role for collagen XI alpha 1 in BMP-2-mediated osteoblast differentiation and also suggest a role for PTHrP in regulating collagen XI alpha splicing and expression during ATDC5 chondrocyte differentiation.

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## INTRODUCTION

The skeleton starts forming during the second month of human development. Initially, mesenchymal cells condense and define the position and basic shape of the future skeletal element. Once the initial mesenchyme condensations are formed, depending on the growth factors present in the immediate environment, cells either differentiate into osteoblasts to form bone or into chondrocytes to form cartilage. Wnt signaling drives mesenchymal cells to differentiate into osteoblasts. In the absence of Wnt signaling, SRY (Sex Determining Region Y)-Box 9 (Sox9) levels increase and mesenchymal cells differentiate into prechondrogenic cells. Once committed, prechondrogenic cells start producing major cartilage extracellular matrix proteins, including collagens II and XI. Once prechondrocytes are formed, cartilage starts growing rapidly in an appositional and interstitial manner, a process known as endochondral ossification. Interstitial growth occurs as prechondrocytes differentiate into chondrocytes, which then enlarge and apoptose. As chondrocytes hypertrophy, the perichondrium is infiltrated with blood vessels and the periosteum is formed. The periosteum houses mesenchymal cells that upon getting a signal from hypertrophic cells differentiate into osteoblasts. Bone is formed as osteoblasts start producing a non-mineralizing fibrillar extracellular matrix, which is then organized into mineralized matrix. Proper bone growth is dependent upon a complex network of systemic and local growth factors. Sox9 is essential in the initial commitment into cartilage. Prechondrocytes and cells of the perichondrium both synthesize Parathyroid hormone related Peptide (PTHrP) to maintain

chondrocytes in a proliferative state. Prehypertrophic cells on the other hand produce Indian Hedgehog (IHH), which promotes hypertrophy. Simultaneously, cells of the periosteum respond to growth factors released by hypertrophic chondrocytes and promote differentiation of mesenchymal cells into osteoblasts. Bone morphogenetic protein-2 (BMP-2) is crucial for promoting chondrocyte hypertrophy as well as osteoblast differentiation in the growth plate. Interstitial and appositional growth processes are linked and the accurate orchestration of different mediators achieves proper bone growth. Fibrillar collagens are abundant extracellular matrix proteins that provide structure and support for cells within the growth plate. During endochondral ossification, collagens are expressed in a tightly regulated spatiotemporal manner. The major fibrillar collagen present in cartilage is collagen type II, whereas collagen type I is predominantly found in bone. Collagen XI is a minor yet essential component of collagen fibrils in the extracellular matrix. Collagens are formed as three procollagen chains come together and form a heter- or homo-trimer in the extracellular matrix. Collagen XI alpha 1 undergoes alternative splicing during development and the different spliceforms show a distinct spatiotemporal expression pattern. My goal in this project was to characterize the expression of collagen XI alpha I alternative spliceforms in mouse chondrocytes and pluripotent stem cells. Further, I assessed the effects of two essential growth factors PTHrP and BMP-2 on collagen XI expression during endochondral ossification. I hypothesized that PTHrP and BMP-2 regulate collagen XI alpha 1 expression.

To test my hypothesis, I used two different cell models of pluripotent mouse C2C12 cells and prechondrogenic mouse ATDC5 cells to assess PTHrP effects on the expression of collagens XI utilizing quantitative real-time PCR. My findings indicate that

PTHrP regulates collagen XI alpha 1 spliceform expression during chondrogenesis. I also show that PTHrP alone does not change the expression of collagen XI alpha 1 in pluripotent C2C12 cells.

Further, I used the mouse pluripotent C2C12 cells and assessed the role of BMP-2 in collagen XI alpha 1 expression during osteoblast differentiation. The results of this study clearly show that BMP-2 regulates collagen XI alpha 1 expression and that collagen XI is essential for canonical BMP-2-induced signaling and thus osteoblast differentiation in C2C12 cells.

In conclusion, my work here shows that 1) continuous treatment of pluripotent C2C12 cells with PTHrP does not affect collagen XI expression; 2) PTHrP changes the expression of collagen XI spliceforms during ATDC5 chondrogenesis *in vitro*; and 3) Collagen XI alpha 1 spliceforms regulate osteoblast differentiation in a BMP-2-dependent manner.

CHAPTER ONE: Col11a1 REGULATES OSTEOBLAST PROLIFERATION AND  
DIFFERENTIATION AND IS ESSENTIAL FOR PERIOSTEAL BONE FORMATION

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**Col11a1 regulates osteoblast proliferation and differentiation and is essential for periosteal bone formation**

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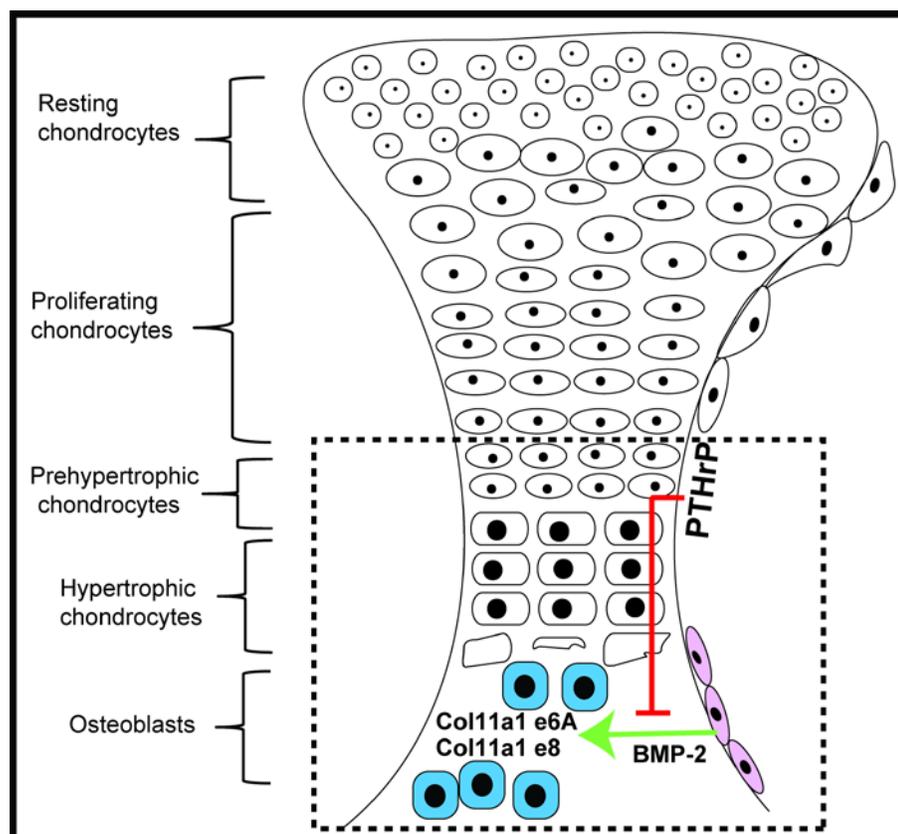
## Abstract

Collagen XI is an extracellular matrix protein required for embryonic development with a role in both nucleating the formation of fibrils and regulating the diameter of heterotypic fibrils during collagen fibrillar assembly. Although found in many different vertebrate tissues throughout the body, Col11a1 is best known for its role in cartilage during endochondral ossification. To further understand the function of Col11a1 in the process of bone formation, we compared skeletal mineralization in wildtype (WT) mice and Col11a1-deficient mice. Overgrowth of the periosteal bone collar of the developing long bones was observed in the case of Col11a1-deficient mice compared to WT littermates. The overgrowth was observed on the outside surface of the bone. To investigate the role of Col11a1 in osteoblast differentiation, we analyzed the regulation of Col11a1 expression in multipotent C2C12 cells. We found that BMP-2 and PTHrP regulate both the level of expression as well as the pre-mRNA splicing of Col11a1. BMP-2 promoted the inclusion of alternatively spliced exons 6A and 8 of the variable region; however, PTHrP inhibited this effect. While Col11a1 knockdown in pre-osteoblasts initially reduced the BMP-2-induced expression of osteoblast markers osteocalcin (OCN), runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), and Collagen I alpha I (Col1a1), at later time points, the opposite effect was observed, and the knockdown of Col11a1 promoted the BMP-2-induced expression of osteoblast markers. Introduction of recombinant Col11a1 fragments during osteoblast differentiation also osteoblast-specific isoforms of recombinant Col11a1 promoted BMP signaling. In contrast, chondrocyte-specific recombinant isoform Col11a1 [p6b-7] inhibited BMP-2-stimulated expression of osteoblast markers and BMP activity. Lastly,

Col11a1 regulated C2C12 proliferation in a spliceform-specific manner. These results show for the first time that Col11a1 expression and alternative splicing is regulated by BMP-2 and suggests a novel role for Col11a1 spliceforms in osteoblast differentiation and bone collar formation during endochondral ossification.

**Keywords:** Col11a1, BMP-2, C2C12, PTHrP, alternative splicing, gene expression.

**Graphical abstract:**

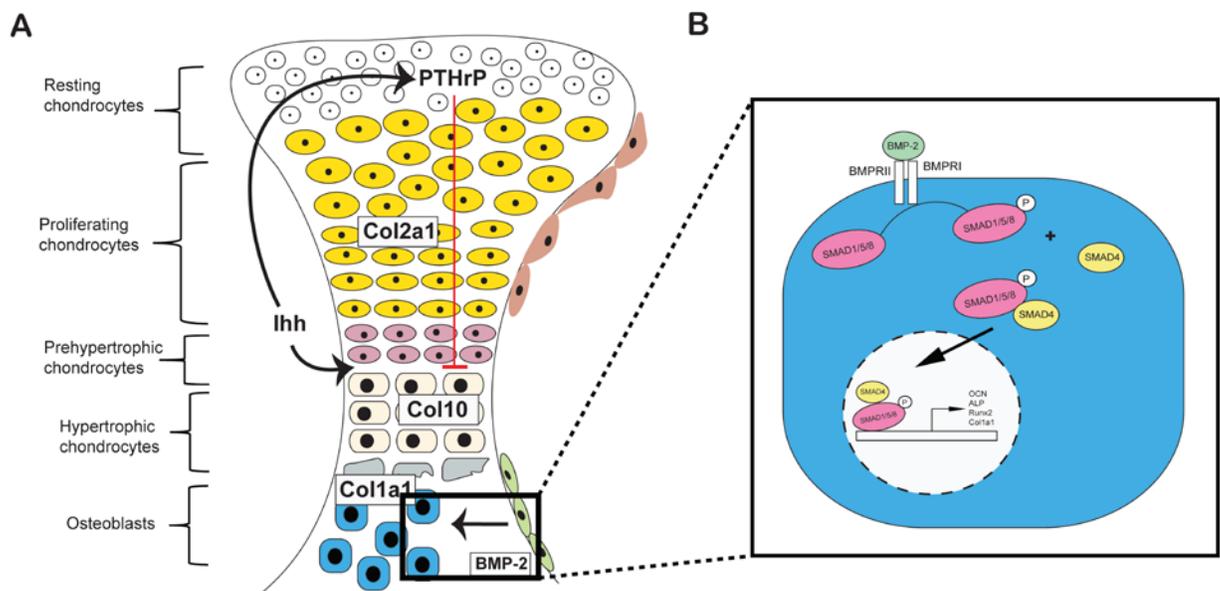


**Highlights**

- Col11a1-deficient mice exhibit increased bone collar thickness compared to wildtype.
- BMP-2 and PTHrP regulate the expression of *Col11a1* during osteoblast differentiation.
- *Col11a1* isoforms regulate the BMP-2-induced differentiation of C2C12 cells into osteoblasts.

## Introduction

The skeleton develops via processes of endochondral and intramembranous ossification. Long bones elongate by means of interstitial growth and widen via appositional growth. Interstitial growth of long bones occurs via endochondral ossification in which cartilage is replaced by bone whereas appositional growth occurs as progenitor cells of the periosteum differentiate into osteoblasts and deposit bone. The process of bone development starts during fetal life and persists until puberty when growth ceases. In a typical long bone, endochondral ossification occurs at the growth plate, which consists of specific zones (Figure 1A) [1].



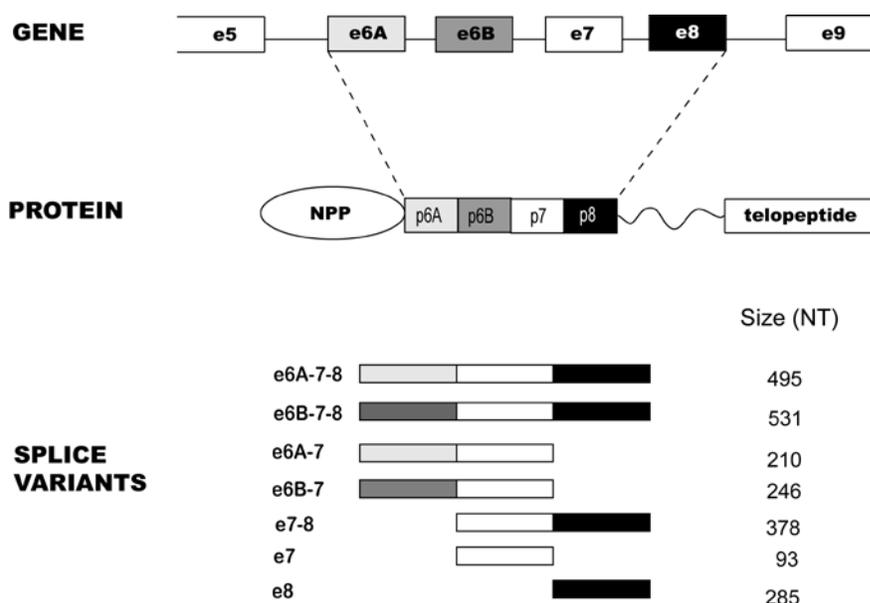
**Figure 1. Endochondral ossification in the growth plate starts with prechondrocytes that undergo initial differentiation into chondrocytes.** Proliferative chondrocytes apoptose and cartilage is replaced by bone as mesenchymal stem cells of the periosteum differentiate into osteoblasts. PTHrP and BMP-2 are key players in the process of endochondral ossification. While PTHrP is widely known for its role in maintaining chondrocyte proliferation and inhibiting terminal differentiation, BMP-2 is an inducer of chondrocyte hypertrophy as well as osteoblast differentiation via the canonical SMAD signaling pathway.

The resting zone contains pre-chondrocytes that differentiate into mature chondrocytes forming the proliferative zone. Proliferating chondrocytes then align into columns, terminally differentiate into hypertrophic chondrocytes, and undergo apoptosis. As hypertrophic chondrocytes apoptose, pre-osteoblast progenitor cells of the periosteum differentiate into osteoblasts, the newly formed bone tissue is innervated, blood vessels infiltrate, and calcification occurs. The highly dynamic environment of the growth plate involves a complex network of hormones, paracrine molecules, and growth factors that work together to facilitate processes of cell proliferation and differentiation that lead to proper tissue development.

Collagens are abundant extracellular matrix proteins that maintain tissue structure and control the environment in which cells find themselves [2]. Mutations in collagen XI have been linked with rare and detrimental human diseases including autosomal dominant Marshall's and Stickler syndromes, the more severe autosomal-recessive fibrochondrogenesis, and otospondylomegaepiphyseal dysplasia (OSMED) [3–8]. In cartilage, mature collagen XI protein consists of three different alpha chains: Col11a1, Col11a2, and Col11a3 (an overglycosylated form of Col2a1). However, in bone, a collagen V/XI hybrid molecule constitutes the minor fibrillar collagen, consisting of Col5a1, Col5a2, and Col11a1. Targeted mutations of Col11a1 in mice closely reflect characteristics of the human diseases. Mice lacking Col11a1 die neonatally and exhibit a phenotype that includes facial dysmorphism and wider but shorter metaphyses in the long bones, suggesting a role for Col11a1 in proper growth plate development [9–11]. While a direct effect on growth plate cartilage has been defined, the consequences of absent or

reduced levels of Col11a1 expressed by osteoblasts has not been investigated. Col11a2 null mice exhibit a milder cartilage phenotype similar to OSMED patients [12, 13].

The Col11a1 alpha chain of collagen XI contains a non-collagenous amino terminal domain (NTD) composed of an amino propeptide (Npp) and a variable region (VR) [14, 15] (Figure 2 ).



**Figure 2.** The variable region within the NTD domain of collagen XI alpha 1 undergoes alternative splicing that can result in several different isoform. Each spliceform is expressed in a distinct spatiotemporal manner during development. The exact function of alternative splicing in collagen XI alpha 1 is yet to be determined (Adapted from [19]).

The NTD is found on the surface of heterotypic collagen fibrils and sterically hinders further addition of collagen molecules, thus regulating fibril diameter [16, 17]. Interestingly, alpha chains of collagen XI undergo alternative splicing. Col11a2 splice variants quickly converge to produce a single splice isoform during development [18]. Conversely, the variable region of Col11a1 undergoes alternative splicing in a spatiotemporal manner and the different spliceforms persist as mature proteins in the ECM, suggesting a role in maintaining or directing matrix events by not only mediating

fibrillogenesis but also by other mechanisms such as interaction with other extracellular matrix molecules [19–22].

## **Materials and Methods**

### Mice

The embryos used in this study were provided by Dr. Robert Seegmiller (Brigham Young University). The mice were housed and euthanized as approved by the Institute of Animal Care and Use Committee of Brigham Young University. All embryos used in this study were at embryonic day 17.5. A total of six wildtype (WT) (+/+) and three homozygous (-/-) on a C57Bl6 background were analyzed.

### Micro-CT Analysis

Embryos were scanned with a SkyScan 1172 high-resolution micro-CT scanner (MicroPhotonics, Aartselaar, Belgium) to generate data sets with a  $1.7 \mu\text{m}^3$  isotropic voxel size using an acquisition protocol that consisted of X-ray tube settings of 60 kV and 250  $\mu\text{A}$ , exposure time of 0.147 seconds, six-frame averaging, a rotation step of 0.300 degrees, and associated scan times were approximately 7 hours. Following scanning, two-dimensional reconstructions were used to produce 6000 serial 4000 x 4000 pixel cross-sectional images. Three-dimensional models were reconstructed using a fixed threshold to analyze the mineralized bone phase using ImageVis 3D software (University of Utah, Center for Integrative Biomedical Computing, Salt Lake City, UT). A Gaussian filter ( $\sigma=1.0$ , kernel=3) was used to remove high frequency noise followed by an adaptive threshold to segment the 3D images, which were visually checked to confirm inclusion of complete volume of interest.

Gross geometric measurements were performed using Skyscan CT Analyzer (CTAn) Software (MicroPhotonics, Aartselaar, Belgium). Comparisons of shape and cross-sectional area were conducted for long bones. CTAn was used to determine trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp). Bone volume (BV) and bone surface (BS) were calculated based on the hexahedral marching cubes volume model of the binarized objects within the volume of interest and the faceted surface of the marching cubes volume model, respectively. Total tissue volume (TV) was defined as the volume-of-interest. Trabecular bone volume fraction (BV/TV) was calculated from BV and TV values. Cross-sectional reconstructions were color-coded according to three density ranges: high-density range (white), intermediate-density range (blue), and low-density range (green).

#### Cell Culture and Differentiation

The mouse pluripotent cell line, C2C12 was purchased from ATCC and maintained in DMEM (Sigma Chem. Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY) and antibiotics (100 units/mL of penicillin-G and 100 µg/mL streptomycin). Cells were kept at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. To differentiate C2C12 cells into osteoblasts, cells were plated at  $2 \times 10^4$  cells/cm<sup>2</sup> in DMEM supplemented with 5% FBS, 100U/mL penicillin and 100 µg/mL streptomycin and recombinant human BMP-2 (300 ng/mL) (R&D Systems, Minneapolis, MN). Control cells were kept in DMEM supplemented with 5% FBS and antibiotics. Medium was changed every 72 hours and supplemented with fresh BMP-2. For BMP-2 and PTHrP experiments, cells were treated with BMP-2 for five days after which PTHrP ( $10^{-7}$  M) (Sigma Aldrich; St. Louis, MO) was added on day 5 for 24

hours, and RNA was harvested on day 6. For recombinant Col11a1 experiments, DNA encoding fragments including exons 2-5, 6B, 7, 8, and 9 were amplified, ligated into expression vectors and expressed as previously described [17]. Recombinant proteins were purified, refolded, and characterized as previously described [17, 61]. Col11a1 N-terminal fragments were added to C2C12 cells in culture at a concentration of 10 – 60  $\mu\text{g/mL}$ .

