

Article

Collagen alpha 1(XI) is required for chondrocyte differentiation through the AKT/GSK3 β / β -catenin signaling axis in ATDC5 cells

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Abstract: Cartilage development is a tightly regulated process that involves multiple molecules and signaling pathways. The loss of expression of a single gene can alter cell behavior and disrupt the development of the cartilage. *Col11a1* encodes the alpha one chain of the minor fibrillar collagen type XI that is essential in skeletal development. We used an RNAi mediated knockdown approach to investigate the role of *Col11a1* expression during chondrogenesis in ATDC5 cells. *Col11a1* expression promotes the transition of mesenchymal cells to the chondrogenic phenotype, and reduction of *Col11a1* expression interferes with cellular differentiation. Our results indicate that collagen $\alpha 1(XI)$ protein is required for chondrocyte cell shape, matrix production, mineralization and gene expression through a mechanism that involves AKT/GSK3 β / β -catenin and TCF/LEF activity in ATDC5 cells.

Keywords: chondrogenesis; cartilage; col11a1; collagen $\alpha 1(XI)$, AKT; GSK3b; Wnt; ATDC5

1. Introduction

Endochondral ossification requires that a cartilage template is formed prior to initiating ossification [1]. The process of endochondral ossification is the primary bone forming mechanism during vertebrate development [2]. Therefore, successful vertebrate development requires the formation of a cartilage template through chondrogenesis. To successfully form the cartilage analgen, the cells must proceed through a series of maturation steps that begin with prechondrogenic mesenchymal cells forming a condensation and initiating the expression of genes involved in chondrocyte differentiation and matrix formation. The chondrocytes continue to mature and further differentiate into hypertrophic chondrocytes that contribute to matrix remodeling and mineralization. Chondrocytes are also responsible for longitudinal growth as residents of the growth plate of the bone [2,3]. Errors occurring during the formation of the cartilage can cause skeletal dysplasia or increase the susceptibility to the early onset of connective tissue diseases such as osteoarthritis.

COL11A1 gene expression is essential for normal skeletal development, evident by human dysplasias and severe tissue disorganization in mice [4,5]. Fibrochondrogenesis and Stickler's syndrome type II are human skeletal dysplasias associated with mutations in the *COL11A1* gene [6,7]. Additionally, genetic mutations in *COL11A1* contributes to intervertebral disc disease and degradation of the articular cartilage [8,9,10].

Collagen type XI is composed of three alpha chains, $\alpha 1(XI)$, $\alpha 2(XI)$, and $\alpha 3(XI)$ [11,12]. Collagen type XI both nucleates fibril formation and regulates the fibril diameter [13,14,15]. The amino terminal domain of the $\alpha 1(XI)$ chain is a unique domain to clade B fibrillar collagens that interacts with multiple extracellular molecules, making it essential in providing integrity to the matrix network [16,17,18,19]. Despite our knowledge of the

structural role of collagen type XI in the extracellular matrix of cartilage, the biological impact of *COL11A1* gene mutations on cell behavior and molecular phenotype remain unknown.

Previous studies have established the importance of collagen $\alpha 1(XI)$ protein in skeletal development, maintenance and health, but they have not investigated how decreased *COL11A1* expression affects the chondroprogenitor cell behavior during chondrogenesis. The chondroprogenitors are derived from mesenchymal stem cells and maturation into overt chondrocytes is a tightly regulated, sequential process [20]. The chondrocytes undergo proliferation and upregulate chondrogenic gene expression of *COL2A1*, *ACAN*, and *SOX9*. These cells further change their expression to include upregulation of the hypertrophic chondrocyte markers *COL10A1*, *RUNX2*, and *MMP13*. Deviation from the normal expression pattern may alter the extracellular matrix environment permanently, causing chondrodysplasia and susceptibility to degenerative cartilage disease.

We investigated the role that collagen $\alpha 1(XI)$ plays during chondroprogenitor cell differentiation and cell signaling events during chondrogenesis using the mouse ATDC5 chondrogenic cell line. ATDC5 cells are a well-established murine cell line used for studying the process of chondrogenesis and the transcriptome has been well documented [21,22,23]. To investigate the early role of collagen $\alpha 1(XI)$ in chondroprogenitor cells, we used an RNAi approach to inhibit *Col11a1* expression during chondrocyte differentiation. We analyzed the expression of chondrogenic markers in the knockdown cell culture as well as the activity of the β -catenin signaling pathway. We found that in the presence of siRNA for *Col11a1*, chondrocyte cellular morphology was inhibited compared to control cells. We identified alteration in the phosphorylation of AKT, GSK3 β , β -catenin, and activation levels of the TCF/LEF activity. The results of these studies indicate a role for collagen $\alpha 1(XI)$ in cell signaling pathways that are required for chondrocyte differentiation of ATDC5 cells.

2. Results

2.1. Characterization of *Col11a1* expression during chondrogenesis in ATDC5 cells.

We characterized the expression profile of the *Col11a1* gene in the mouse chondrogenic ATDC5 cell line induced to differentiate with ITS and A2P (Figure 1). Day 0 of the experiment was designated as the time at which culture medium was exchanged for differentiation medium in confluent cultures. Using quantitative RT-PCR, we determined that *Col11a1* mRNA was increased 7.6-fold at day 7 relative to day 0, and 156-fold by day 14. We also demonstrated that *Col11a1* mRNA levels significantly decreased between day 14 and 21 to 20% of the levels observed on day 14 (Figure 1A). Western blot analysis using two separate rabbit polyclonal antibody directed to the collagen $\alpha 1(XI)$ chain epitopes confirmed protein production at each of the investigated time points (Figure 1B). Collagen $\alpha 1(XI)$ protein was detectable at day 0 and day 7 and, in agreement with the mRNA levels, collagen $\alpha 1(XI)$ protein reached maximally detectable levels in cell lysates by day 14. The protein detected on day 21 remained high despite the significant decrease in mRNA levels, a result that is not surprising given the long half-life of collagen proteins [24,25].

In addition to *Col11a1*, we confirmed the expression of chondrogenic differentiation markers in culture (Figure 1C). The expression patterns that we observed were consistent with previously reported results in ATDC5 cells using ITS and A2P to induce chondrogenic differentiation [21,22]. *Col2a1* and *Acan* mRNA were increased at day 7 of differentiation. We did not observe significant changes in *Sox9* mRNA between day 0 and 7 (Figure 1C).