#### Transfection of Cells with Small Interfering RNA

C2C12 cells were grown to 70-80% confluency. Media was then removed and cells were rinsed with Phosphate Buffer Saline (PBS) twice and 2 mL of serum-free DMEM was added into each well of a 6-well plate. Col11a1 or SMAD4 siRNA and scrambled control siRNA (10  $\mu\text{M}$ ) (Life Technologies, Carlsbad, CA) were diluted in 300  $\mu\text{L}$  serum free OPTIMEM (Life Technologies, Carlsbad, CA) and mixed with nine microliters of RNAiMAX Lipofectamine (Life Technologies, Carlsbad, CA) diluted in 300  $\mu\text{L}$  serum-free OPTIMEM. The mixture was incubated at room temperature for 15 min and then 250  $\mu\text{L}$  of mixture was added into each well of a 6-well plate that already contained 1.75 mL of serum-free DMEM. Cells were incubated with siRNA-containing medium for 24 hours at 37°C in a humidified chamber containing 5% CO<sub>2</sub>. The final concentration of the siRNA was 15 nM. A fluorescent siRNA oligonucleotide (siGLO, Dharmacon, Lafayette, CO) was used to confirm efficient delivery of siRNA to 71%  $\pm$  5.1 (SD) of the cells. After treatment, RNA was extracted as described below to confirm Col11a1 knockdown and to determine the expression levels of osteoblast markers. Scramble control siRNA experiments were carried out in an identical manner to account for any non-specific effects. Cells were treated with BMP-2 (300 ng/mL) for 24h

and 72h after the 24h transfection to analyze the effect of diminished Col11a1 levels on BMP-2 signaling activity. For BMP-2 treatment, after the 24h Col11a1 siRNA transfection, media was replaced with DMEM containing 5% FBS and BMP-2 (300 ng/mL) and incubated for 24h and 72h. RNA was extracted as described below.

#### Semi-Quantitative Polymerase Chain Reaction

Total RNA was extracted from cells using TriZol (Gibco-BRL; Grand Island, NY) and 2 µg of RNA was used to synthesize cDNA using High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Life Technologies, Carlsbad, CA). Twenty-five microliter PCR reactions were prepared using 12.5 µL GoTaq Colorless Master Mix (Promega, Madison, WI), 1 µL of each forward and reverse primer (10 µM), 3 uL undiluted cDNA, and 7.5 uL nuclease-free water. Samples were amplified for 32 cycles, with denaturation at 95°C (3 min), annealing at 57°C (1 min), and extension at 72°C (30 sec). Col11a1 forward primer was designed to anneal within exon 5 and the reverse to anneal within exon 9, flanking the variable region: 5'- CAG GAG CCG CAC ATA GAT GAG-3' (forward), 5'- TTT CTC TCC ATA TGC GCC AT-3' (reverse), generating a defined set of PCR products reflecting the alternative splicing patterns of the specific cell type. To account for any difference in the amount of RNA, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen as endogenous control. The amplification products were separated by electrophoresis through 4% Nusieve 3:1 Agarose (Lonza, Basel, Switzerland) gels according to manufacturer's instructions and visualized under UV light after staining with ethidium bromide.

### Primer Design and Quantitative Real-Time PCR

Primers were designed for each exon using Primer Blast (NCBI) (**Table 1**). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies; Carlsbad, CA). Each 20  $\mu$ L PCR reaction consisted of 10  $\mu$ L SYBR Green PCR Master Mix, 500 nM of each forward and reverse primer, and 1  $\mu$ L of undiluted template cDNA plus nuclease-free water. Targets were amplified using an Eppendorf real-time PCR Mastercycler as following: 50°C (2 min), 95°C (10 min) followed by 40 cycles of 95°C (15 sec) and 60°C (1 min) followed by one cycle of 72°C (1 min). Expression levels were quantified relative to housekeeping gene levels of expression and presented as  $2^{-\Delta Ct}$  values to reflect the ratio of expression level of the gene of interest to that of the housekeeping gene. PCR products were separated by electrophoresis through 4% Nusieve 3:1 Agarose (Lonza, Basel, Switzerland) to verify that the primer pair produced a single product of the expected size. A 'no-template' control was also included in the assay substituting water for template as well as a control omitting reverse transcriptase to control for the possibility of genomic DNA contamination.

**Table 1: Real-time PCR primers were designed to amplify different Col11a1 exons within the variable region of the NTD using NCBI Primer Blast. Primers were purchased from Integrated DNA Technologies and resuspended in nuclease-free water. All primers were used at Annealing temperature of 60 °C. For maximum efficiency, PCR amplicon sizes were kept below 200bp and products were ran through agarose electrophoresis to validate amplification.**

| <b>GENE</b> | <b>SENSE PRIMER</b>     | <b>ANTISENSE PRIMER</b>  |
|-------------|-------------------------|--------------------------|
| ALP         | GTGCCCTGACTGAGGCTGTC    | GGATCATCGTGTCCTGCTCAC    |
| COL1A1      | GCATGGCCAAGAAGACATCC    | CCTCGGGTTTCCACGTCTC      |
| COL11A1 e6A | AGGCTGAGAGTGTAACAGAGA   | TCTGTTTGTGCTACTGTTTCTTCA |
| COL11A1 e6B | GTTACATCCCCAAATCTGA     | CCCCTAGTTTGGCTTTGGCT     |
| COL11A1 e7  | GGAACAATGGAACCTTACCAGAC | ATTGATCCTGATACCCGCC      |
| COL11A1 e8  | AGGAGTAGACGGCAGGGATT    | GGAGGTCGTAGTCCTTTCTTCA   |
| OSTEOCALCIN | CCGGGAGCAGTGTGAGCTTA    | TAGATGCGTTTGTAGGCGGTC    |
| PPIA        | CGCGTCTCCTTCGAGCTGTTTG  | TGTAAAGTCACCACCCTGGCACAT |
| RUNX2       | GTGCGGTGCAAACCTTCTCC    | AATGACTCGGTTGGTCTCGG     |

#### Western Blot Analysis

To confirm SMAD phosphorylation upon BMP-2 treatment, C2C12 cells were plated at  $2 \times 10^4$  cells/cm<sup>2</sup> and grown overnight. The next day, medium was changed to DMEM containing 5% FBS and BMP-2, and incubated for 30 minutes. Cells were then rinsed twice with cold PBS and lysed using 250  $\mu$ L of cold M-PER lysis buffer (Pierce, Rockford, IL) containing protease and phosphatase inhibitors (Halt Protease Inhibitor<sup>TM</sup>, Pierce, Rockford, IL). A 25  $\mu$ l aliquot was used to determine protein concentration using a BCA assay (Bio Rad; Hercules, CA). Twenty micrograms of protein samples were combined with NuPAGE LDS sample buffer and  $\beta$ -mercaptoethanol (Life Technologies; Carlsbad, CA) and incubated at 70°C for 10 minutes. Proteins were then separated by 4-12% SDS-PAGE gradient gel and transferred to nitrocellulose membranes using iBlot® 7-minute iblotting system (Life Technologies; Carlsbad, CA). Membranes were blocked for 1 hr at room temperature using SuperBlock solution (Life Technologies, Carlsbad,

CA), washed 3 x 5 min with 10 mM Tris, pH 8 containing 150 mM NaCl, and 0.05% Tween-20 (TBST) while gently rocking and probed with antibodies as described (**Table 2**) overnight at 4°C. After 3 x 5 min washes with TBST, horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5000 dilution in blocking buffer, Cell Signaling, Danvers, MA) was added for 1 hr at room temperature. Blots were then washed 3 x 5 min with TBST and incubated in SuperSignal West Femto Maximum Sensitivity Substrate<sup>TM</sup> (Pierce, Rockford, IL) for 1 min and exposed for 5 min on a Kodak Imager 4000R imaging system. Constant substrate concentration was achieved by acquiring images while the nitrocellulose membrane was completely submerged in ECL solution. ECL images were taken in the native format using the system's standard software KODAK and exported to 16-bit TIFF format. Analysis of ECL images was performed using the public domain *ImageJ* program (developed at the National Institutes of Health and available at <http://rsb.info.nih.gov.libproxy.boisestate.edu/ij/>), using the “Gel Analysis” functions. Background correction was done using “background subtractor” tool. Integrated Density Value (IDV) of each band was used to quantify the signals.

**Table 2: Antibodies were purchased from Cell Signaling and used at the indicated dilutions in blocking buffer overnight at 4°C.**

| NAME                       | COMPANY        | CAT# | ISOTYPE    | DILUTION | MW    | SPECIFICITY |
|----------------------------|----------------|------|------------|----------|-------|-------------|
| Beta-Actin                 | Cell Signaling | 4967 | Rabbit     | 1:5000   | 45kDa | Polyclonal  |
| Smad1<br>(D59D7)           | Cell Signaling | 6944 | Rabbit IgG | 1:1000   | 60kDa | Monoclonal  |
| Phospho-Smad1/5<br>(41D10) | Cell Signaling | 9516 | Rabbit IgG | 1:1000   | 60kDa | Monoclonal  |

### Immunofluorescence

C2C12 cells were plated on sterile glass coverslips in the presence and absence of BMP-2 (300 ng/mL). Media was removed and cells were rinsed twice with PBS. Cells were then fixed with 1:1 ice-cold acetone:methanol for 15 min and rinsed twice with PBS. Coverslips were then washed 3 x 5 min with PBS while gently rocking and then blocked with 2% BSA for 1 hour at room temperature. Antibodies specific for SMAD1 and Phospho-SMAD1/5/8 were used at the indicated dilutions (**Table 2**) in 0.5% BSA in PBS (PBB) and coverslips were incubated at 4°C overnight while gently rocking. Cells were subsequently washed 5 x 5 min in PBB. A Rhodamine (TRITC)-conjugated AffiniPure Donkey Anti-rabbit IgG (2.5 µg/mL) (Jackson ImmunoResearch Laboratories) in PBB was added to the cells and incubated at room temperature in the dark for 1 hour. Coverslips were then washed 5 x 5 min with PBB and mounted on slides using ProLong® with DAPI (Life Technologies; Carlsbad, CA).

### Confocal Microscopy

Images were acquired using an LSM Meta 510 scanning confocal microscope (Zeiss, Germany) and ZEN 2009 imaging software (Carl Zeiss, Inc., Thornwood, NY). We used a pinhole of 1.5 Airy units and objective of 63x oil (NA 1.4). Excitation was provided by the 405 nm line of an Argon laser for DAPI and the 543 nm line of a HeNe laser for Rhodamine red-X. Images were acquired as confocal stacks (7-12 optical sections) with an optical section separation (z-interval) of 1.180 µm. Optical sections were acquired at a digital size of 512 x 512 pixels and averaged 4 times to reduce noise. Equivalent settings, including pinhole, filters and bandpass were used for all images.

### Luciferase Reporter Assays

To determine the effect of Col11a1 on BMP activity, cells were plated at 65,000 cells/well of a 24-well plate and incubated overnight. The following day, cells were washed twice with PBS and transfected with LT1 liposomes (Mirus, Madison, WI) containing 10 ng/well of CMV  $\beta$ -galactosidase control plasmid and 300 ng/well of BMP reporter element luciferase plasmid (a kind gift from Dr. Allan Albig, Boise, ID) for 24 hr. In designated experiments, cells were also transfected with 14 ng/well of Silencer Select Col11a1 siRNA (Life Technologies; Carlsbad, CA). The next day, cells were washed with PBS and medium was changed to DMEM supplemented with 5% FBS and treated with Col11a1 recombinant protein fragments and BMP-2 (300 ng/mL) for 24 hr. The following day, C2C12-BRE-luc cells were lysed in Reporter Lysis Buffer (Promega, Madison, WI) and frozen overnight. Lysates were then collected and enzyme activity was measured using the Luciferase Assay System and  $\beta$ -Galactosidase Enzyme Assay System (Promega, Madison, WI). Enzyme activities were measured in a microplate luminometer. The ratio of luciferase to  $\beta$ -galactosidase activity was calculated, and fold-induction was determined relative to control. Each data point represents the mean of results from three independent transfections.

### WST-1 Proliferation Assay

C2C12 cells were plated at a density of  $5 \times 10^3$  cells/100 $\mu$ L of growth medium in 96-well plates and grown overnight at 37°C. The next day, cells were rinsed twice with PBS and then treated with fresh medium containing Col11a1 recombinant protein fragments for 24 hr, 48 hr, and 72 hr. At each time point, ten microliters of WST-1 reagent (Clontech, Mountain View, CA) was added to each well and cells were incubated

for an additional 2 hr at 37°C in the dark. As a control, WST-1 was also added to empty and untreated wells to establish a blank baseline. The absorbance was measured using a BioTek microplate reader at a test wavelength of 450 nm and a reference wavelength of 630 nm.

## Results

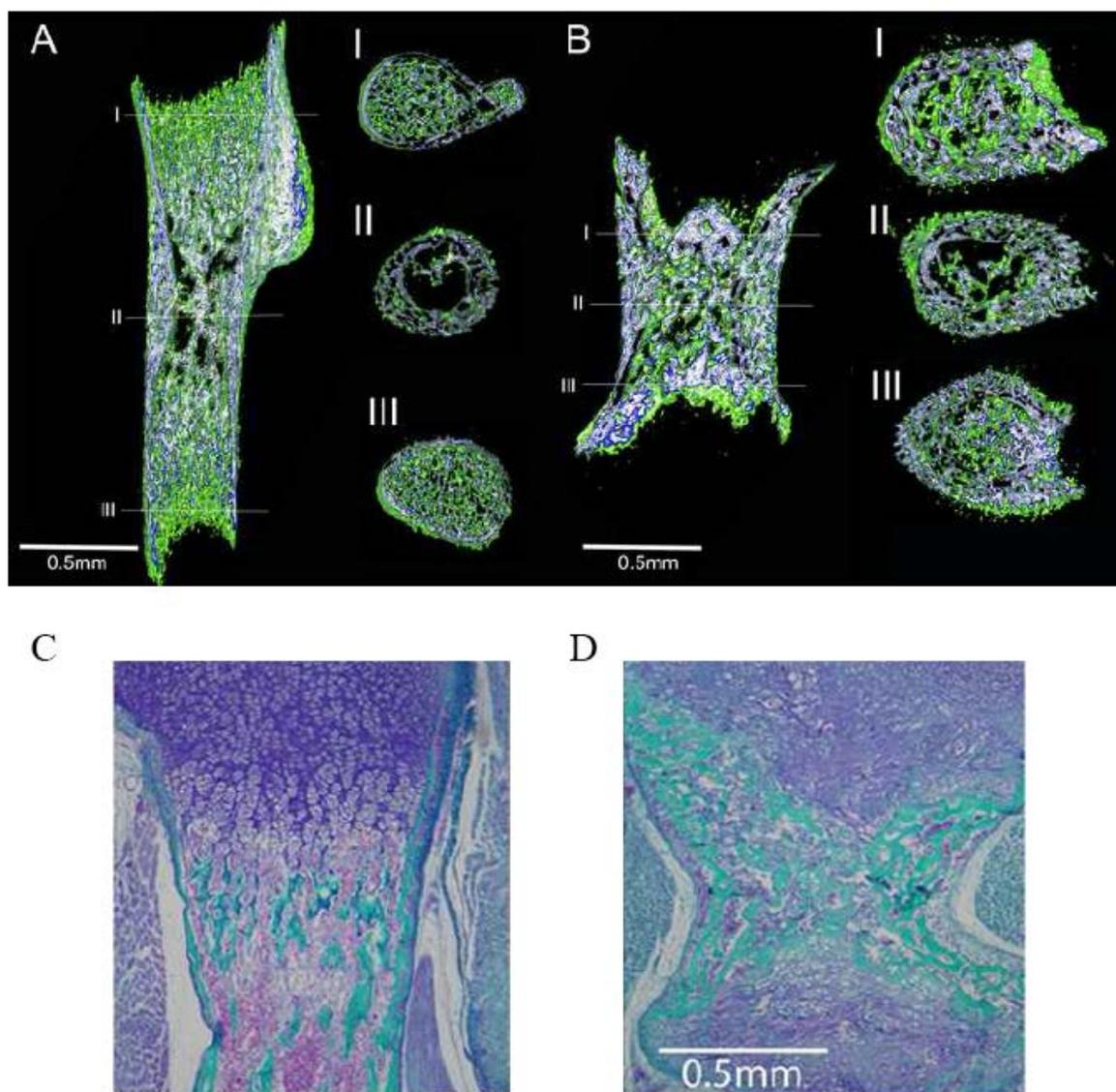
### Col11a1-Deficient Mice Exhibit Overgrowth of Bone Collar

Measurements obtained from X-ray  $\mu$ CT images indicated that the mineralizing portion of Col11a1-deficient mice long bones were an average of 41% shorter than the WT humerus and femur, which was in agreement with previously reported results obtained from whole embryos stained with Alizarin red and Alcian blue (**Table 3**).

**Table 3:** Structural indices for humeri are reported as mean +SD for WT and Col11a1<sup>-/-</sup>

| Genotype               | Length ( $\mu$ m) | Proximal Metaphysis diameter ( $\mu$ m) | Diaphysis diameter ( $\mu$ m) | Distal Metaphysis diameter ( $\mu$ m) |
|------------------------|-------------------|---|-------------------------------|---------------------------------------|
| WT                     | 2409 $\pm$ 33.6   | 806 $\pm$ 16.9                          | 592 $\pm$ 11.6                | 528 $\pm$ 15.2                        |
| Col11a1 <sup>-/-</sup> | 1422 $\pm$ 65.5   | 930 $\pm$ 38.4                          | 735 $\pm$ 37.4                | 778 $\pm$ 86.1                        |
| % difference           | 41.0              | 15.4                                    | 24.2                          | 47.4                                  |
|                        | $p < 0.0001$      | $p < 0.05$                              | $p < 0.05$                    | $p < 0.05$                            |

The bones of the Col11a1-deficient mice were wider at all points along the length (**Figures 3A** and **3B**) and on average were 24% wider at the diaphysis, 15% wider at the proximal metaphysis, and 47% wider at the distal metaphysis (**Table 3**). Interestingly, the Col11a1-deficient long bones displayed an overgrowth of mineralized tissue on the outer surface of the bone collar (**Figures 3C** and **3D**).



**Figure 3. Cross-section of humeri at diaphysis, distal, and proximal metaphyses.** Mineralized tissue was assigned a color dependent upon three density ranges: low density range (green), intermediate density (blue), and high density (white). *Col11a1*-deficient humerus was wider and more cylindrical than WT. C-D) Trabecular bone was more dense in *Col11a1*-deficient mice compared to WT at proximal metaphysis. Trabecular bone is less dense in *Col11a1*-deficient mice compared to WT at distal metaphysis. Bone collar is less dense but thicker in *Col11a1*-deficient mice compared to WT littermate. Scale bars = 0.5 mm. C-D) Histological differences in the humeri of WT and *Col11a1*-deficient mice. Trichrome staining was used to identify mineralized tissue (green), compared to cartilage tissue (blue). Proliferative and hypertrophic zone of the growth plate in *Col11a1*-deficient mice showed altered cellular density, cell size, and organization within the cartilage. Scale bar A and B = 0.5 mm; scale bars C, D, E, and F = 0.1 mm.

Trabecular thickness, trabecular separation, and trabecular percent bone volume were increased in the forelimb bones of the Col11a1-deficient mice compared to wildtype. Analysis of microarchitectural indices at the proximal metaphysis of the humerus showed differences in trabecular thickness (92% increase in Tb.Th), trabecular separation (17% increase in Tb.Sp), and trabecular percent bone volume (73% increase in BV/TV) in the absence of Col11a1 expression. No significant difference was detected for isotropy values or structure model index, indicating similar relative prevalence of rods and plates in the three-dimensional structure of the trabecular bone for WT and Col11a1-deficient mice (**Table 4**).

**Table 4: Densitometric indices for the humeri (mean±SD) WT and Col11a1<sup>-/-</sup>.** Trabecular bone volume fraction (BV/TV) was calculated from Bone Volume (BV) and Trabecular Volume (TV) values. Skyscan CT Analyzer was used to determine trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp). Statistical differences are reported as p values unless determined to be not significant (ns). Control mice (n = 6), Col11a1-deficient mice (n = 3).