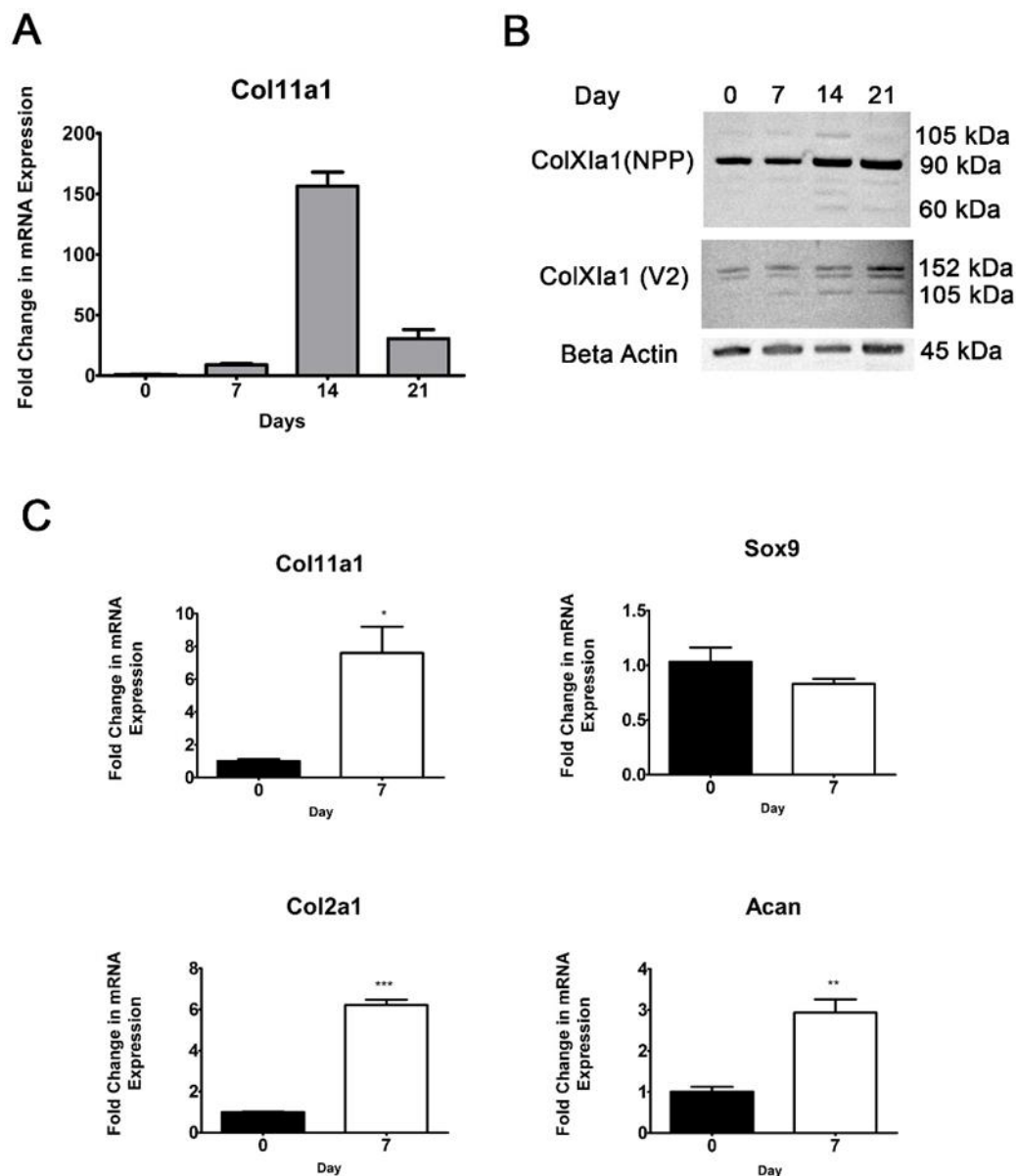


Figure 1. *Col11a1* gene expression during chondrogenesis in ATDC5 cells. Differentiation media was added to confluent cell cultures at day 0. Quantitative RT-PCR was performed to detect *Col11a1* mRNA in ATDC5 during chondrogenesis (A). Western blot analysis using rabbit polyclonal antibodies that recognized distinct epitopes within collagen $\alpha 1(XI)$ were used to detect protein expression over the same time course. The antibody to the Npp domain epitope recognized the 90 kDa fragment of collagen $\alpha 1(XI)$ with minor fragments recognized at 105 kDa, 90 kDa, 80 kDa, and 60 kDa, while the V2 antibody recognized bands migrating with apparent molecular weights of 152 kDa, 150 kDa, and 105 kDa. (B). *Col11a1* mRNA expression correlated with the expression of established chondrogenic markers *Sox9*, *Col2a1*, and *Acan* (C). Data is represented as mean and the standard deviation. Significance was determined using the t-test, $n=3$ * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

2.2. *Col11a1* knockdown induces fibroblast-like morphology, inhibits chondrogenesis, and increases mineralization

To investigate the requirement for *Col11a1* expression during the transition of pre-chondrogenic cells to differentiated chondrocytes, we transfected ATDC5 cells with double stranded siRNA complexes targeting *Col11a1* mRNA. We optimized and verified the effectiveness of this approach by monitoring mRNA and protein levels in ATDC5 cells. RNAi transfection prevented the increase in *Col11a1* mRNA previously observed on day 7 (Figure 2). Expression on day 3 was decreased by 90% compared to negative control

transfected cells and by more than 75% on day 7 (Figure 2A). Western blot analysis confirmed a decrease in total collagen $\alpha 1(XI)$ chain protein levels in the cell lysates on day 7, although a low level of protein was detectable (Figure 2B). New protein translation was blocked through RNAi degradation complexes as we could not detect collagen $\alpha 1(XI)$ chain by immunofluorescence in transfected cells (Figure 2C). The actin cytoskeleton showed marked changes in organization 3 days after *Col11a1* siRNA transfection with cells appearing more elongated and fibroblast-like than control cells undergoing chondrogenesis (Figure 2C).

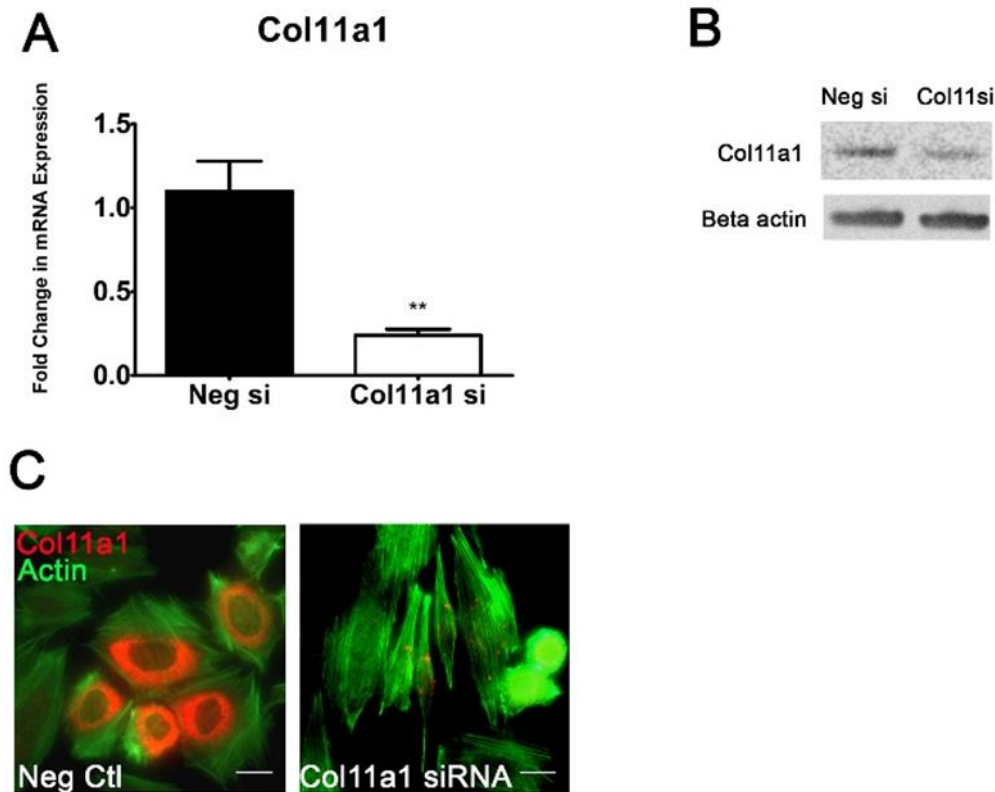


Figure 2. *Col11a1* knockdown causes actin cytoskeletal reorganization and loss of chondrocyte cellular morphology in ATDC5 cells. qRT-PCR was used to quantify the decrease of *Col11a1* mRNA and collagen $\alpha 1(XI)$ protein 7 days after siRNA transfection. *Col11a1* mRNA was decreased, relative to negative control siRNA transfections at day 7 (A). Protein levels detected using a collagen $\alpha 1(XI)$ antibody showed a decrease in collagen $\alpha 1(XI)$ protein. Beta actin was used to demonstrate equal loading per lane (B). Immunofluorescence detecting collagen $\alpha 1(XI)$ protein (red) and actin (green) verified the loss of detectable collagen $\alpha 1(XI)$ protein and changes in the actin cytoskeleton from primarily cortical to containing stress fibers, focal adhesions, lamellipodia or filopodia at 3-days post transfection. Scale bar in is 20 μm . Data is represented as mean and SD., data analyzed using the t-test, ** $p < 0.005$. $n=3$.

The production of the cartilage extracellular matrix can be monitored by the detection of sulfated glycosaminoglycans in developing cartilage nodules in micromass culture. Additionally, late stage chondrogenesis is marked by increased matrix calcification. Micromass cultures transfected with *Col11a1* siRNA and subsequently grown for 7 days produced less extracellular matrix than cells transfected with negative control siRNA as indicated by a decreased Alcian Blue staining (Figure 3A and B). Additionally, increased Alizarin red staining was detectable in micromass cultures treated with siRNA to knockdown *Col11a1* expression. The increase in Alizarin red staining is consistent with an increase in calcification or mineralization after 7 days in differentiation media (Figure 3C and D).