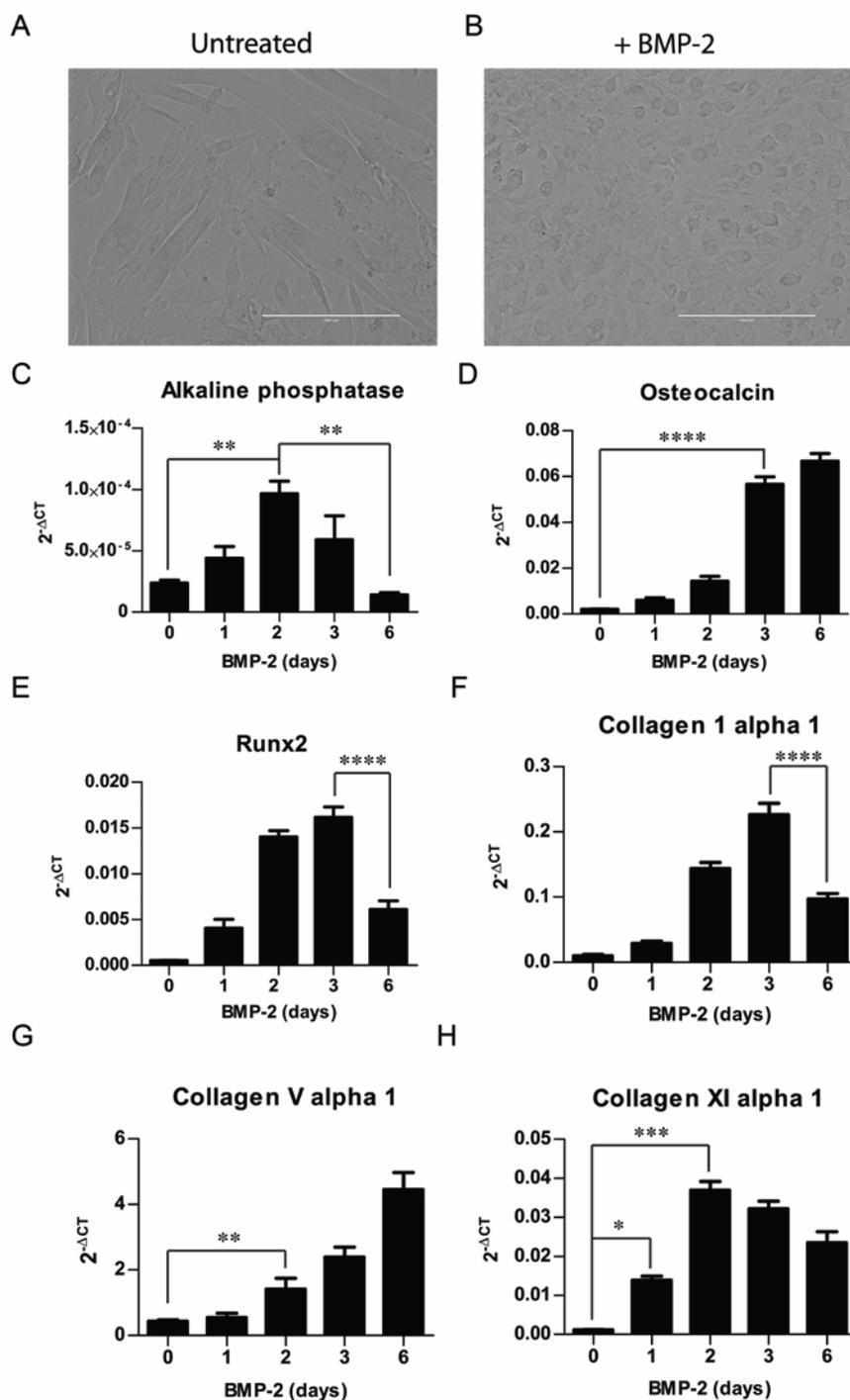
| Genotype               | BV/TV (%)               | DA           | Tb.Th (μm)              | Tb.N (1/mm) | Tb.Sp (μm)              |
|------------------------|-------------------------|--------------|-------------------------|-------------|-------------------------|
| WT                     | 25.3 ± 2.9              | 0.90 ± 0.025 | 18.32 ± 0.22            | 13.8 ± 1.5  | 33.2 ± 1.81             |
| Col11a1 <sup>-/-</sup> | 43.7 ± 4.1              | 0.84 ± 0.082 | 35.3 ± 3.7              | 12.4 ± 0.81 | 38.9 ± 1.19             |
| % difference           | 72.7<br><i>p</i> < 0.05 | 0.1<br>ns    | 91.6<br><i>p</i> < 0.05 | 10.2<br>ns  | 17.2<br><i>p</i> < 0.05 |

#### BMP-2 Induces the Expression of Col11a1 During Osteoblast Differentiation in a SMAD-Dependent Manner

It has been shown that BMP-2 regulates periosteal bone formation during development and we have previously shown that Col11a1 plays a role in BMP-2-mediated alkaline phosphatase (ALP) expression [30, 56]. To evaluate a potential role for

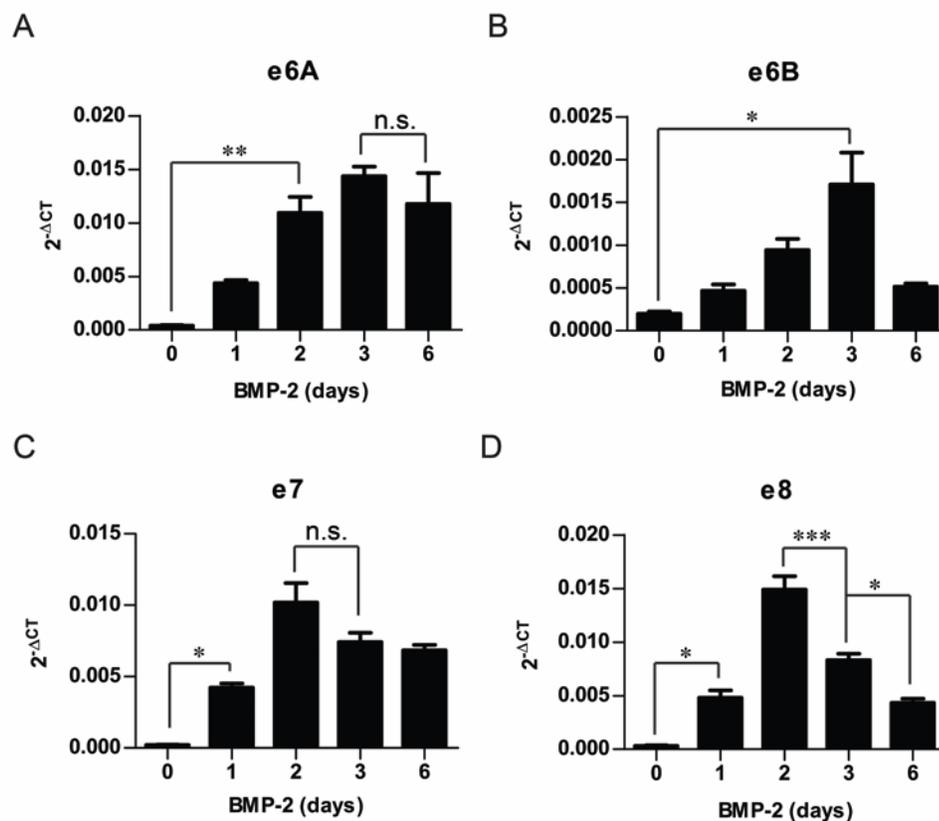
BMP-2 in the regulation of Col11a1 during osteoblast differentiation, we used the pluripotent mesenchymal C2C12 cell line. When treated with BMP-2, C2C12 cells changed their morphology from spindle to cuboidal-shaped cells (**Figure 4A** and **4B**). To confirm that BMP-2 induced osteoblast differentiation under our culture conditions, we assessed the expression of well-established osteoblast markers alkaline phosphatase (ALP), osteocalcin (OCN), runt-related transcription factor 2 (Runx2), and collagen I alpha 1 (Col1a1). Our results showed an initial increase in early osteoblast differentiation marker ALP up to day 2 that was followed by a decrease (**Figure 4C**). Similarly, Runx2 and Col1a1 expression increased up to day 3 followed by a decrease on day 6, while late osteoblast marker OCN levels persistently increased reaching maximum expression on day 6 (**Figures 4D, 4E, and 4F**). Further, BMP-2 induced the expression of Col5a1 in a time-dependent manner during osteoblast differentiation (**Figure 4G**).

To determine if BMP-2 induces Col11a1 expression in C2C12 cells during osteoblast differentiation, we treated cells with BMP-2 over a time course of 6 days. We analyzed expression levels of Col11a1 as well as alternative exon expression on days 1, 2, 3, and 6 by quantitative real-time PCR. Our results demonstrate an induction of Col11a1 expression by BMP-2 (**Figure 3H**) and an early and persistent increase in exon 6A up to day 6 (**Figure 5A**). The expression of exons 7 and 8 increased up to 2-3 days and then decreased or plateaued (**Figures 5C and 5D**). Exon 6B expression was relatively low compared to other exons and only showed a slight increase on day 3 (**Figure 5B**).



**Figure 4. BMP-2 induces osteoblast differentiation in pluripotent C2C12 cells.**

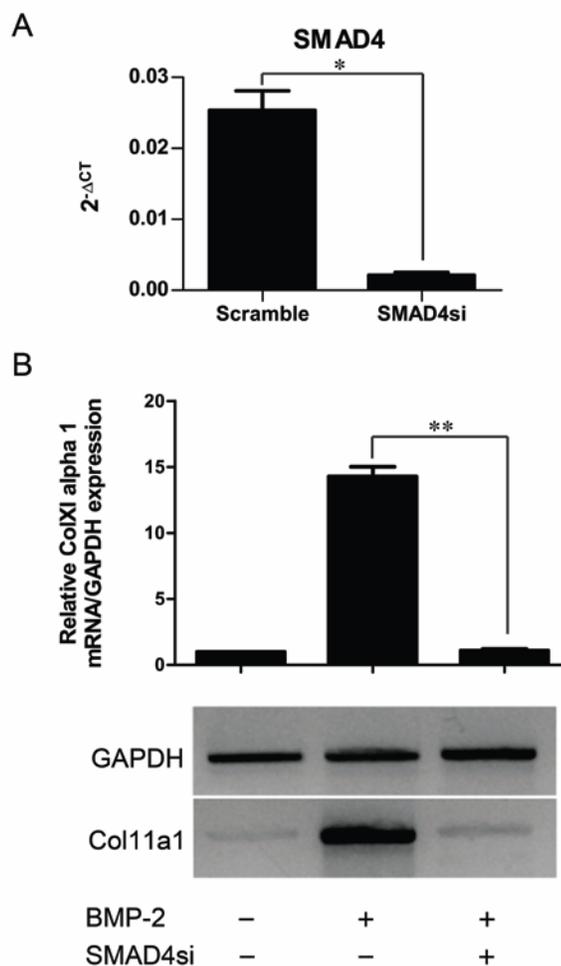
A) On day 6, C2C12 had changed morphology from mesenchymal spindle-shaped cells to osteoblastic cuboidal shaped cells. B-F) On day 2, the expression of ALP, Runx2, and Coll1a1 was markedly increased in BMP-2-treated samples compared to control. ALP mRNA levels decreased after day 2 and Runx2 and Coll1a1 levels dropped after day 3. However, OCN mRNA levels increased persistently up to day 6. G-H) The expression of minor collagens V and XI also increased during osteoblast differentiation.



**Figure 5. BMP-2 regulates Col11a1 mRNA levels in a time-dependent manner.** Total RNA was isolated from pluripotent mesenchymal C2C12 cells treated with BMP-2 (300 ng/mL) for the indicated number of days. Relative expression is reported as  $2^{-\Delta CT}$ . All samples were normalized to housekeeping gene peptidylprolyl isomerase A (PPIA). A-D) Upregulated expression of exons 6A peaked on day 3 and remained increased up to day 6; whereas, exons 7 and 8 mRNA levels peaked on day 2 and remained upregulated until day 6. In contrast, BMP-2 induced a spike in exon 6B expression on day 3 that was followed by a drop that was comparable to control cells. D) Expression of Col11a1 spliceform containing exons 6A- 7-8 was reduced. Statistical significance was calculated using one-way ANOVA with Bonferroni's Multiple Comparison post hoc test. Results represent mean $\pm$ SEM n = 3 in each group. \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ , \*\*\* indicates  $p < 0.001$ , and \*\*\*\* indicates  $p < 0.0001$ , and n.s. is not significant.

To elucidate whether induction of Col11a1 gene expression by BMP-2 was dependent upon the canonical SMAD signaling pathway, we knocked down SMAD4 in C2C12 cells using SMAD4 siRNA and lipofectamine (**Figure 6A**). SMAD4 knockdown diminished BMP-2 induced Col11a1 gene expression (**Figure 6B**) demonstrating that

regulation of Col11a1 expression by BMP-2 is dependent on the canonical SMAD1/5/8 signaling pathway.

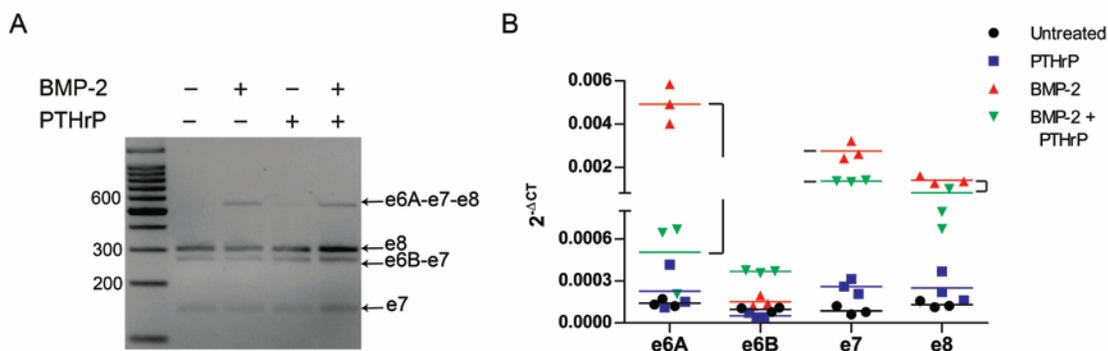


**Figure 6. BMP-2 acts in a SMAD4-dependent mechanism to regulate collagen XI alpha 1 expression in C2C12 cells.** A) SMAD4 was knocked down using siRNA and B) the expression of collagen XI alpha 1 was assessed using semi-quantitative PCR. C2C12 cells pre-incubated in SMAD4 siRNA showed a reduced BMP-2-mediated regulation of collagen XI alpha 1 expression.

#### PTHrP Modulates BMP-2-Induced Changes in Col11a1 Expression

Previous studies confirmed an inhibitory role for PTHrP (1-36) on BMP-2-induced osteoblast marker expression [37] and thus we sought to investigate the effects of PTHrP on BMP-2-induced Col11a1 expression. Quantitative real-time PCR demonstrated

that PTHrP attenuated the BMP-2-induced splicing to include exon 6A and 8, and to a lesser extent for exon 7 (**Figures 7A and 7B**). Combinations of alternatively spliced exons included four predominant spliceforms; variable region exons 1) e6a-7-8, 2) e6b-7, 3) e8, and 4) e7, expressed by differentiating osteoblasts (**Figure 7**). PTHrP alone did not have significant effects on Col11a1 expression at 24h continuous treatment.



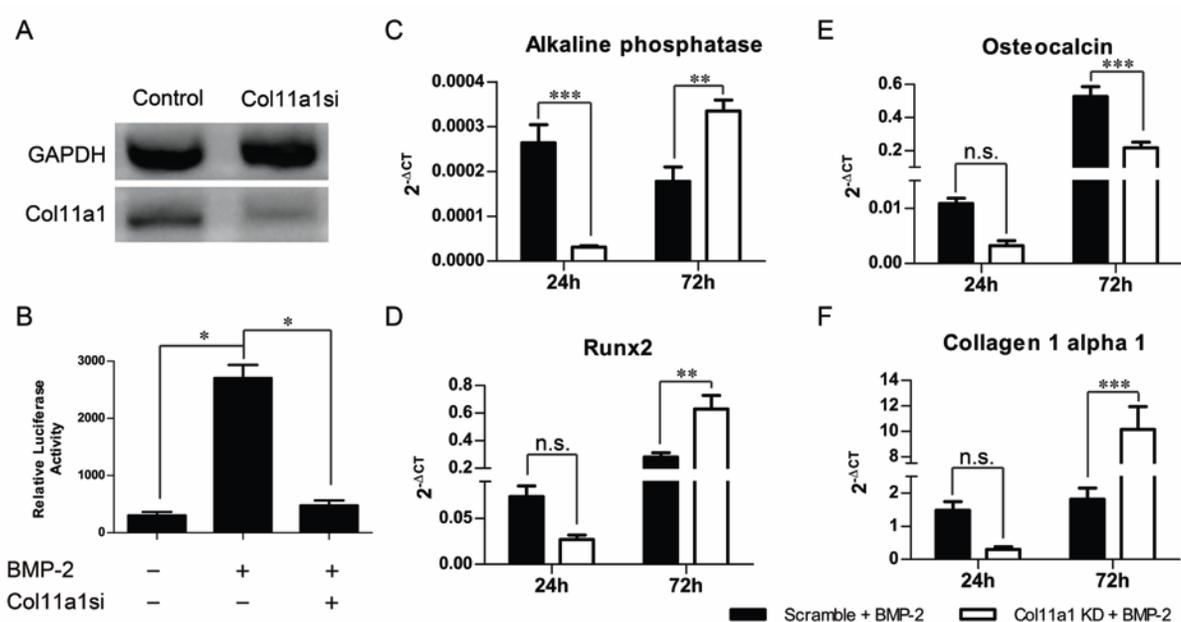
**Figure 7. PTHrP alters BMP-2-induced expression of Col11a1 alternative exons.** Pluripotent mesenchymal C2C12 cells were treated with BMP-2 (300 ng/mL) for five days and PTHrP ( $10^{-7}$  M) was added to select samples for 24 hr. As control, cells were incubated in the absence of growth factors or presence of PTHrP only. Col11a1 exon expression was assessed by semiquantitative and quantitative real-time PCR using different sets of primers. Relative expression is reported as  $2^{-\Delta CT}$ . All samples were normalized to housekeeping gene peptidylprolyl isomerase A (PPIA). A) and B) PTHrP alone did not induce any significant changes in Col11a1 exon expression. In contrast, combined with BMP-2, PTHrP reduced BMP-2-stimulated expression of exons 6A and 7. Further, PTHrP was able to synergistically increase exon 6B expression with BMP-2, although this effect was statistically not significant. Statistical significance was calculated using two-way ANOVA with Bonferroni's Multiple Comparison post hoc test. Results represent mean $\pm$ SEM n = 3 in each group. \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ , \*\*\* indicates  $p < 0.001$ , and \*\*\*\* indicates  $p < 0.0001$ .

### Col11a1 Is Required for BMP-2 Induction of Osteoblast Markers During the First 24

#### Hours

To further elucidate the biological relevance of Col11a1 regulation by BMP-2 during osteoblast differentiation, we assessed the effects of Col11a1 knockdown on BMP-2-induced expression of osteoblast markers ALP, OCN, Runx2, and Col1a1. Upon

transfecting C2C12 cells with either Col11a1 siRNA or control scramble siRNA, we confirmed our knockdown by RT-PCR (**Figure 8A**). We then determined the expression levels of osteoblast markers using quantitative real-time PCR. Our results demonstrated that Col11a1 knockdown caused a decrease in 24 hr BMP-2-stimulated osteoblast marker expression, most markedly for ALP (**Figures 8C-F**). In contrast, this effect was reversed later at 72 hr osteoblast differentiation (discussed below).