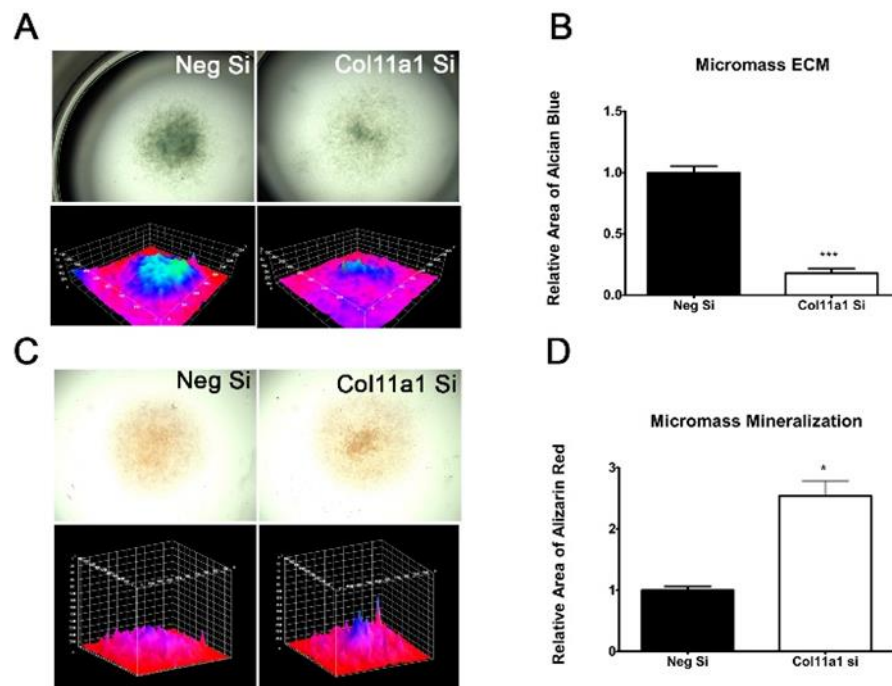


Figure 3. Knockdown of *Col11a1* expression decreased Alcian blue staining and increased Alizarin Red staining. Alcian blue staining was used to quantify the proteoglycan production in 7-day micromasses. *Col11a1* siRNA decreased the intensity and area of proteoglycan content of micromass cultures (A and B). Surface area maps provide a visual representation of the staining intensity and distribution within the micromass culture. *Col11a1* siRNA increased the calcium content of micromass cultures. The relative area and intensity of Alizarin red staining increased in the presence of siRNA-mediated inhibition of *Col11a1* expression in micromass cultures. (C and D). Data were analyzed using t-test and represented as the mean with standard deviation (n=3).

2.3. Inhibition of *Col11a1* expression does not affect genes involved in mesenchymal condensation but does affect genes involved in matrix production and remodeling

The expression of mesenchymal condensation and chondrogenic differentiation markers were analyzed by quantitative RT-PCR. Condensation of mesenchymal prechondrogenic cells is a prerequisite for chondrogenic differentiation and subsequent chondrocyte maturation. The expression of versican (*Vcan*), tenascin (*Tnc*), N-cadherin (*Cdh2*) and N-CAM (*Ncam*) are required for successful condensation to occur [1]. The expression of *Vcan*, *Tnc*, *Cdh2*, and *Ncam* were unaffected by *Col11a1* knockdown in ATDC5 cells (Figure 4). However, in the absence of *Col11a1* expression, the mRNA levels of collagen $\alpha 1(\text{II})$ chain (*Col2a1*) and matrix metalloproteinase-13 (*Mmp13*) were increased, while the expression of *Sox9*, *Acan*, and *Col10a1* did not significantly change (Figure 5).

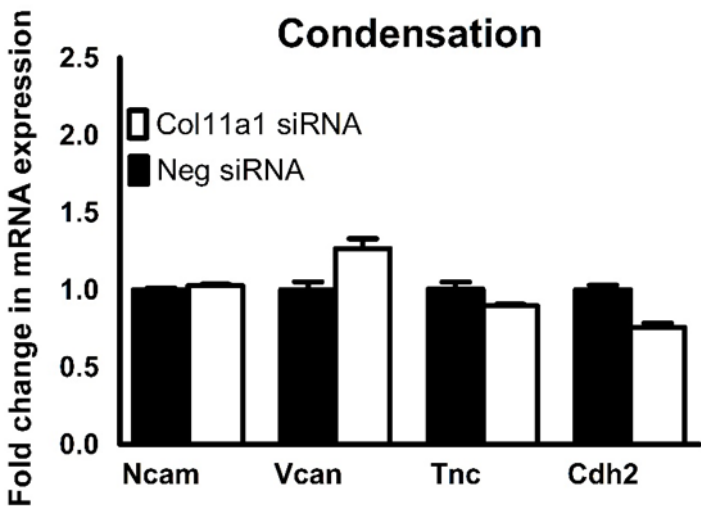


Figure 4. *Col11a1* expression was not required for the expression of cellular condensation markers in ATDC5 cells. The mRNA expression of *Ncam*, *Vcan*, *Tnc*, and *Cdh2* were not significantly altered in response to *Col11a1* knockdown during chondrogenesis. Data were analyzed using an unpaired t-test and represented as the mean with the standard deviation (n=3).

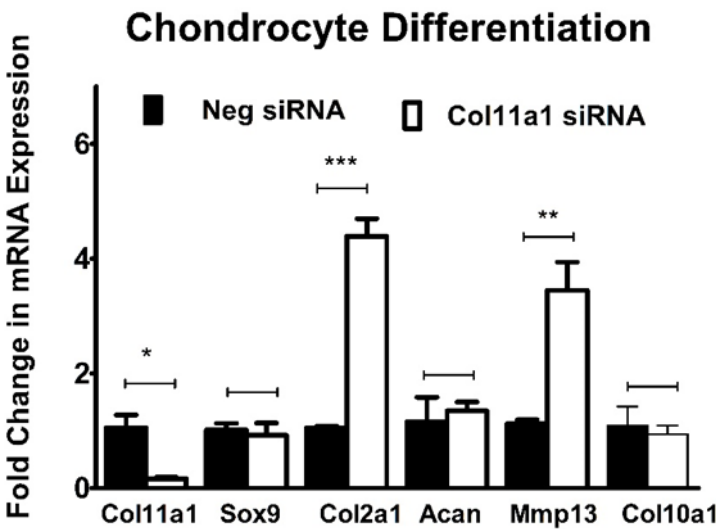


Figure 5. The expression of *Col2a1* and *Mmp13* are regulated by *Col11a1* knockdown. ATDC5 cells were transfected with either Neg siRNA or *Col11a1* siRNA prior to chondrogenic differentiation. Expression of *Sox9*, *Acan*, and *Col10a1* mRNA was not significantly different following inhibition of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

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

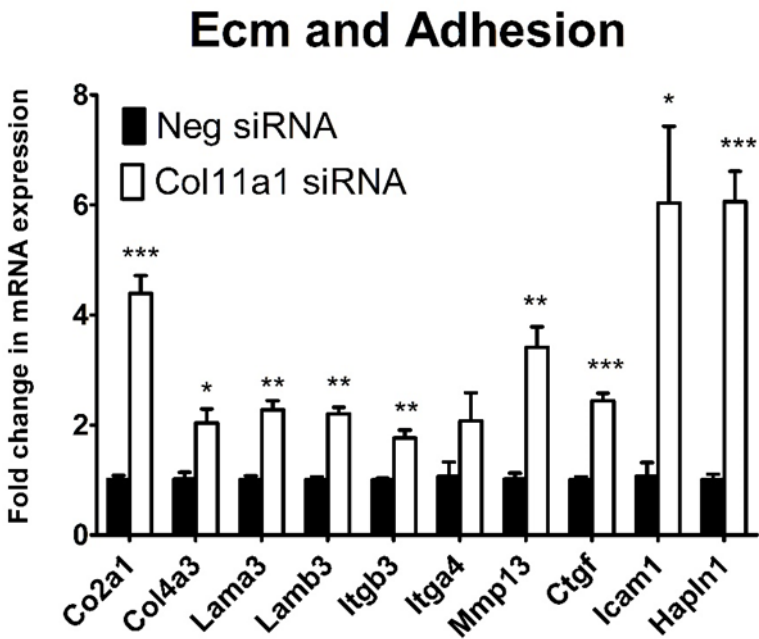


Figure 6. Extracellular matrix and adhesion gene transcription was regulated by *Col11a1* expression. Changes in mRNA expression levels were determined by comparing the expression level of mRNA for each target gene relative to β -actin as housekeeping gene. The relative expression of the *Col11a1* siRNA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

2.4. *β -catenin signaling pathway activity is increased when *Col11a1* expression is inhibited during chondrogenesis and is independent of Wnt induction*