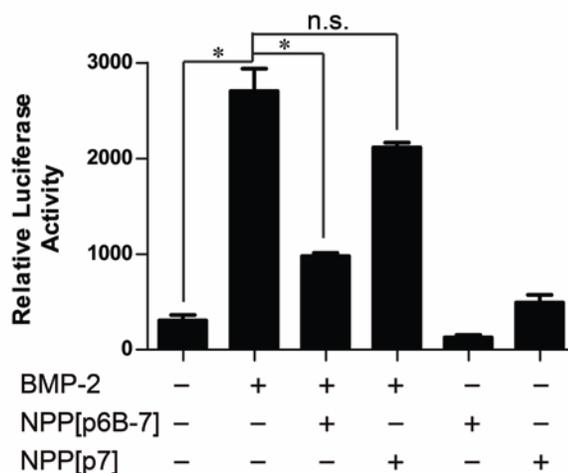


**Figure 8. Effects of Col11a1 KD on the expression of ALP, OCN, Runx2, and Col1a1 is time-dependent.** Pluripotent C2C12 cells were transfected with either Col11a1 or scramble siRNA for 24 hr. Next, cells were stimulated with BMP-2 (300 ng/mL) for 24 hr and 72hr. Relative expression is reported as  $2^{-\Delta\text{CT}}$ . All samples were normalized to housekeeping gene peptidylprolyl isomerase A (PPIA). A) At 24 hr BMP-2 stimulation, ALP expression was significantly decreased in Col11a1 deficient cells as compared to control. In contrast, at 72 hr BMP-2 stimulation, Col11a1 deficient cells showed a marked increase in ALP expression as compared to control. B) OCN mRNA levels were not significantly different in Col11a1 deficient cells as compared to control at 24 hr BMP-2 treatment. By 72 hr, however, Col11a1-deficient cells expressed lower levels of OCN as compared to control. C) and D) Both Runx2 and Col1a1 mRNA levels were not significantly affected by Col11a1 KD at 24 hr BMP-2 stimulation; however, at 72 hr, both Runx2 and Col1a1 mRNA levels were significantly higher in Col11a1 deficient cells as compared to control. Statistical significance was calculated using two-way ANOVA with Bonferroni's Multiple Comparison post hoc test. Results are reported

as the mean $\pm$ SEM n = 3 in each group. \* indicates p < 0.05, \*\* indicates p < 0.01, \*\*\* indicates p < 0.001, and \*\*\*\* indicates p < 0.0001, and n.s. is not significant.

### Col11a1 siRNA Reduced BMP Activity in C2C12 Cells During the First 24 Hours

Because we found that Col11a1 was required for BMP-2 induction of osteoblast markers during the first 24 hours, we tested the effects of Col11a1 on BMP signaling, using a luciferase BMP-response element reporter construct. Treatment of C2C12 cells with BMP-2 induced an increase in luciferase activity, indicating activation of BMP signaling compared to untreated control cells (**Figure 9**). Col11a1 siRNA reduced the BMP activity by 82% (p-value 0.0161) compared to controls in C2C12 cells during the first 24 hours.

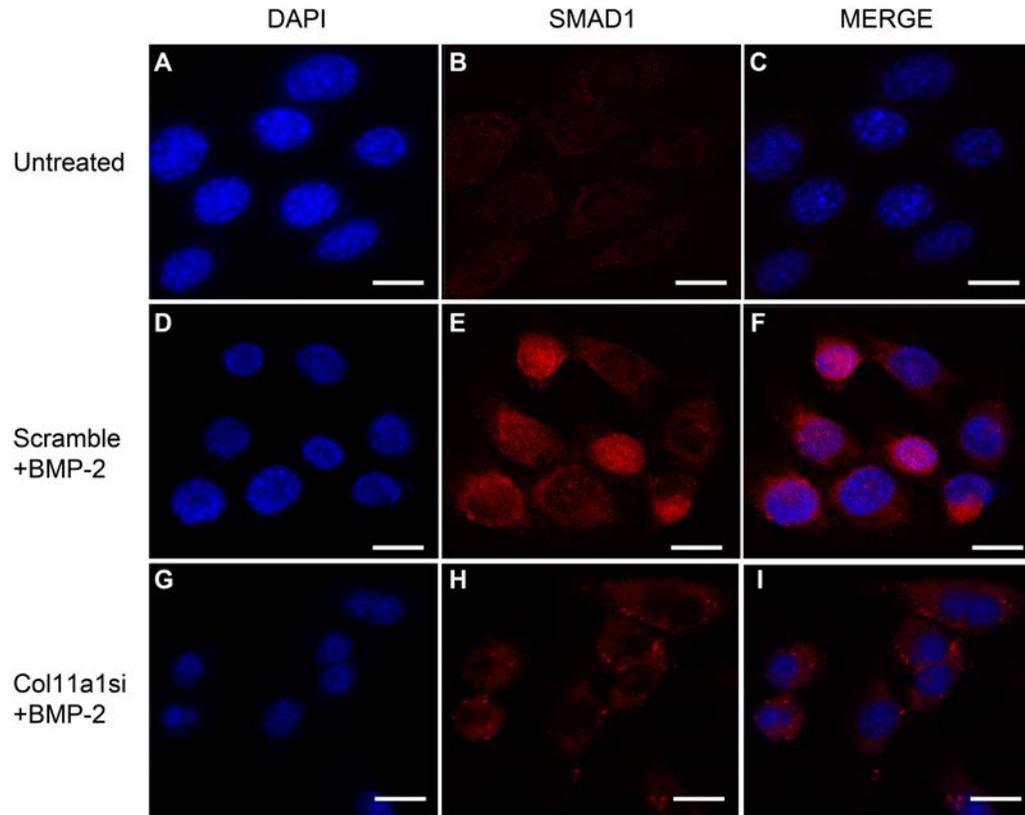


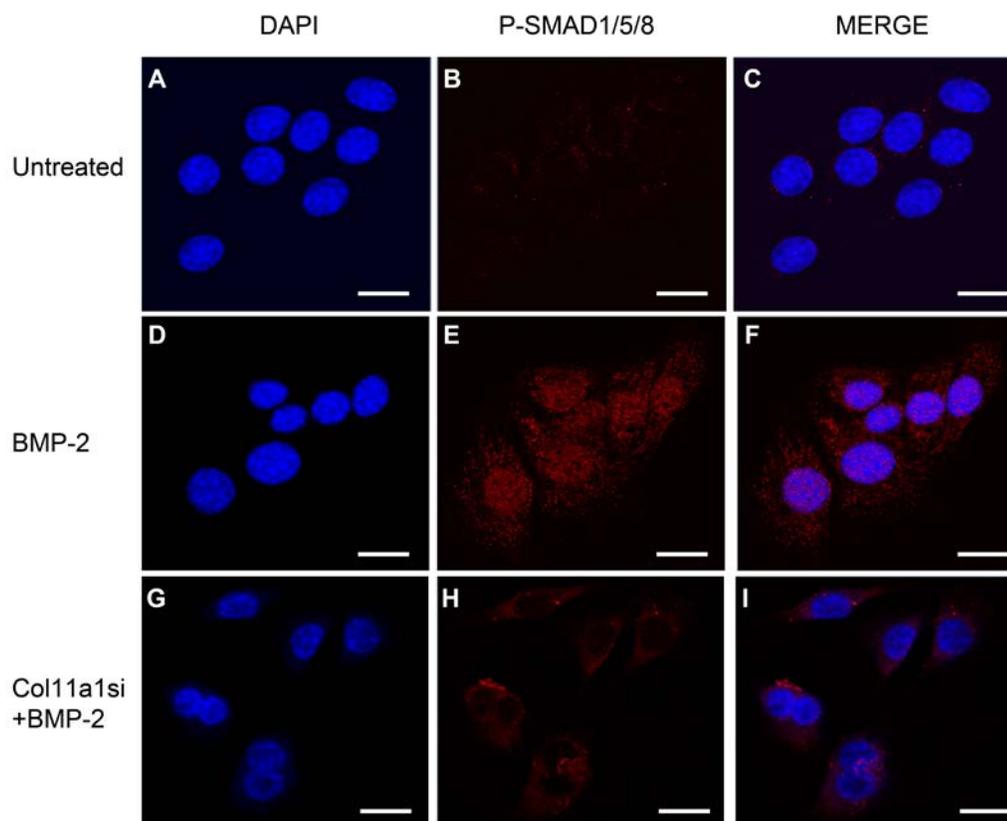
**Figure 9. Col11a1 recombinant p6B but not p7 reduces BMP-dependent luciferase activation in C2C12 cells.** C2C12 cells were transfected with a BMP-responsive firefly luciferase reporter plasmid and a control pCMV- $\beta$ -gal reporter plasmid for 24 hr. The next day, BMP-2 (300 ng/mL) and/or recombinant Col11a1 p6B (37.5 ug/mL) and p7 (62.5 ug/mL) were added to cultures and incubated for another 24 hr. Cell lysates were analyzed for luciferase activity. Relative luciferase activity was calculated as the ratio of luciferase to  $\beta$ -galactosidase activity, to control for transfection efficiency, and is expressed as a multiple of the activity of unstimulated cells transfected with reporter alone (control). Col11a1 p6B significantly reduced BMP-2-induced relative luciferase activity. Similarly, Col11a1 KD diminished BMP-2-induced luciferase activity. In contrast, Col11a1 p7 did neither enhance nor reduce BMP-2-induced luciferase activity. Statistical significance was calculated using student's paired t-test. Results

represent mean $\pm$ SEM n = 3 in each group. \* indicates p < 0.05, \*\* indicates p < 0.01, \*\*\* indicates p < 0.001, and \*\*\*\* indicates p < 0.0001, and n.s. is not significant.

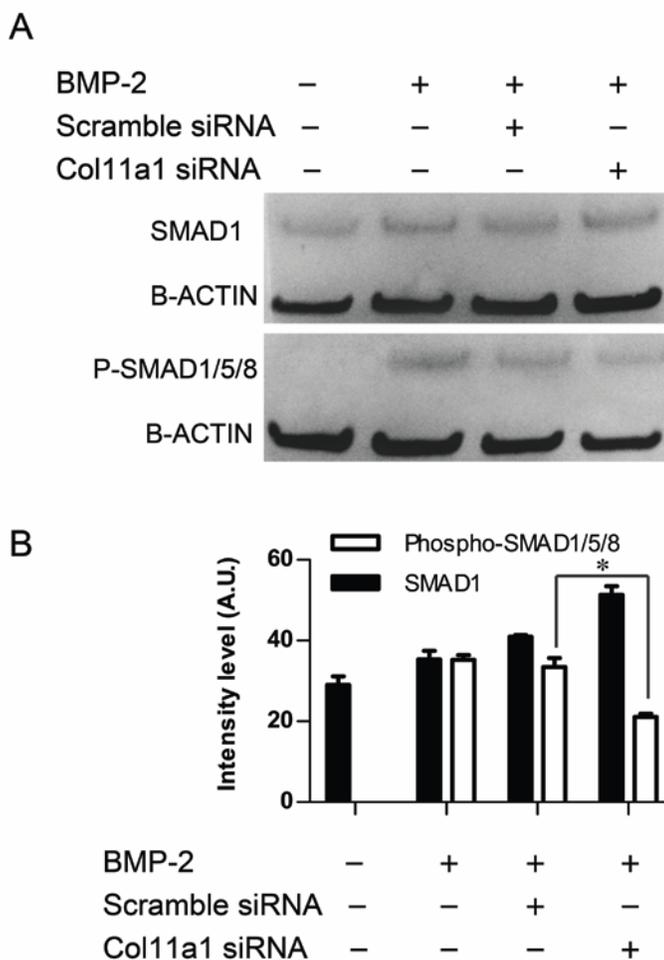
### Col11a1 Is Required for BMP-2-Induced Smad1/5/8 Phosphorylation

To elucidate the mechanism by which Col11a1 regulates BMP-2-induced expression of osteoblast markers, we investigated the role of Col11a1 in SMAD1/5/8 phosphorylation and nuclear translocation in C2C12 cells by western blot and immunocytochemistry. Treatment with BMP-2 induced SMAD1/5/8 phosphorylation, which was not detected in control cells (**Figures 10A-F**). Further, phospho-SMAD1/5/8 co-localized with DAPI, indicating its presence in the nucleus (**Figure 10F**). Pre-treatment with Col11a1 siRNA for 24 hours resulted in a reduction of phospho-SMAD1/5/8 by approximately 50% (p-value <0.001) (**Figure 11A and 11B**) and inhibited the SMAD1/5/8 complex translocation to the nucleus (**Figure 11G-I**). Interestingly, a 45% (p-value <0.01) increase in SMAD1 protein was observed in the Col11a1-deficient cells compared to cells treated with the control scramble siRNA (**Figure 11B**).





**Figure 10. Col11a1 knockdown negatively affects canonical BMP-2 signaling.** C2C12 cells were plated on glass coverslips at  $2 \times 10^4$  cells/cm<sup>2</sup> and incubated overnight. Next, cells were transfected with either Col11a1 or scramble siRNA for 24 hr and then stimulated with BMP-2 for 30 minutes. Antibodies against Smad1 and Phospho-Smad1/5/8 were used to assess the effects of Col11a1 knockdown on BMP-2 mediated Smad1/5/8 phosphorylation. A-I) Immunofluorescence staining was performed to look at the effects of Col11a1 knockdown on Phospho-Smad 1/5/8 localization. No Phospho-Smad1/5/8 was detected in the nuclei of Col11a1 deficient cells as compared to control. Instead, Col11a1 deficient cells showed a distinct punctuate pattern of staining characterized by discrete “spots” close but not inside the nuclei.



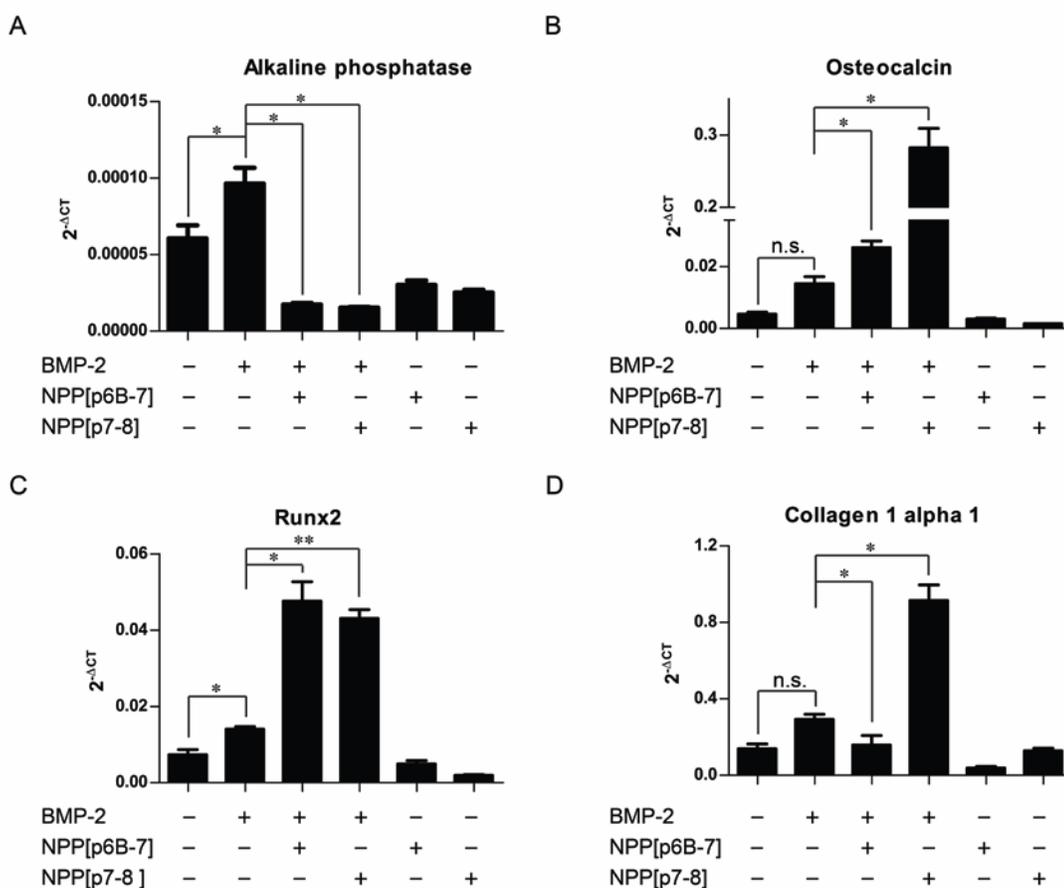
**Figure 11. Col11a1 knockdown negatively affects SMAD1/5/8 phosphorylation.** C2C12 cells were plated at  $2 \times 10^4$  cells/cm<sup>2</sup> and incubated overnight. Next, cells were transfected with either Col11a1 or scramble siRNA for 24 hr and then stimulated with BMP-2 for 30 minutes. Antibodies against Smad1 and Phospho-Smad1/5/8 were used to assess the effects of Col11a1 knockdown on BMP-2 mediated Smad1/5/8 phosphorylation. Western blot analyses of C2C12 cells transfected with Col11a1 siRNA or scramble siRNA was performed to quantify Smad1 and Phospho-Smad1/5/8 levels. Col11a1 deficient cells showed a 40% decrease in Phospho-Smad1/5/8 levels as compared to control. In contrast, Smad1 levels were 27% higher in Col11a1 deficient cells as compared to control.

#### Recombinant Col11a1p8 NTD Fragment Enhances BMP-2-Induction of Osteoblast

##### Markers at 24 Hours

An essential role for Col11a1 during the first 24 hr of BMP-2 treatment was further demonstrated by treating cells with recombinant Col11a1p8 NTD fragment,

representing one of the spliceforms synthesized by C2C12 cells as shown above. This treatment resulted in an increase in OCN (19.5 fold, p-value 0.0103), Runx2 (3.0 fold, p-value 0.0099), and Coll1a1 (3.1 fold, p-value 0.0284) (**Figures 12B-D**). In contrast, treatment with recombinant Coll1a1p8 resulted in a 6.3 fold decrease (p-value 0.0143) in ALP expression (**Figure 12A**).



**Figure 12. BMP-2-induced expression of ALP, OCN, Runx2, and Coll1a1 is altered by variable Coll1a1 fragments.** A) BMP-2 upregulated ALP expression was significantly reduced upon incubation with Coll1a1 p6B and p8. B) and C) OCN and Runx2 mRNA levels were markedly increased when Coll1a1 p6B and p8 were added to BMP-2 treated cells as compared to control. D) Coll1a1 expression was enhanced by addition of Coll1a1 p8 but reduced by Coll1a1 p6B. Statistical significance was calculated using student's paired t-test. Results represent mean±SEM n = 3 in each group. \* indicates p < 0.05, \*\* indicates p < 0.01, \*\*\* indicates p < 0.001, and \*\*\*\* indicates p < 0.0001, and n.s. is not significant.

## Col11a1 Inhibits BMP-2 Induction at Later Time Points During Osteoblast

### Differentiation

In contrast to our observations after 24 hr BMP-2 treatment, the effect of Col11a1 siRNA was reversed later in osteoblast differentiation. After 72 hours of BMP-2 treatment, Col11a1 siRNA increased the expression of ALP, Runx2, and Col1a1 genes by 1.8 fold (p-value <0.01), 2.2 fold (p-value <0.01), and 5.6 fold (p-value <0.001), respectively compared to scramble siRNA (**Figures 8C, 8D, and 8F**). Interestingly, OCN expression level remained suppressed in the presence of Col11a1 siRNA compared to scramble siRNA at 72 hr, similar to the effect seen at 24 hours (**Figure 8E**). This may reflect the fact that osteocalcin is considered a late osteoblast differentiation marker.

### Chondrocyte-Specific Isoform Col11a1p6b Inhibits BMP Activity in C2C12 Cells

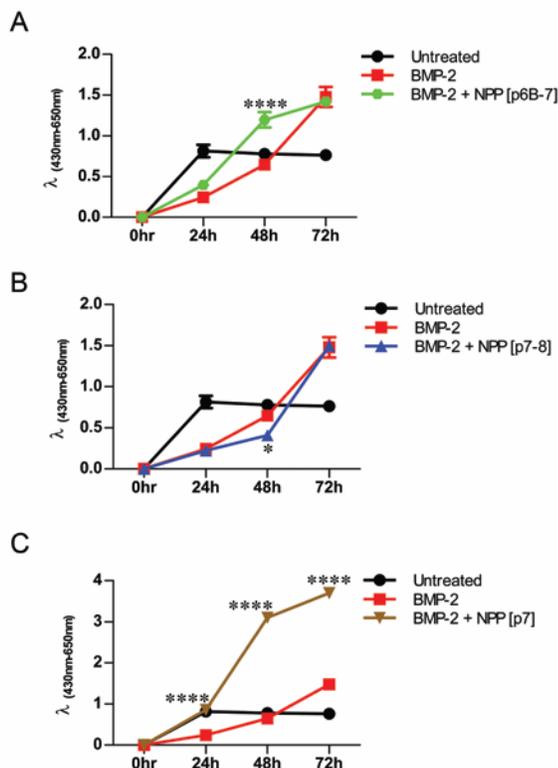
To investigate the potential role of neighboring cartilage tissue on the formation of the bone collar, we introduced a fragment from the isoform of Col11a1 that is expressed by chondrocytes at the boundary between cartilage and the developing bone collar on BMP signaling in osteoblasts, using the luciferase BMP-response element reporter construct. Recombinant Col11a1p6b NTD fragment reduced the BMP activity by 64% (p-value 0.0136) while treatment of C2C12 cells with BMP-2 induced an increase in luciferase activity compared to untreated control cells (**Figure 9**). Conversely, this effect was not observed when cells were treated with a recombinant Col11a1p7 NTD fragment that differs only by the absence of 51 amino acids encoded by exon 6b, indicating a specific function for this 51 amino acid region in the regulation of growth plate maturation, specifically bone collar formation.