Wnt/ β -catenin signaling and the regulation of TCF/LEF transcriptional activity is essential in the development of cartilage [26,27]. Wnt/ β -catenin unregulated activity in cells of the chondrocyte lineage negatively influence tissue morphogenesis and consequently leads to disease [28]. GSK3 β is a constitutively expressed, multifunction, protein kinase involved in a variety of cellular mechanisms, including homeostasis of the cartilage [29,30]. Phosphorylation of GSK3 β at serine 9 uniquely inhibits the molecule’s kinase activity [29,31]. Active GSK3 β can phosphorylate β -catenin when in complex with Axin2, leading to β -catenin degradation by an E3 ubiquitin ligase complex and proteasomes and a resulting decrease in the cytoplasmic levels of β -catenin [32,33]. In contrast, inactive GSK3 β would not phosphorylate β -catenin, and not lead to β -catenin degradation by the proteasome.

GSK3 β is switched off by phosphorylation at serine 9 in response to stimuli that include Wnt3a and insulin. We observed that inhibition of *Col11a1* expression increased the phosphorylation of GSK3 β at serine 9, corresponding to a decrease in GSK3 β kinase activity. Additionally, we observed a corresponding decrease in the amino terminal β -catenin phosphorylation (Figure 7A). Interestingly, we also observed increased AKT phosphorylation at serine 473 upon treatment with *Col11a1* siRNA (Figure 7B).

In addition to changes in AKT, GSK3 β , and β -catenin phosphorylation, we observed increased TCF/LEF activity when measured by luciferase assay with the TOP:FLASH reporter construct (Figure 7C), supporting the idea that *Col11a1* knockdown resulted in increased Wnt/ β -catenin signaling.

To identify changes in cell shape and localization of β -catenin caused by *Col11a1* expression we used immunofluorescence microscopy. Actin fibers in control chondrocytes had a cortical actin distribution consistent with a chondrocyte phenotype. In contrast, inhibition of *Col11a1* expression prevented cortical actin and favored cell spreading and

stress fiber formation. Additionally, β -catenin was localized perinuclearly and β -catenin appeared throughout the cytoplasm of the cell (Figure 8).

Stimulation of the canonical Wnt signaling pathway with recombinant Wnt3a protein was not inhibited by *Col11a1* knockdown as indicated by substantial GSK3 β phosphorylation and decreased β -catenin phosphorylation in both the negative control and *Col11a1* siRNA treated samples (Figure 9). Wnt3a protein stimulated the Wnt/ β -catenin pathway independent of *Col11a1*.