### Chondrocyte-Specific Isoform Col11a1p6b Inhibits Expression of Osteoblast Differentiation Markers in C2C12 Cells

To further test the role of Col11a1 as an osteoblast differentiation regulator, we first treated C2C12 cells with BMP-2 and then cultured them in medium containing recombinant Col11a16b NTD fragment to assess the expression of the markers of osteoblast differentiation. Recombinant Col11a16b decreased the BMP-2-induced ALP expression by 5.5 fold (p-value 0.0132) and Col1a1 expression by 1.8 fold (p-value 0.032) consistent with an inhibitory role for early marker expression (**Figures 12A and 12D**). Conversely, recombinant Col11a1p6B promoted the expression of OCN by 1.8 fold (p-value 0.017) and Runx2 by 3.4 fold (p-value 0.022) in BMP-2-induced cells consistent with a positive regulatory role for later osteoblast differentiation (**Figures 12B and 12C**).

### Col11a1 Regulates C2C12 Proliferation in a Spliceform-Dependent Manner

Cellular proliferation of cells giving rise to the bone collar is an essential aspect of bone development and is stimulated by BMP-2 in C2C12 cells. Proliferation and differentiation events are highly coordinated in biological systems and thus we wanted to look at the effects of Col11a1 on cellular proliferation in our model system. We found that recombinant Col11a1p7 NTD fragment significantly increased the rate and extent of proliferation of C2C12 cells treated with BMP-2. Two other spliceforms tested had only minimal effects (**Figure 13**). Col11a1p7 was shown to be expressed at low levels in C2C12 cells (see **Figure 4C** above), and increasing the level by the addition of exogenous protein may have revealed a unique function for the smallest of the spliceforms of Col11a1, Col11a1p7.



**Figure 13. Col11a1 regulates cell proliferation during osteoblast differentiation.** C2C12 cells were cultured overnight in 96-well plates and recombinant Col11a1 protein fragments p6B (37.5 ug/mL), p7 (62.5 ug/mL), and p8 (30 ug/mL) were added the next day for 24 hr, 48 hr, and 72 hr. At each time point, 10 ul of wst-1 was added to each well and incubated for 2 hr at 37°C. Absorbance is reported as test wavelength of 450 nm minus a reference wavelength of 630 nm. WST-1 was also added to empty and untreated wells to establish a blank baseline. A) Addition of Col11a1 p6B to BMP-2 treated cells did not significantly change cell proliferation at 24 hr and 72hr as compared to control cells. However, at 48 hr, Col11a1 p6B enhanced cell proliferation as compared to both untreated and BMP-2 only treated cells. B) Col11a1 p7 significantly enhanced cell proliferation in C2C12 cells during osteoblast differentiation at all three time points. C) Col11a1 p8 did not affect BMP-2 mediated cell proliferation at 24 hr and 72 hr but significantly decreased cell proliferation at 48 hr time point. Statistical significance was calculated using two-way ANOVA with Bonferroni's Multiple Comparison post hoc test. Results are reported as the mean $\pm$ SEM n = 3 in each group. \* indicates p < 0.05, \*\* indicates p < 0.01, \*\*\* indicates p < 0.001, and \*\*\*\* indicates p < 0.0001, and n.s. is not significant.

## Discussion

Taken together, these findings suggest that Col11a1 spliceforms play specific roles in the regulation of C2C12 osteoblast differentiation and that the effects of

individual spliceforms may be in opposition with respect to the other spliceforms.

Taking into consideration the results presented for the functional knockout mouse, these findings are likely to translate to in vivo osteoblast differentiation and bone development.

Col11a1 is an extracellular matrix protein that is essential for proper skeletal development. Marshall and Stickler syndrome patients carry heterozygous mutations in *Coll1a1* and suffer from short stature and skeletal abnormalities. Previous groups have shown that Col11a1-deficient mice exhibit increased skeletal mineralization compared to their wildtype littermates [9, 11]. Based on recent microarray studies showing increased Col11a1 presence during differentiation, we hypothesized that Col11a1 would affect periosteal bone architecture and further alter osteoblast differentiation in a BMP-dependent manner.

Our findings support a role for Col11a1 in bone formation consistent with alterations to the bone collar during skeletal development. Further, we have presented information that suggests intersection between BMP-2 signaling and Col11a1-regulation of bone formation. Our results indicate that BMP-2 regulates *Coll1a1* transcription and alternative splicing of pre-mRNA characterized by the expression of exons 6A, 7, and 8 in osteoblasts. BMP-2-stimulated expression of exon 6B on day 3 coincided with a significant decrease in the expression levels of osteoblast markers *ALP*, *Runx2*, and *Coll1a1*, suggesting a negative regulatory role for exon 6B during osteoblast differentiation. We also showed that exogenous treatment of cells with Col11a1[p6b-7] recombinant protein fragment significantly reduced BMP activity. One possible explanation for less BMP activity upon exposure to Col11a1[p6B-7] is its direct binding to heparan sulfates on the surface of osteoblasts, which are responsible for trapping and

internalizing BMP-2 into the cell [15 ,28, 29]. Our laboratory has previously shown that Col11a1 binds specifically to heparan sulfate and heparan sulfate proteoglycans via the p6b region and Npp domain [30].

Treatment of C2C12 cells with Col11a1[p6B-7] resulted in a significant decrease in *ALP* and *Col1a1* expression but not *OCN* and *Runx2*. Instead Col11a1[p6B-7] induced increased expression of *OCN* and *Runx2*. This finding is intriguing because although *OCN* is up-regulated by BMP-2 and is essential for osteoblast differentiation, *OCN*-deficient mice exhibit increased bone formation, suggesting a limiting role for *OCN* in bone formation [31]. Furthermore, previous studies have shown that osteocalcin is under direct regulation of *Runx2* in osteoblasts [32]. Thus, it is possible that Col11a1p6b acts upstream of *Runx2* and induces the expression of *Runx2* and *OCN* to limit bone formation. Alternatively, since *Runx2* is a shared target of BMP and TGF- $\beta$  signaling pathways [33], Col11a1[p6B-7]-mediated inhibition of BMP signaling at the receptor level may signal the cells to increase TGF- $\beta$ -induced expression of *Runx2* and subsequently *OCN*. Since BMP-2 increased the expression of exon 8, we postulated a positive regulatory role for regions of the protein encoded by exons 6A, 7, and 8 during osteoblast differentiation. Indeed, when we treated BMP-2-stimulated cells with Col11a1p8, mRNA levels of *OCN*, *Runx2*, and *Col1a1* increased significantly. Surprisingly though, *ALP* expression was decreased by Col11a1p8.

Upon knocking down Col11a1, we initially observed a decrease in the expression of osteoblast markers, suggesting that Col11a1 is required for BMP-2 mediated induction of osteoblast marker expression. However, by 72 hr, we noted the opposite trend and detected higher levels of osteoblast marker expression (except for *OCN*) in Col11a1-

deficient cells as compared to control. This difference in the trend may be due to 1) compensatory mechanism used by cells to increase their expression of osteoblast markers and overcome the *Col11a1* knockdown and/or 2) early induction of other genes that have yet to be identified, perhaps other BMPs or receptors.

Continuous treatment with PTHrP has been shown to inhibit osteoblast differentiation [25]. Previous teams have shown a feedback mechanism between BMP-2 and PTHrP during osteoblast differentiation, in which PTHrP attenuates BMP-2-induced increases in osteoblast markers *Runx2* and *osterix* [24]. Our findings indicate a regulatory role for PTHrP in BMP-2-induced alternative splicing further supporting our postulation of exons 6A, 7, and 8 as positive regulators of BMP-2 mediated osteoblast differentiation. We also reported an increase in *Col11a1* exon 6B expression when PTHrP was added to BMP-2 treated cells although this effect did not reach statistical significance.

Canonical BMP signaling requires phosphorylation of SMAD1/5/8 and thus we wanted to test whether *Col11a1* knockdown affects this process. Our results clearly show that *Col11a1*-deficient cells exhibit lower levels of phospho-SMAD1/5/8 as compared to control cells. Furthermore, phospho-SMAD1/5/8 proteins in *Col11a1*-deficient cells are primarily localized to the cytoplasm and not localized to the nucleus.

Considering the opposing relationship between cellular proliferation and differentiation, we wanted to determine the effect of *Col11a1* on BMP-2 mediated regulation of cellular proliferation during osteoblast differentiation. Cellular proliferation plays an important role in growth of long bone at the growth plate and bone collar. Our findings demonstrate a proliferative role for *Col11a1* p6b and p7 when combined with BMP-2 as compared to BMP-2 only treated cells. Conversely, *Col11a1* p8 acts in an anti-

proliferative manner, reducing BMP-2 mediated cellular proliferation. It is possible that while Col11a1p6B acts as an inhibitor of osteoblast differentiation, it promotes cellular proliferation instead. Conversely, Col11a1p8 might inhibit cell proliferation in favor of cellular differentiation. Future studies on cell cycle analyses may answer questions about the role of Col11a1 in cell proliferation.

In conclusion, our study clearly demonstrates a novel function for Col11a1 as a regulator of BMP-2 induced osteoblast signaling and osteoblast differentiation. These findings are consistent with our earlier studies suggesting that Col11a1p6b acts to inhibit *ALP* expression during osteoblast differentiation [26]. Based on our observations, we propose a model in which BMP-2 regulates Col11a1 in a spliceform-specific manner and in return different spliceforms of Col11a1 act to either inhibit or enhance BMP-2 signaling and downstream expression of osteoblast markers correlated with the spatial and temporal expression patterns within the developing long bone.

Future studies will address the role of Col11a1 in cell proliferation, direct binding interactions between different collagen spliceforms and BMP-2, and the effects of Col11a1 on integrin-mediated BMP-2 induction of osteoblast differentiation [34]. These additional studies will offer more insight as to how Col11a1 plays a role in bone development.

### **Authorship**

Conceived and designed the experiments: JTO, NS. Performed the experiments: NS and AH. Analyzed the data: NS, JTO. Drafted manuscript: NS, JTO.

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CHAPTER TWO: A ROLE FOR PTHrP in ColXI ( $\alpha$ 1) ISOFORM EXPRESSION  
DURING ATDC5 CHONDROGENIC DIFFERENTIATION

Submitted to “International Journal of Molecular Sciences”

*Article*

## **A role for PTHrP in ColXI ( $\alpha$ 1) isoform expression during ATDC5 chondrogenic differentiation**

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### **Abstract**

Alternative splicing is essential for protein diversity in eukaryotes. Long bones develop as prechondrocytes differentiate and mature into hypertrophic chondrocytes, which are eventually replaced by osteoblasts. Cellular differentiation and maturation during chondrogenesis is tightly linked with pre-mRNA alternative splicing of important extracellular matrix molecules including collages II and XI. ColXI ( $\alpha$ 1) splicing results in up to eight different isoforms that are expressed in a spatiotemporal manner during chondrogenesis. Here, we studied ColXI ( $\alpha$ 1) splicing during chondrogenesis using mouse ATDC5 cells and pluripotent C2C12 cells. We also determined the effects of

PTHrP on alternative ColXI ( $\alpha$ 1) transcript levels during chondrogenesis. Our results showed that the expression of ColXI ( $\alpha$ 1) exons 6A and 8 did not change significantly during the initial stages of chondrogenesis but reached peak levels on day 16. Further, while exons 7 and 8 plateaued after day 16, exon 6A expression decreased. In contrast ColXI ( $\alpha$ 1) exon 6B expression levels remained unchanged until day 30 of differentiation. PTHrP had inductive effects on exon 6A and 8 expression levels during early chondrogenesis but did not change exon 6B expression until day 30. Overall, this study shows for the first time that PTHrP regulates ColXI ( $\alpha$ 1) alternative splicing during chondrogenesis.

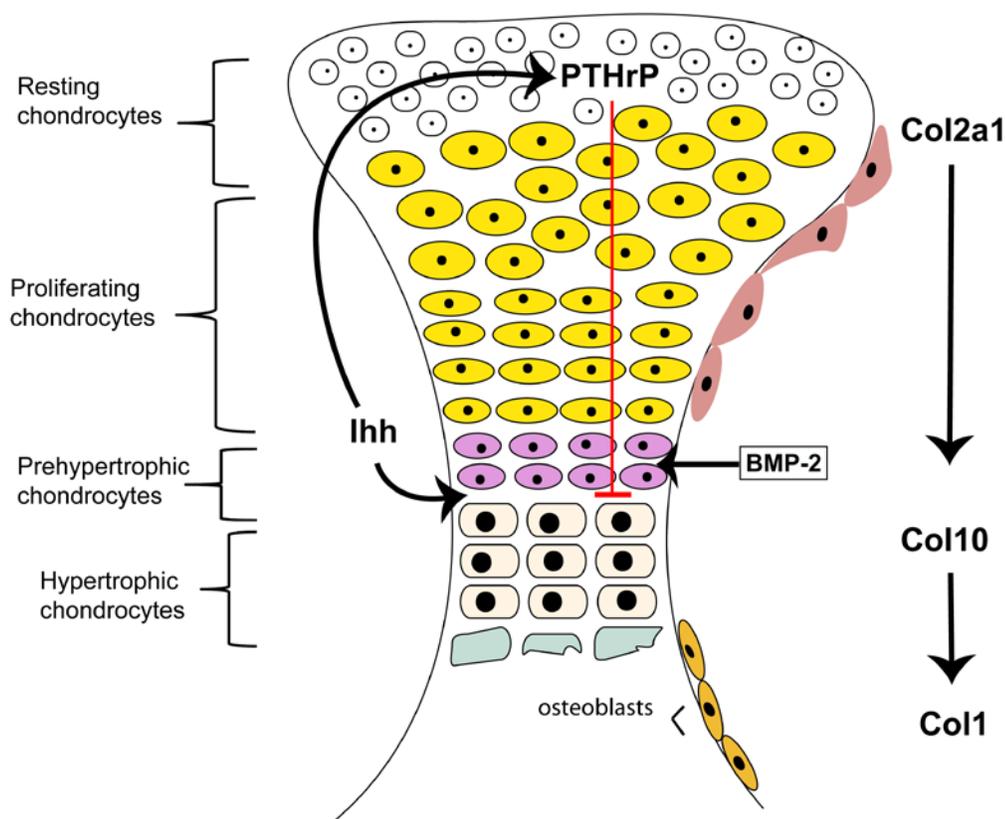
**Keywords:** ColXI ( $\alpha$ 1); alternative splicing; ATDC5; PTHrP; chondrogenesis; differentiation.

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## 1. Introduction

The skeleton starts forming during the second month of human development. Initially, mesenchymal cells condense and define the position and basic shape of the future skeletal element. Once the initial mesenchyme condensations are formed, depending on the growth factors present in the immediate environment, cells either differentiate into osteoblasts to form bone or into chondrocytes to form cartilage [1]. Wnt signaling drives mesenchymal cells to differentiate into osteoblasts [2]. In the absence of Wnt signaling, SRY (Sex Determining Region Y)-Box 9 (Sox9) levels increase and mesenchymal cells differentiate into prechondrogenic cells. Once committed, prechondrogenic cells start producing cartilage extracellular matrix proteins including collagens II and XI. Sox9 binds directly to the promoter of *Col2a1* gene and induces its

expression [3]. Although studies have shown Sox9 binding sites on collagen XI promoter, Sox9-induced expression of *collagen XI* has not been reported [4]. Once prechondrocytes are formed, cartilage starts growing rapidly in an appositional as well as interstitial manner, a process known as endochondral ossification (**Figure 1**). Interstitial growth occurs as prechondrocytes differentiate into chondrocytes, which then enlarge and apoptose [5]. As chondrocytes hypertrophy, the perichondrium is infiltrated with blood vessels induced by Vascular endothelial growth factor (VEGF) and the periosteum is formed [6]. The periosteum houses mesenchymal cells that upon getting a signal from hypertrophic cells differentiate into osteoblasts. Bone is formed as osteoblasts start producing a non-mineralizing fibrillar extracellular matrix, which is then organized into mineralized matrix. Proper bone growth is dependent upon a complex network of systemic and local growth factors.



**Figure 1. Long bones elongate as resting chondrocytes undergo differentiation into proliferative chondrocytes.** PTHrP produced by perichondrial and resting chondrocytes maintains cells in a proliferative state. Proliferative chondrocytes then hypertrophy and undergo apoptosis as cells of the bony collar become osteoblasts. BMP-2 is an inducer of chondrocyte hypertrophy, whereas IHH produced by pre-hypertrophic chondrocytes signals to synthesize more PTHrP and thus inhibit hypertrophy.

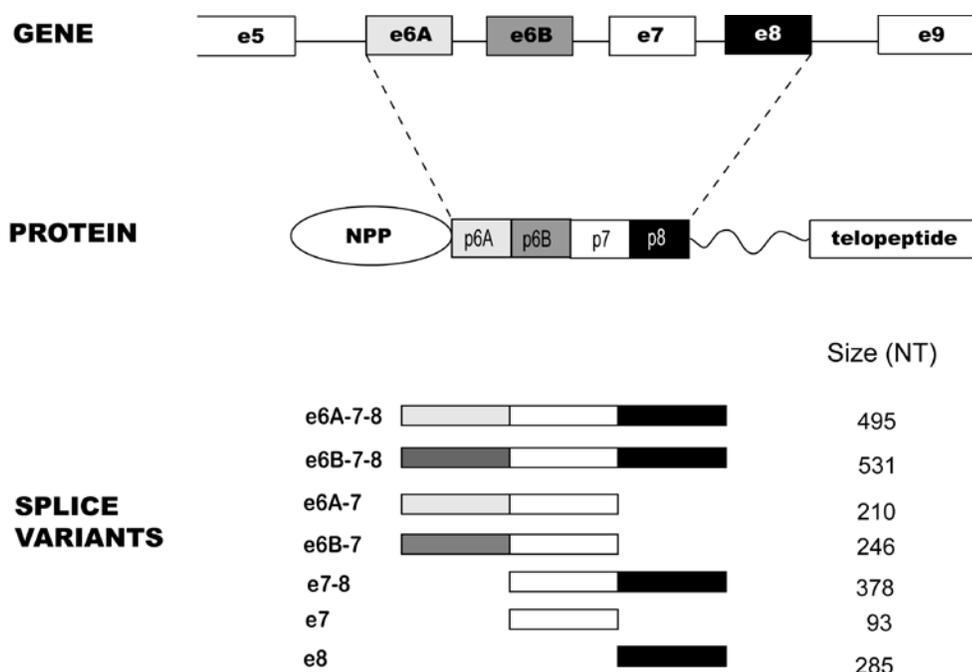
Initially Sox9 is essential for commitment into cartilage. Prechondrocytes and cells of the perichondrium both synthesize Parathyroid hormone-related Peptide (PTHrP) to maintain chondrocytes in a proliferative state [7]. Prehypertrophic cells on the other hand produce Indian Hedgehog (IHH), which promotes hypertrophy [8]. Simultaneously, cells of the periosteum respond to growth factors released by hypertrophic chondrocytes and promote differentiation of mesenchymal cells into osteoblasts. Bone morphogenetic protein-2 (BMP-2) is crucial for promoting chondrocyte hypertrophy as well as osteoblast differentiation in the growth plate [9]. Interstitial and appositional growth processes are

linked and the accurate orchestration of different mediators is crucial in achieving proper bone growth.

Fibrillar collagens are abundant extracellular matrix proteins that provide structure and support for cells [10, 11]. During endochondral ossification, collagens are expressed in a spatiotemporal manner [12]. The major fibrillar collagen present in cartilage is collagen type II, whereas collagen type I is predominantly found in bone. Collagens V and XI are minor in abundance relative to collagens I and II; however, their presence is required for proper fibril formation [13]. Collagens are formed as procollagen chains and three alpha chains come together to form a heter- or homo-trimer protein in the extracellular matrix. Previous studies have shown that collagen V is often associated with collagen type I, whereas collagen XI is associated with collagen type II in cartilage.

Collagen XI is an important component of the collagen network in cartilage and is essential for growth plate development [14, 15]. Interestingly, exons within the variable region of the amino terminal domain of all three collagen XI alpha chains are alternatively spliced [16]. This is intriguing because the globular amino-terminal domain of collagen XI containing the variable region is not completely embedded in the collagen fibrils but instead projects away from the fibril surface [17]. Alternative splicing of collagen XI alpha I chain involves exons 6A, 6B, 7, and 8 encoding the variable region [18, 19] (**Figure 2**). Previous work has shown that there is a strong link between chondrogenesis and ColXI ( $\alpha 1$ ) alternative splicing in fetal rat limb buds [19]. Similarly, there is compelling evidence that the different ColXI ( $\alpha 1$ ) spliceforms are regulated in a spatiotemporal manner during growth plate development [12].

Mutations in Parathyroid hormone- 1- receptor (PTH1R) result in skeletal dysplasias including Blomstrand's chondrodysplasia and Jansen Metaphyseal Chondrodysplasia characterized by short-limbed dwarfism due to abnormal growth plate development [20–23]. Similarly, ColXI ( $\alpha$ 1) mutations exhibit short-limbed dwarfism and improper growth plate development [24–26]. Based on the overlap in phenotype and co-localization in the growth plate for ColXI ( $\alpha$ 1), PTHrP, and PTH1R, we hypothesized that PTHrP regulates ColXI ( $\alpha$ 1) alternative splicing and expression in a differentiation-specific manner. We predicted that treatment with PTHrP would alter the pattern of ColXI ( $\alpha$ 1) spliceform expression in chondrocytes and pluripotent cells. We tested our hypothesis using commercially purchased PTHrP and prechondrogenic ATDC5 cells and pluripotent C2C12 cells in culture. We first characterized the expression ColXI ( $\alpha$ 1) alternative spliceforms during ATDC5 chondrogenesis and then assessed the effects of PTHrP on the expression of ColXI ( $\alpha$ 1) alternative spliceforms in both ATDC5 and pluripotent C2C12 cells.



**Figure 2.** The variable region within the NTD domain of collagen XI alpha 1 undergoes alternative splicing that can result in several different isoform. Each spliceform is expressed in a distinct spatiotemporal manner during development. The exact function of alternative splicing in collagen XI alpha 1 is yet to be determined (Adapted from [19]).

Our results clearly demonstrate that PTHrP modulates ColXI ( $\alpha 1$ ) alternative splicing during ATDC5 chondrogenesis. In contrast, the expression of ColXI ( $\alpha 1$ ) in pluripotent C2C12 cells does not change with PTHrP treatment. Taken together, our results suggest a regulatory role for PTHrP on ColXI ( $\alpha 1$ ) expression during chondrogenesis promoting the expression of exons 6A and 8 during early chondrogenesis and exon 6B at later stages of chondrocyte differentiation. Our study provides insight into the role of PTHrP on ColXI ( $\alpha 1$ ) alternative splicing and may be important for future studies on the interaction between the molecules.

## 2. Results and Discussion

### 2.1. Characterization of Collagen XI Spliceform Expression During ATDC5

#### Chondrogenic Differentiation

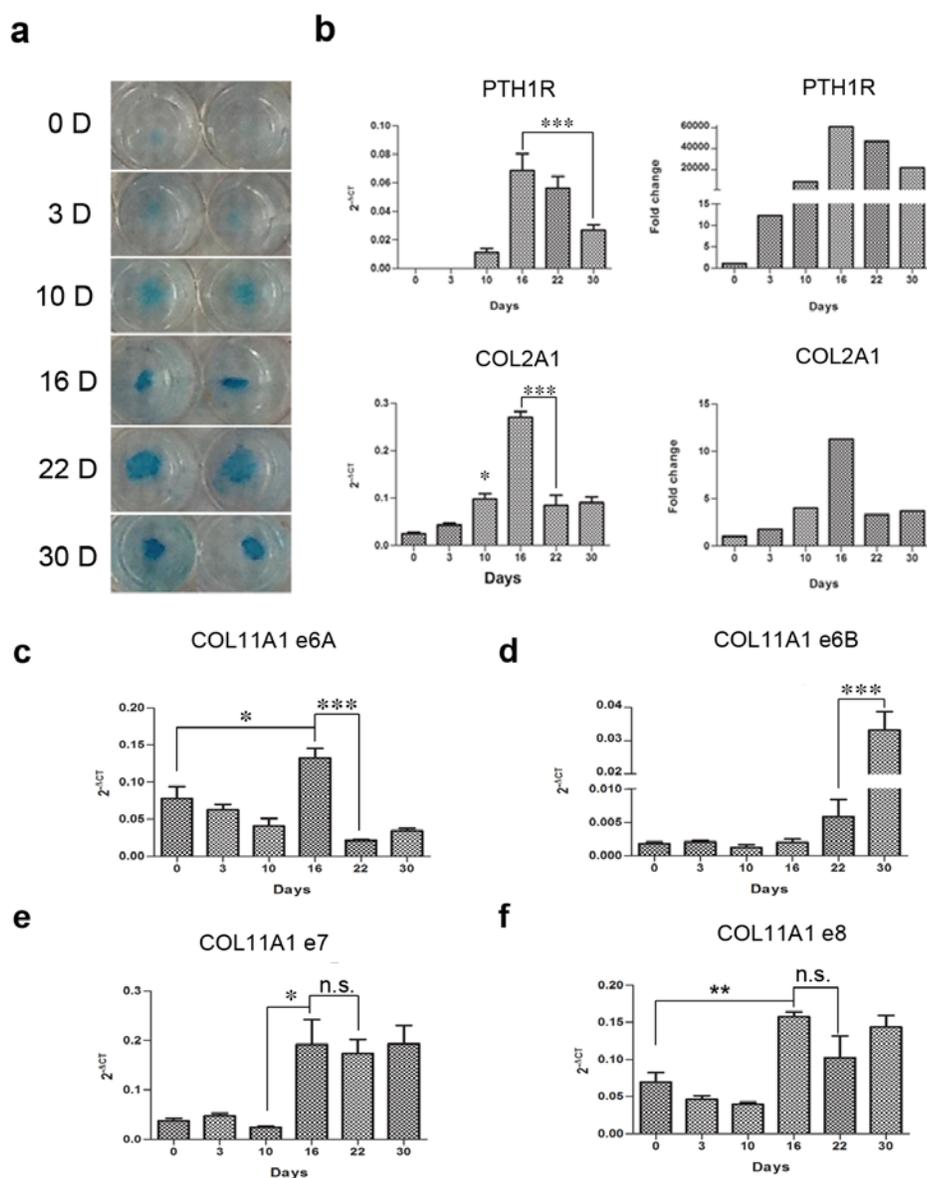
To examine the expression pattern of different ColXI ( $\alpha$ 1) spliceforms during chondrogenesis, we differentiated mouse ATDC5 cells in culture to recapitulate the process of chondrogenesis *in vitro*. ATDC5 cells provide an outstanding model to study processes of chondrogenic differentiation *in vitro* [27]. Cells stimulated with insulin-transferrin-selenium (ITS) showed positive Alcian blue staining for proteoglycans suggesting chondrogenesis (**Figure 3a**). We also confirmed chondrocyte differentiation of ATDC5 cells on a gene level by assessing the expression of well-known chondrogenic markers *Col2a1* and *PTH1R*. Our results showed a 4-fold increase in *Col2a1* expression on day 10 (p-value <0.05) and an 11-fold increase on day 16 (p-value <0.0001). Similarly, although at much higher levels, *PTH1R* expression increased reaching peak levels on day 16. Both *Col2a1* and *PTH1R* expression levels decreased after day 16 in agreement with their roles as early differentiation markers [28] (**Figure 3b**).

To determine the extent of ColXI ( $\alpha$ 1) alternative splicing during chondrogenesis, we designed primers specific to each alternatively spliced exon within the variable region of ColXI ( $\alpha$ 1) (**Table 1**). Real-time PCR analysis demonstrated that ColXI ( $\alpha$ 1) exons 6A, 7, and 8 peaked at early to mid stages of chondrocyte differentiation (**Figure 3c, 3e, 3f**), whereas ColXI ( $\alpha$ 1) exon 6B was more prevalent during later stages of chondrogenic differentiation (**Figure 3d**). These results are intriguing because previous work has shown that peptides p6A and p8 encoded by exons 6A and 8 are predominantly found during early stages of chondrocyte differentiation in rat long bones [12, 19] Similarly, our

laboratory has previously shown that the predominant spliceform during early stages of zebrafish development included exons 6A, 7, and 8 [29]. Conversely, ColXI ( $\alpha$ 1) peptide p6B encoded by exon 6B is almost exclusively found at the periphery of cartilage underlying the perichondrium preceding the bony collar. In addition, ColXI ( $\alpha$ 1) p6B overlaps with collagen type X in fetal rat long bones suggesting a role for p6B in terminal chondrocyte differentiation [12].

**Table 1: List of real-time primers designed using NCBI's Primer Blast Program.**

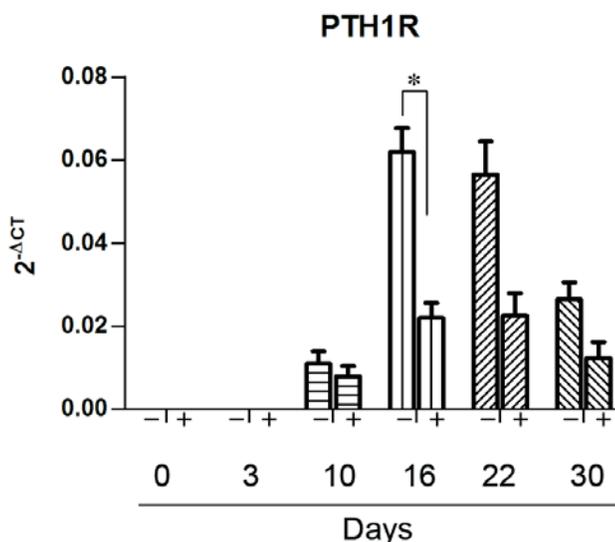
| GENE        | SENSE PRIMER            | ANTISENSE PRIMER         | SIZE(bp) |
|-------------|-------------------------|--------------------------|----------|
| COL2A1      | ACGAAGCGGCTGGCAACCTCA   | CCCTCGGCCCTCATCTCTACATCA | 73       |
| COL11A1 e6A | AGGCTGAGAGTGTAACAGAGA   | TCTGTTTGTGCTACTGTTTCTTCA | 58       |
| COL11A1 e6B | G TTCACATCCCCCAAATCTGA  | CCCCTAGTTTGGCTTTGGCT     | 80       |
| COL11A1 e7  | GGAACAATGGAACCTTACCAGAC | ATTCGATCCTGATACCCGCC     | 54       |
| COL11A1 e8  | AGGAGTAGACGGCAGGGATT    | GGAGGTCGTAGTCCTTTCTTCA   | 100      |
| PPIA        | CGCGTCTCCTTCGAGCTGTTTG  | TGTAAAGTCACCACCCTGGCACAT | 150      |
| PTH1R       | GGGCACAAGAAGTGGATCAT    | GGCCATGAAGACGGTGTAGT     | 210      |



**Figure 3.** (a) Prechondrogenic ATDC5 cells were treated with differentiation medium as described in experimental and incubated in culture for 30 days. Chondrogenesis was monitored using Alcian Blue Stain. (b) The expression of PTH1R and Col2a1 increased up to day 16 and then dropped up to day 30 in culture. (c) Collagen XI alpha is alternatively spliced during ATDC5 chondrogenic differentiation. Exon 6A and 8 expression levels peak on day 16 of chondrogenic differentiation, whereas exon 7 peaks on day 16 and remains increased. In contrast exon 6B expression reaches highest level on day 30 of differentiation.

## 2.2. A Role for PTHrP (1-36) in Collagen XI Alpha 1 Splice Form Expression in ATDC5 Chondrocytes

In differentiated chondrocytes, PTHrP (1-36) downregulated *PTH1R* expression by 3.3-fold (p-value <0.05) on day 16 (**Figure 4**). PTHrP (1-36)- induced downregulation of *PTH1R* was also observed on days 22 and 30, albeit at lower levels, 2.6-fold and 2.3-fold respectively. These results are interesting because other groups have shown an antagonistic role for PTHrP on *PTH1R* expression in human mesenchymal stem cells and mouse vascular smooth muscle cells [30, 31]. In addition, previous studies have shown a complementary expression pattern for *PTHrP* and *PTH1R* in osteoblasts [32]. In the growth plate, it is known that PTHrP secreted by perichondrial cells acts on proliferating chondrocytes to limit their terminal differentiation into hypertrophy. *PTH1R* on the other hand is widely expressed in pre-hypertrophic chondrocytes of the growth plate and promotes hypertrophy [33]. It is thus possible that PTHrP downregulation of *PTH1R* in our study reflects the effects of PTHrP on *PTH1R* expression in growth plate chondrocytes during endochondral ossification.



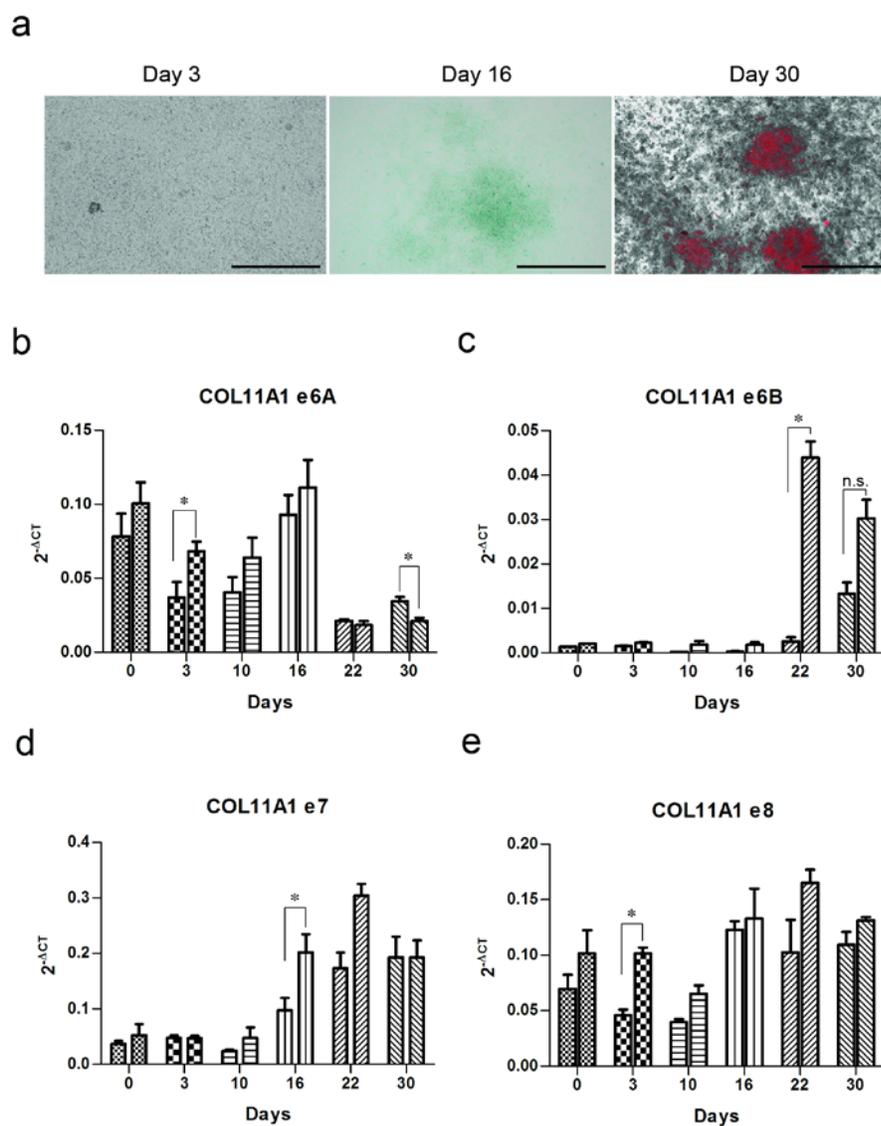
**Figure 4.** ATDC5 cells were differentiated into chondrocytes over 30 days cells were treated with PTHrP ( $10^{-7}$  M) for 24 hours on days 0, 3, 10, 16, 22, and 30. 2 ug of total RNA was used to run real-time PCR and assess the expression of PTH1R as a result of PTHrP treatment. Here, it is shown that PTHrP has no significant effects on PTH1R expression at the early stages of chondrogenic differentiation. After 16 days in incubation medium however, PTHrP reduces *PTH1R* expression levels by 3.3-fold compared to untreated cells. PTHrP-induced decrease in *PTH1R* expression is sustained throughout the chondrogenic differentiation stages reaching peak levels on day 16.

To determine whether PTHrP (1-36) modulates ColXI ( $\alpha$ 1) alternative splicing and expression levels during chondrogenesis, we treated ATDC5 cells at different stages of chondrocyte differentiation with PTHrP (1-36) for 24 hours and analyzed gene expression using real-time PCR. PTHrP (1-36) increased the expression levels of ColXI ( $\alpha$ 1) exons 6A and 8 by 2-fold (p-value <0.05) and 2.2-fold (p-value <0.05), respectively, on day 3. At later stages, PTHrP (1-36) had the opposing effects on ColXI ( $\alpha$ 1) e6A expression and decreased its expression by two-fold (p-value <0.05) on day 30. In contrast, PTHrP (1-36) treatment did not significantly change ColXI ( $\alpha$ 1) e6B expression during early stages of chondrogenesis but increased e6B expression by 3-fold (p-value <0.05) and 2-fold (p-value <0.05) on days 22 and 30, respectively. These findings support the concept of ColXI ( $\alpha$ 1) exons 6A and 8 as early chondrogenic markers that are increased by PTHrP to maintain cells in a proliferative state. Similarly, it is possible that PTHrP up-regulates ColXI ( $\alpha$ 1) exon 6B expression only at later stages of differentiation at the bone collar to promote terminal differentiation.

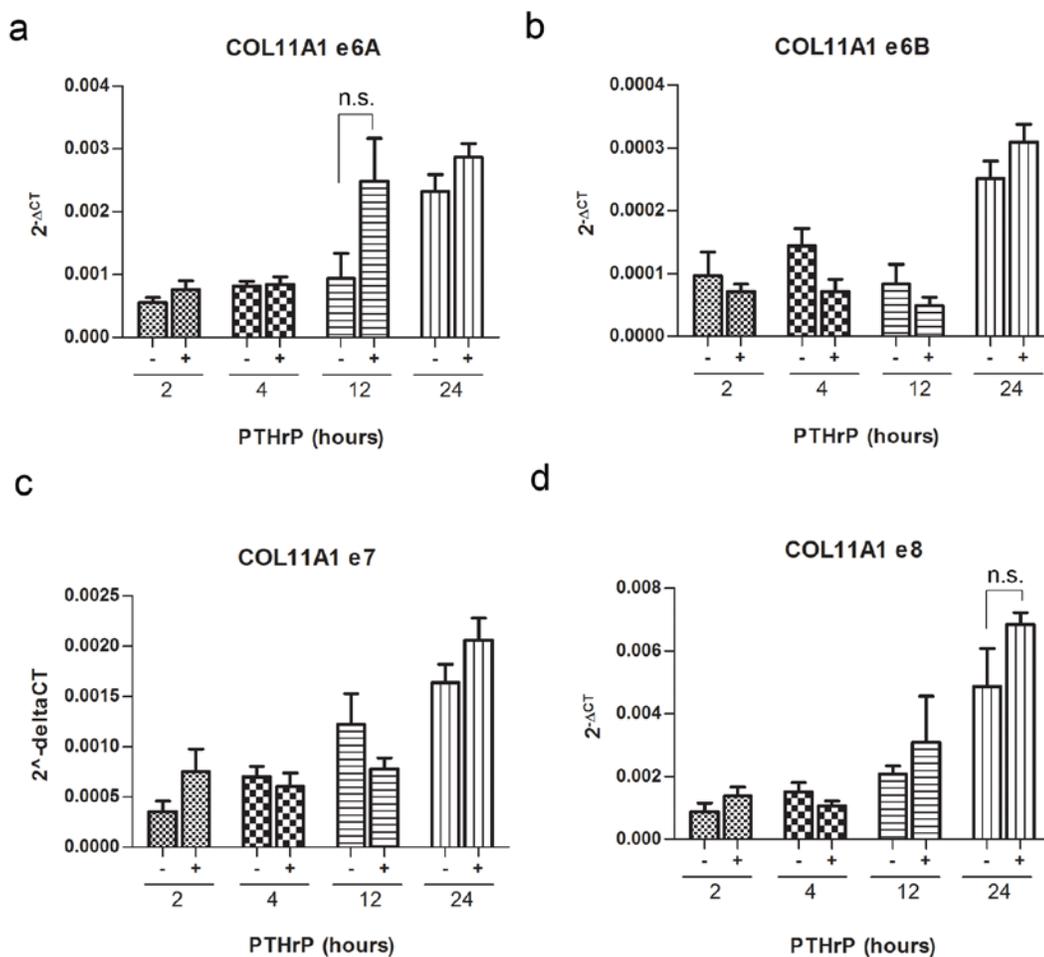
### 2.3. Effects of PTHrP (1-36) on Collagen XI alpha 1 Expression in Pluripotent C2C12 Cells

To determine whether PTHrP (1-36) regulates ColXI ( $\alpha$ 1) expression in a pluripotent stem cell model, we used mouse C2C12 cells, which can be used as a model for cells found at the bone collar. C2C12 cells can differentiate into myoblasts,

osteoblasts, adipocytes, and neurons. Here, we used C2C12 cells as a pluripotent stem cell model and assessed ColXI ( $\alpha 1$ ) expression upon PTHrP (1-36) treatment. Our results showed no significant changes in ColXI ( $\alpha 1$ ) alternative spliceform expression at any time point treatment (**Figures 6 a-d**). To confirm our findings, we co-treated PTHrP-treated cells with a PKA inhibitor H89 and as expected did not observe any significant changes to the expression levels (data not shown). It is important to note that we used PTHrP in a continuous fashion to treat C2C12 cells. Previous studies have shown opposing roles for PTHrP (1-36) depending on whether it was used in a continuous PTHrP or intermittent manner. Continuous PTHrP (1-36) treatment results in bone loss, whereas intermittent exposure can increase bone formation [34].



**Figure 5.** (a) ATDC5 cells were differentiated into chondrocytes and chondrogenesis was assessed using Alcian Blue and Alizarin red. (b) PTHrP treatment of ATDC5 cells increased ColXI ( $\alpha$ 1) exon 6A expression during early stages of chondrogenesis but decreased its expression on day 30. (c) PTHrP increased ColXI ( $\alpha$ 1) exon 6B expression during late stages of chondrogenesis. (d) PTHrP did not affect exon 7 expression during early chondrogenesis but increased its expression on days 16 and 22. On day 30, PTHrP did not change ColXI ( $\alpha$ 1) exon 7 expression. (e) ColXI ( $\alpha$ 1) exon 8 expression is increased by PTHrP on day 3 similar to exon 6A but remains unchanged with treatment at later stages of chondrogenesis.



**Figure 6.** (a-d) PTHrP did not affect the expression and alternative splicing of ColXI ( $\alpha 1$ ) in pluripotent C2C12 cells. Cells were plated at  $2 \times 10^4$  cells/cm<sup>2</sup> and grown overnight in maintenance medium. The next day, cells were treated with PTHrP for 2 hr, 4 hr, 12 hr, and 24 hr continuously. RNA was extracted and the expression of ColXI ( $\alpha 1$ ) spliceforms was assessed using real-time PCR.

### 3. Experimental Section

#### 3.1. Cell Culture, Differentiation, and Treatment

The mouse pluripotent cell line, C2C12 was purchased from ATCC and maintained in DMEM (Sigma Chem. Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY) and antibiotics (100 units/mL of Penicillin-G and 100  $\mu$ g/mL of Streptomycin). Cells were kept at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. To assess PTHrP effects, cells were plated at  $2 \times 10^4$

cells/cm<sup>2</sup> in DMEM supplemented with 10% FBS, 100 U/mL Penicillin and 100 µg/mL Streptomycin and hPTH1H (10<sup>-7</sup> M) (Sigma Aldrich; St. Louis, MO) for the indicated amount of time. At each time point, total RNA was extracted from the cells using TriZol (Gibco-BRL) and real-time PCR was performed as described below.

For chondrogenesis experiments, prechondrogenic cells mouse ATDC5 cells were purchased from ATCC and maintained in DMEM: F12 (1:1) supplemented with 5% fetal bovine serum (FBS) (GIBCO, Grand Island, NY) and antibiotics (100 units/mL of Penicillin-G and 100 µg/mL of Streptomycin). For differentiation studies, ATDC5 cells were grown in micromass cultures as following: cells were suspended at 1.0 x 10<sup>7</sup> cells/mL in maintenance medium supplement with ITS-G (Insulin-Transferrin-Selenium, Life Technologies, Carlsbad, CA). Three 10 µl drops were placed into a well of a 6-well plate and let adhere for 2 hr in the incubator. Two milliliters of media was added to each well. Media was changed every two days. PTHrP was added on days 0, 3, 10, 16, 22, and 30. RNA was extracted using 1 mL TriZol per well of a 6-well plate.

### 3.2. Alcian Blue and Alizarin Red Staining

ATDC5 cells were plated in micromass cultures as described above in 12-well plates and cultured in differentiation medium for 30 days. At each time point, cells were rinsed with PBS and fixed with 95% methanol for 15 minutes. Then, cells were stained with 0.1% Alcian Blue 8GS (Fluka, Buchs, Switzerland) in 0.1M HCl overnight at room temperature. The next day, cells were rinsed with PBS and imaged using an Olympus America's BX53 microscope.

For mineralization, cells were cultured in differentiation medium on glass coverslips as described above and incubated for 30 days. On day 30, cells were rinsed

three times with PBS and fixed in 95% ethanol for 15 min. Cells were then stained with 400  $\mu$ l of 40 mM Alizarin Red (Millipore, Billerica, MA) for 20 minutes. After destaining with deionized water, samples were imaged using an AMG EVOS Fluorescence Microscope.

### 3.3. Primer Design and Real-Time PCR

Primers were designed for each exon using Primer Blast (NCBI) (**Table 1**). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies). Each 20  $\mu$ L PCR reaction consisted of 10  $\mu$ L of SYBR Green PCR Master Mix, 500 nM of each forward and reverse primer, and 1  $\mu$ L of undiluted template cDNA and nuclease-free water. Targets were amplified using Eppendorf Mastercycler as following: 50°C (2 min), 95°C (10 min) followed by 40 cycles of 95°C (15 sec) and 60°C (1 min) followed by one cycle of 72°C (1 min). PCR products were separated by electrophoresis through 4% Nusieve 3:1 Agarose as per manufacturer's instructions (Lonza, Basel, Switzerland) to verify that the used primer pair produced the expected product. A no-template control was also included in the assay substituting water for template.

### 3.4. Statistics

Results are reported as the mean $\pm$ SEM n = 3 in each group. One-way ANOVA followed by *post-hoc* Bonferroni test was used to determine the statistical differences between the various experimental and control groups. \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ , \*\*\* indicates  $p < 0.001$ , and \*\*\*\* indicates  $p < 0.0001$ , and n.s. is not significant.

#### 4. Conclusions

In summary, our findings indicate that collagen XI alpha 1 is alternatively spliced during ATDC5 chondrogenic differentiation *in vitro*. These results will enable further studies on the role of different collagen XI alpha 1 spliceforms during chondrogenesis. In addition, our work provided evidence for a role for PTHrP in collagen XI alpha 1 alternative splicing and expression in ATDC5 cells. We also showed that continuous PTHrP (1-36) did not alter ColXI ( $\alpha 1$ ) spliceform expression in pluripotent C2C12 cells.

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#### Author Contributions

Conceived and designed the experiments: JTO, NS. Performed the experiments: NS. Analyzed the data: NS, JTO. Drafted manuscript: NS, JTO.

#### Conflicts of Interest

The authors declare no conflict of interest.

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## APPENDIX

**Role of Extracellular Matrix Protein and Minor Fibrillar Collagens in Sox-Induced  
Chondrogenesis During Craniofacial Development - A Minireview**

## PUBLISHED MINIREVIEW

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## **Role of extracellular matrix and minor fibrillar collagens in Sox induced chondrogenesis during craniofacial development - A mini-review**

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### **ABSTRACT**

An essential aspect of craniofacial development is chondrogenesis, the process of chondrocyte differentiation from multipotent neural crest cells. These multipotent cells delaminate from the neural tube during neurulation, travel along specific routes and differentiate into various cell types forming different tissues. The formation of this wide range of neural crest cell derivatives is dependent on multiple factors, including transcription factors, extracellular matrix proteins, and growth factors. Sox proteins are transcription factors that play a role during development of various tissues. L-Sox5, Sox6, and Sox9 play a role in the differentiation of neural crest cells into chondrocytes by binding to regulatory elements, enhancing the expression of chondrocyte-specific collagens. Minor fibrillar collagens V and XI contribute to chondrogenesis of the craniofacial skeleton by mechanisms that include the regulation of collagen fibrillogenesis and a bridging function between major fibrillar collagens and other cell or matrix molecules. However, the mechanism of regulation is not fully understood. Disruption of craniofacial development during chondrogenesis can lead to Stickler Syndrome or Campomelic Dysplasia as a result of collagen XI and Sox9 mutations, respectively.

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Here, we focus on potential additional mechanisms by which the extracellular matrix plays a role in Sox-mediated chondrogenesis during craniofacial development. PTHrP may be the link between the expression of extracellular matrix and Sox transcription factor family-mediated regulation of chondrogenesis, creating a regulatory cycle that supports growth and development of cartilage-derived structures.

**KEYWORDS:** Sox9, craniofacial, chondrogenesis, neural crest cells, collagen, PTHrP

## ABBREVIATIONS

Sox (Sry (Sex determining Region Y)- HMG box), ECM (Extracellular Matrix), EMT (Epithelial-Mesenchymal Transition), FGF (Fibroblast Growth Factor), Hox (Homeobox), NCC (Neural Crest Cell), CD (campomelic dysplasia), cyclic-AMP (cAMP) response element-binding (CREB), BMP (Bone Morphogenetic Protein), MAP (Mitogen-Activated Protein), PGC1- $\alpha$  (Proliferation activated receptor gamma co-activator 1- alpha), TRAP (thyroid hormone receptor associated protein), PKA (Protein Kinase A), PNS (Peripheral Nervous System), PTHrP (Parathyroid hormone-related protein).

## INTRODUCTION

Congenital craniofacial abnormalities span a spectrum of deformities including cleft palate, deformed jaw, severe defects of the eye, and mental retardation. Due to the nature of these disorders, treatment is limited and can lead to lifelong physiological and psychological suffering of the concerned individuals.

The craniofacial skeleton is formed via intramembranous and endochondral ossification in which cartilage formation precedes that of bone. Multipotent mesenchymal cells condense and differentiate into chondroblasts, which then proliferate rapidly and differentiate into chondrocytes. Chondrocytes deposit the cartilage-specific extracellular matrix (ECM) proteins, proliferate, and become hypertrophic. Upon hypertrophy, the cells grow in size, alter their collagen expression, and eventually undergo apoptosis. Simultaneously, mesenchymal cells surrounding the hypertrophic cartilage differentiate into osteoblasts and secrete bone matrix, while osteoclasts degrade the hypertrophic cartilage as the cartilage model is replaced with newly formed bone tissue [1].

Collagen is the most prevalent protein found in the ECM, with 29 different types discovered to date [2, 3]. Based upon abundance, collagens are divided into major fibrillar collagens, including collagen types I and II, and minor fibrillar collagens, including types V and XI. Collagens V and XI can form hybrid molecules, therefore they are considered to belong to the same collagen type, which can be referred to as collagen type V/XI. *In vivo* studies of collagen V/XI morphants have demonstrated their role as regulatory proteins that contribute to fibrillogenesis [4, 5]. However, while major fibrillar collagens have been studied extensively, additional roles for minor fibrillar collagens in cellular differentiation and signaling require further study and are still being defined.

The cartilaginous framework of the craniofacial skeleton is formed by differentiation of multipotent neural crest cells (NCCs) into mature chondrocytes. Disruption of chondrogenesis causes severe facial skeletal structure abnormalities and has detrimental effects on the embryo. Deformities of the craniofacial features can be induced by genetic factors. For instance, mutations in minor fibrillar collagens V/XI can cause Marshall, Stickler, or Ehlers-Danlos syndromes, which all present complex phenotypes of flattened

face, hearing loss, eye abnormalities, and joint problems. Likewise a mutation of the *Sox9* gene in humans can result in campomelic dysplasia (CD) [6], a genetic condition characterized by craniofacial defects, severe damage of the endochondral bone, and sex reversal [7]. Similar anomalies are observed *in vivo* when *Sox9* ortholog genes are knocked out in other species including zebrafish (*Danio rerio*), mouse (*Mus musculus*), and frog (*Xenopus laevis*). Interestingly, Parathyroid Hormone-related Protein (PTHrP) homozygous mutant mice also exhibit a chondrodysplastic phenotype characterized by a deformed skull, short snout and mandible, protruding tongue, and disproportionately short limbs, which are all similar to characteristics in human osteochondrodysplasias [8]. In all the above-mentioned disorders, chondrogenesis and bone formation are interrupted in the development of the craniofacial structures.

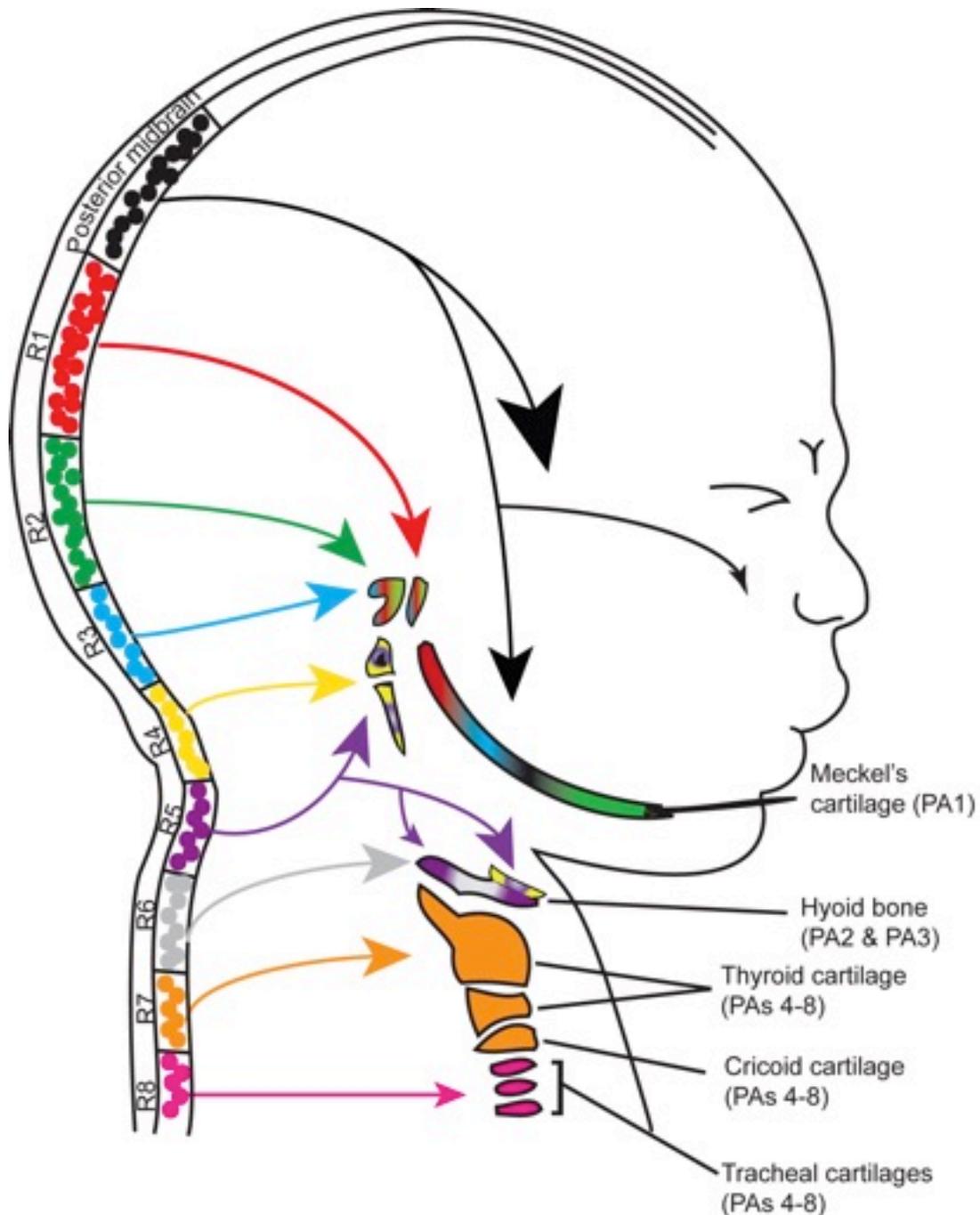
NCCs represent a population of multipotent stem cells that arise from the rhombomeres of the hindbrain in vertebrates. These cells undergo an epithelial-mesenchymal transition (EMT) during the first few weeks of embryogenesis and migrate to different sites in the body, where they differentiate into neuronal cells, cardiac crest cells, pigment cells, chondrocytes, and osteocytes [9, 10, 11]. Differentiation into this wide array of derivative cells is tightly regulated by transcription and growth factors. The different transcription and growth factors work together within a complex system and achieve proper formation of the craniofacial skeleton and other developmental structures during embryogenesis. Growth factors such as fibroblast growth factor (FGF), Wnt, bone morphogenetic protein (BMP), PTHrP, and transcription factors including Hox and Sox proteins influence chondrogenesis and thus the development of the craniofacial skeleton [12]. Within the Sox (SRY- HMG box) transcription family, L-Sox5, Sox6, and Sox9 regulate chondrogenesis [13] shown by *L-Sox5sox6* double mutants, which lack endochondral bones and exhibit severe skeletal abnormalities [14]. Similarly, PTHrP plays a role in proper endochondral ossification as indicated by PTHrP-deficient mice exhibiting abnormalities of the craniofacial cartilages and skeletal deformities [15].

Due to similar anomalies observed in PTHrP, Sox9, and minor fibrillar collagen mutant model organisms, investigation into the potential link between the three factors may provide fundamental knowledge regarding the mechanisms at play during chondrogenesis of neural crest derivatives, a possibility that has not been characterized.

### **Craniofacial development and neural crest cells**

Craniofacial cartilage structures can be divided into seven prominences including the frontonasal, and one pair each of the mandibular, maxillary and lateral nasal (Figure 1) [16]. The frontonasal features arise from NCCs to comprise the forehead, nose, upper lip, and primary palate. Structures of the mandibular and maxillary prominences include the lower jaw, sides of the face, and lips [16, 17].

Upon gastrulation, the ectoderm forms the non-neural ectoderm, the neural plate and the NCCs [18]. Cells of the non-neural ectoderm form the epidermis, while the neural plate folds to give rise to the central nervous system, including the brain and the spinal cord [19]. During the initial stages, the brain is divided into three primary vesicles: the



**Figure A1. Neural crest cell migration from the posterior midbrain and rhombomeres of the hindbrain to various sites of differentiation.** NCCs from the posterior midbrain migrate to the frontonasal region and contribute to nasal structures as well as to the first pharyngeal arch forming Meckel's cartilage. NCCs migrate from rhombomeres 1, 2, and 3 form Meckel's cartilage, whereas rhombomeres 4 and 5 make up ear structures and part of the hyoid bone. NCCs from rhombomeres 5 and 6 form the greater horn of the hyoid bone. Similarly, rhombomere 7 NCCs migrate to form thyroid and cricoids cartilages. NCCs from rhombomere 8 give rise to tracheal cartilages.

forebrain, midbrain and hindbrain. The hindbrain, or rhombencephalon, consists of rhombomeres where NCCs originate. These mesenchymal cells migrate to the frontonasal region and to one or more of the 6 pharyngeal arches (Figure 1). Each pharyngeal arch gives rise to specific structures of the craniofacial and neck skeleton. Pharyngeal arch 1 forms the mandibular and maxillary structures including the maxilla, zygomatic bone, temporal bone, incus, malleus, and Meckel's cartilage. Pharyngeal arch 2 shapes the stapes, styloid process, and hyoid bone. Pharyngeal arch 3 makes up the greater horn of the hyoid bone, and pharyngeal arches 4-6 form the arytenoid cartilage, thyroid cartilage, and other laryngeal cartilages [20].

Collagens V/XI are expressed in pre-migratory NCCs as well as in developed craniofacial cartilages in zebrafish [21, 22]. Interestingly, the expression patterns of the minor fibrillar collagens are highly comparable to that of Sox9 in the craniofacial structures, although there is no direct evidence showing an interaction between Sox9 protein and minor fibrillar collagen genes. Similarly, accelerated endochondral bone formation and chondrocyte maturation in the craniofacial cartilaginous structures in PTHrP-deficient mice indicate expression of PTHrP in the craniofacial structures [23].

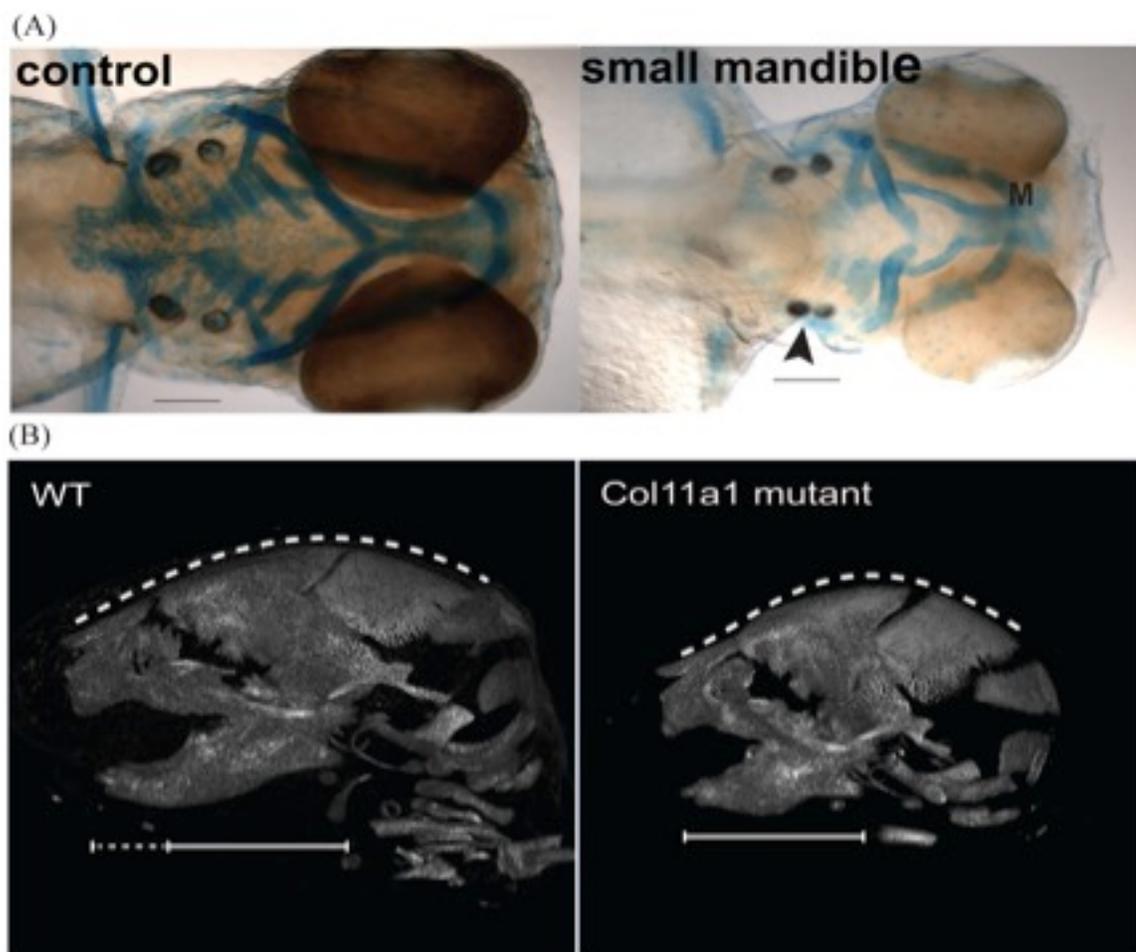
### **Minor fibrillar collagens and SOX proteins**

During endochondral ossification, chondrocytes deposit major collagen type II and minor fibrillar collagen type V/XI [24]. As cartilage is mineralized and converted to bone, chondrocytes change their overall expression pattern to collagen type I and more predominantly minor fibrillar collagen V. Collagens consist of three peptide alpha chains forming either heterotrimers or homotrimers [25, 26, 27]. Three different collagen XI alpha chains have been described: *Col11a1*, *Col11a2* and *Col11a3* [28, 29]; where *Col11a3* is actually a modified form of *Col2a1*. Similarly, three collagen V alpha chains have been described in humans: *Col5a1*, *Col5a2* and *Col5a3* [21]. Minor fibrillar collagens V and XI play a role in the organization of major fibrillar collagens I and II, respectively, by forming heterotrimers with them. Collagens V/XI share strong homology among their respective protein domains and are interchangeable, forming collagen V/XI isoforms [30, 31, 32].

### **What can we learn from animal models?**

The role of collagen V/XI in chondrogenesis of craniofacial skeletal structures has been studied in mice and in zebrafish [4, 5]. Expression of *Col5a1* in developing cranial cartilages, *Col11a2* expression in cephalic mesoderm, and a unique *Col11a1* expression in the hindbrain has been identified in zebrafish [21], suggesting a potential role in the induction and differentiation of NCCs into craniofacial cartilages. This potential is supported by vertebral and craniofacial abnormalities in zebrafish *Col11a1* morphants. The abnormalities include a smaller lower jaw and abnormal otolith genesis (Figure 2A). Analogous defects have been observed in chondrodysplasia (*cho/cho*) mice lacking a functional *Col11a1* protein (Figure 2B). The *cho/cho* mouse demonstrates a morphology characteristic of Marshall's and Stickler syndromes and fibrochondrogenesis in humans

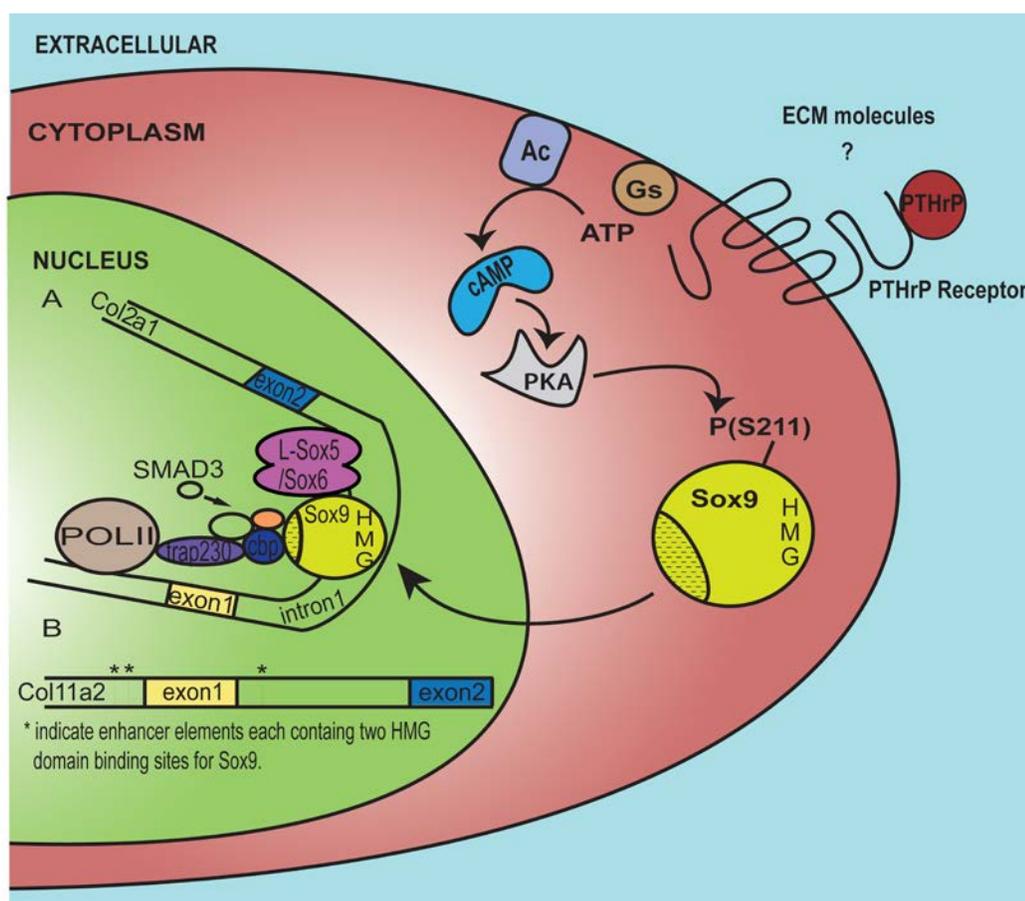
[33]. Likewise, mutations in *Col11a2* have been linked to osteochondrodysplasias in humans [34].



**Figure A2. Effects of *Col11a1* knockdown in mouse and zebrafish. *Col11a1* morphant zebrafish show a smaller mandible (M) and smaller otoliths (arrowhead).** Similarly, microcomputed tomography representation of wildtype (WT) and *Col11a1* mutant chondrodysplastic (cho) mice at embryonic day 17.5 show an overall smaller, deformed cranium (dashed lines), and decreased jaw length (bar), illustrating the role for *Col11a1* in craniofacial development.

Multiple families of transcription factors are responsible for the expression of genes necessary for chondrogenesis. One essential family of transcription factors is represented by the highly conserved Sox family. All Sox proteins share the same high mobility (HMG) domain that is extraordinarily conserved among vertebrates (Figure 3) and bind to a similar minor groove DNA motif of (A/T)(A/T)CAA(A/T)G resulting in bending of the DNA helix [7, 35, 36]. Bending of the DNA facilitates the unwinding of the DNA double helix, which in turn causes the gene promoters to be in a more open conformation and enhance transcriptional activity. Sox transcription factors are divided into 8 subgroups of A-H [37]. Sox9 is a transcription factor that belongs to subgroup E and has a characteristic transcription domain at its C-terminus [38]. *Sox9* is expressed in a variety

of cell types, including mesenchymal NCCs, Sertoli cells of the testes, cardiac neural crest cells, the kidney, and all chondroprogenitor cells [4]. Along with Sox9, other SoxE members such as Sox8 and Sox10 play a role in the development of the NCCs prior to their migration. Once the NCCs start migrating, *Sox9* expression is downregulated and expression of *Sox8* and *Sox10* are lost. However, upon arrival at the site of chondrogenesis, the NCCs up-regulate *Sox9* [16]. Sox9 does not play a role in NCC migration as evidenced by normal cell migration patterns in *Sox9* mutant cells [1]. Nonetheless, at the site of chondrogenesis, *Sox9* plays a crucial role in not only mesenchymal cell condensation, but also proliferation and differentiation into chondrocytes [39, 7].



**Figure A3. Schematic of a potential mechanism by which extracellular matrix proteins can act upstream or downstream of PTHrP in the cell.** Collagens V/XI may act upstream and interact with PTHrP or PTHrP receptor in the extracellular matrix activate the PKA pathway, and regulate chondrogenesis via Sox9. Alternatively, PTHrP may regulate the expression of minor fibrillar collagens V/XI by activating the PKA pathway and phosphorylating Sox9 in the cytoplasm, which can then move to the nucleus and regulate the expression of the minor fibrillar collagens in a similar manner that it regulates Col2a1. Both Col11a1 and Col11a2 have transcription sites for Sox9.

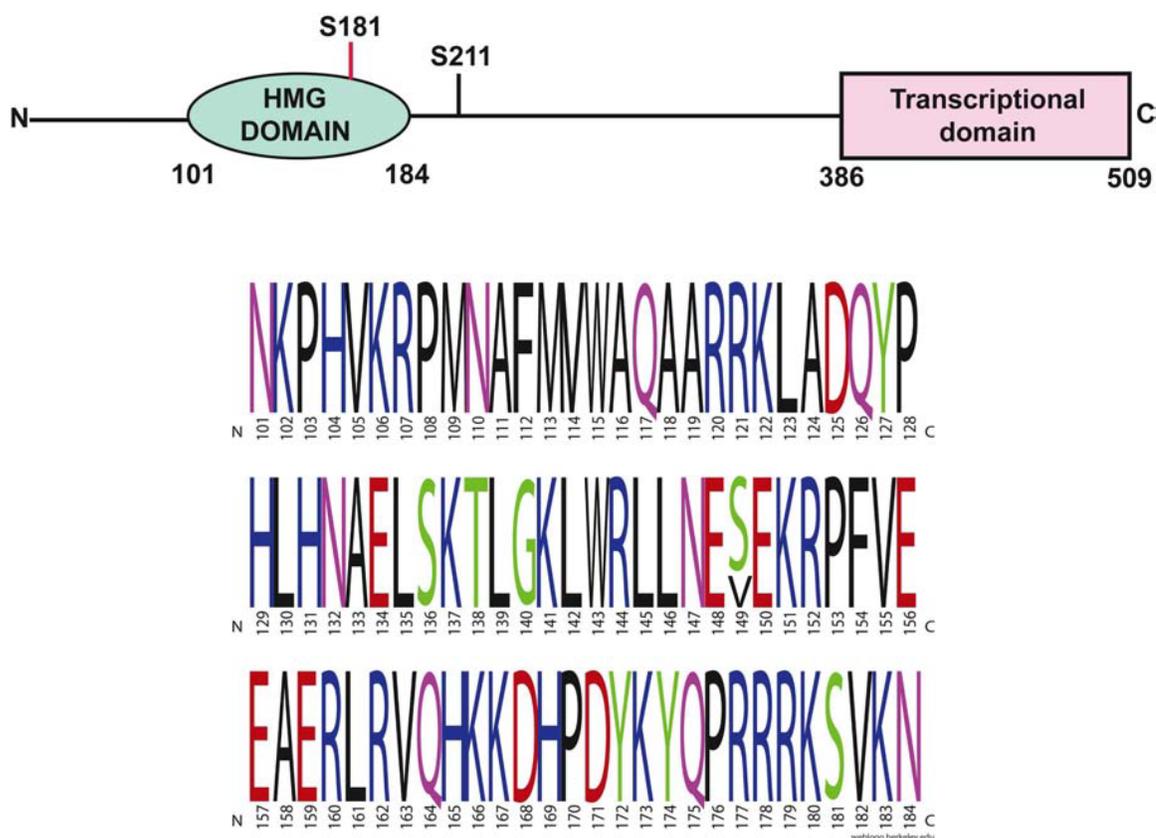
Additionally, L-Sox5 and Sox6 are required for the development of chondroblasts [40]. Both Sox factors *L-Sox5* and *Sox6* are co-expressed with *Sox9* during chondrogenesis except for the period prior to mesenchymal condensation verified by experiments with *Sox5sox6* double mutants demonstrating normal mesenchymal condensations but no chondrogenesis [10, 40]. All three Sox proteins are required for the differentiation of chondroblasts to chondrocytes [14]. L- Sox5 and Sox6 belong to subgroup D and share a similar sequence in their coiled-coil region forming homo- or hetero-dimers [4]. Dimerization of L- Sox5 and Sox6 facilitates their binding to the first intron of *Col2a1*. They share the common HMG domain with Sox9 [41]. As neither L-Sox5 nor Sox6 have a transcription activation domain, they act as architectural factors that do not influence the transcriptional activity but can indirectly modulate gene activity [42].

L-Sox5, Sox6, and Sox9 recognize HMG binding sites on *Col2a1* and *Aggrecan*, the major structural proteoglycan found in the ECM of cartilage. Collagen type II, the major protein of the cartilage, contains a 48 bp sequence in its first intron, which contains 4 binding sites for Sox proteins [12]. Sox proteins can bind to *Col2a1* and *Aggrecan*, enhancing their expression [12]. Similarly, HMG binding sites have been identified on *Coll1a2* that can be recognized by Sox9 [39, 43, 44]. Computational analyses have also identified seven conserved pairs of HMG binding domains in the promoter region of *Coll1a1* in both humans and mice, which could further show a relationship between Sox9 activity and collagen type XI [45]. Recent EMSA studies have determined true Sox9 binding sites on *Coll1a1* [46]. However, to date, regulation of *Coll1a1* expression by Sox9 has not been demonstrated. One possibility is that the growth factors mediating expression of cartilage-specific extracellular matrix proteins may do so through the activity of Sox family transcription factors.

### **Regulation of chondrogenesis**

Binding of L-Sox5, Sox6, and Sox9 to *Col2a1* promotes the assembly of a multiprotein enhancer complex and activates the basal transcriptional machinery required for chondrogenesis (11). L-Sox5, Sox6, and Sox9 regulate transcription in chondrogenesis using a cyclic-AMP (cAMP) response element-binding (CREB) protein mediated by DNA acetylation [37]. Sox9 activity can be regulated by multiple factors. For instance, Smad3 increases the interaction between Sox9 and CREB-Binding Protein (CBP/p300), which in turn increases Sox9 transcriptional activity [47]. Similarly, proliferation activated receptor gamma co-activator 1-alpha (PGC1- $\alpha$ ) co-activates Sox9 during chondrogenesis [48]. The interaction between Sox9 and thyroid hormone receptor-associated protein 230/Med12 (TRAP) also significantly increases Sox9 transcriptional activity [49]. Since *Coll1a1* and *Coll1a2* both contain multiple Sox9 binding sites, it is possible that the same transcriptional machinery may be used to regulate their expression in cartilage (Figure 4). Further studies are required to verify the role for Sox9 in *Coll1a1* and *Coll1a2* expression. *Sox9* expression is regulated by many growth factor signaling pathways. For instance, FGFs use the Mitogen Activated Protein (MAP) pathway to increase *Sox9* expression in chondrocytes and mesenchymal cells. Both chondrocytes and mesenchymal cells express FGF receptors [50]. Likewise, PTHrP increases cAMP levels

and phosphorylates Sox9 by activating the Protein Kinase A (PKA) pathway *in vivo* [51]. Phosphorylation of Sox9 enhances its activity and leads to increased *Col2a1* expression in the prehypertrophic zone of the growth plate [51] (Figure 4).



**Figure A4. Sox9 protein has highly conserved high mobility group (HMG) domain and sites of phosphorylation.** The HMG domain is a conserved domain (amino acids 101-184) within human (*H. sapiens*), mouse (*M. musculus*), rabbit (*O. cuniculus*) and zebrafish (*D. rerio*). Serine 181 (S181) and serine 184 (S211) are PTHrP dependent sites of phosphorylation on Sox9. Sequence logo was generated using WebLogo 3.0 [54, 55].

Both Sox9 and PTHrP prevent proliferating chondrocytes from becoming hypertrophic [45]. As a result, cartilage is conserved and bone formation is inhibited. Inhibited differentiation of proliferating chondrocytes into hypertrophic chondrocytes could alternatively be due to the lack of ECM components as suggested by Akiyama. Indeed, mice lacking *Col11a1* show premature ossification [5] (Figure 2B). *Col11a1* binds to heparin sulfate proteoglycans and through this interaction may facilitate FGF signaling that is dependent on heparan sulfate [52, 53]. FGF can subsequently stimulate chondrogenesis via Sox9 activation. Similarly, Sox9, PTHrP, and *Col11a1* all show similar or overlapping effects in preventing chondrocytes from becoming hypertrophic and thus mineralizing. It is notable to mention that pre-hypertrophic chondrocytes express Sox9 and the PTHrP receptor [46]. Extracellular matrix may modulate PTHrP-mediation of chondrogenesis and the transition from proliferative chondrocyte to hypertrophic

chondrocyte. Alternatively, PTHrP may induce changes in the expression of extracellular matrix molecules that are critical for the formation of cartilage. To date, the analysis of PTHrP-mediated changes during skeletal development has not included an analysis of the minor fibrillar collagens. It is possible that minor fibrillar collagens in the ECM bind to PTHrP or alternatively to its receptor and regulate the PTHrP pathway, acting upstream of the cellular signaling pathway during chondrogenesis. Studies on the regulation of PTHrP-Sox mediated chondrogenesis may provide us with a better understanding of the molecular mechanism of craniofacial development.

## CONCLUSION

The role of minor fibrillar collagens V/XI in L-Sox5, Sox6, and Sox9-mediated chondrogenesis and the interaction with growth factors that provide external cues during craniofacial development is a relatively new area of investigation. Since the cartilage abnormalities in *Col11a1 cho/cho* mice, and *Col11a1* zebrafish mutants are similar to those induced in *Sox9* mutant mice and zebrafish, and PTHrP-deficient mice, an investigation of the interaction between these molecular constituents may provide fundamental information. Studies are needed to look at the role of PTHrP in Sox-mediated chondrogenesis and the relationship to minor fibrillar collagens. The multipotency of NCCs is of great interest to basic science as well as to the development of regenerative medicine approaches for the treatment of skeletal injuries. Understanding the interplay between minor fibrillar collagens, Sox proteins, and PTHrP in NCC-derived structures could explain the multiple levels of regulation of chondrogenesis and thus proper cartilage formation during craniofacial development of the embryo. The results could help us understand the mechanism by which minor fibrillar collagens regulate chondrogenesis as well as how minor fibrillar collagens are regulated during this process. Having a better understanding of the mechanisms by which chondrogenesis and cartilage formation occur may provide us with new tools to diagnose and prevent disorders of the craniofacial skeleton including cleft palate and osteochondrodysplasias *in utero*.

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