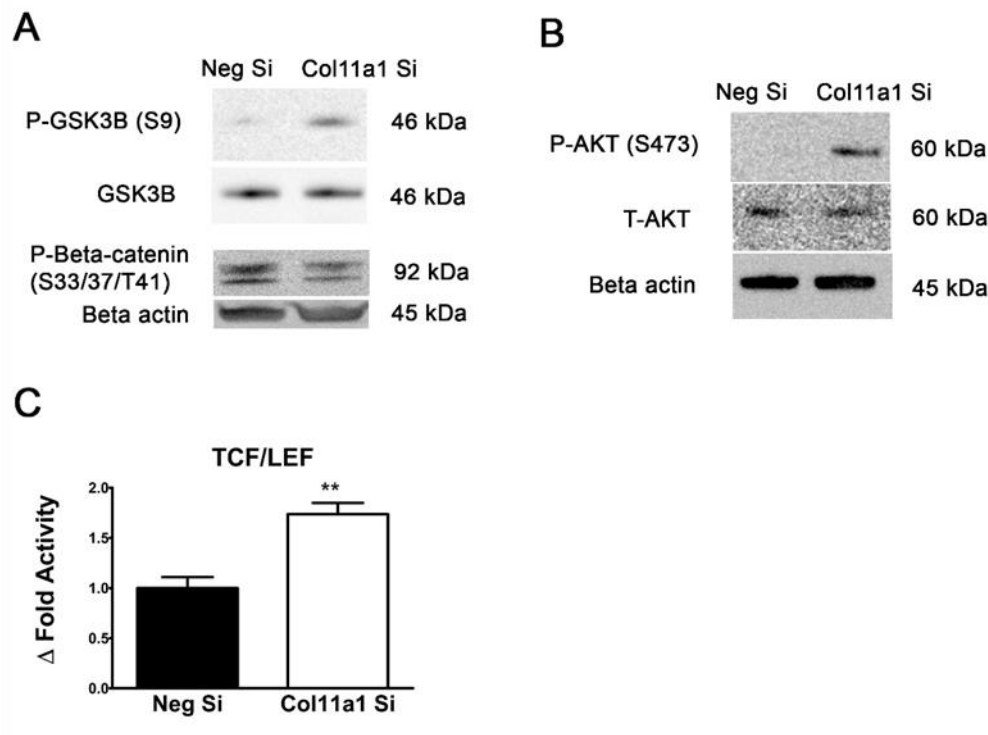


Figure 7. *Col11a1* knockdown increased AKT/GSK3 β / β -catenin signaling activity and increased TCF/LEF activity as an indication of Wnt/ β -catenin activation. *Col11a1* siRNA treatment increased the phosphorylation of GSK3 β , inactivating the kinase activity, while decreasing phosphorylation levels of β -catenin, resulting in stabilization of the β -catenin (A). Additionally, *Col11a1* siRNA induced phosphorylation of AKT at serine 473 (B). *Col11a1* knockdown significantly increased TCF/LEF transcription factor activity indicative of Wnt/ β -catenin signaling compared to negative control siRNA transfected cells. (C).

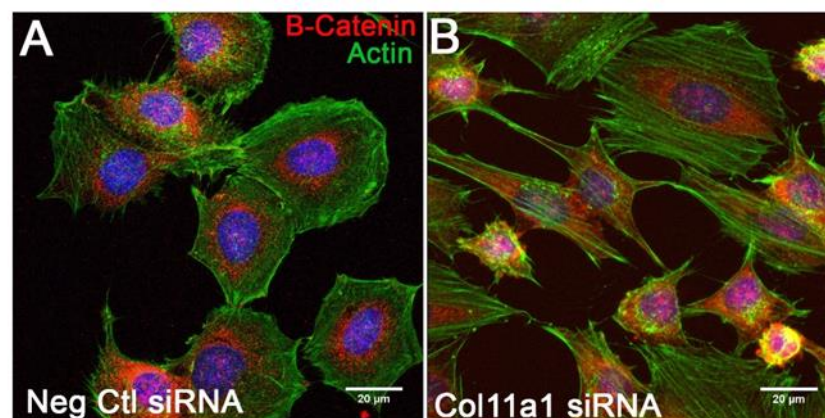


Figure 8. Immunofluorescence imaging of the actin cytoskeleton and β -catenin localization upon *Col11a1* knockdown. Increased cell spreading and increased β -catenin nuclear localization was found following *Col11a1* knockdown. Immunofluorescence of the actin cytoskeleton (green) showed cortical actin in Neg Ctl siRNA transfected cells (A) and an increase in actin stress fibers and

increased cell spreading in cells transfected with *Col11a1* siRNA (B). β -catenin (red) was primarily perinuclear in the Neg Ctl transfected cells (A) and was present throughout the cytoplasm and localized to the nucleus. (B). Scale bars are 20 μ m.

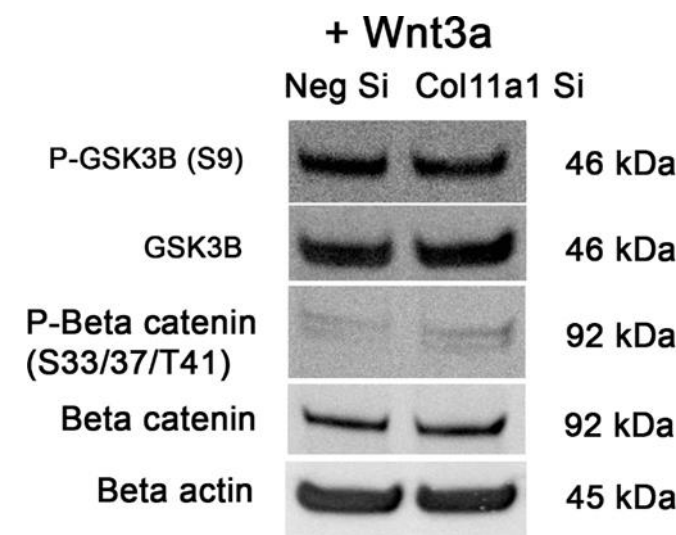


Figure 9. Wnt3a induced phosphorylation of GSK3 β as well as inhibition of β -catenin phosphorylation independent of *Col11a1* knockdown. The addition of 100 ng/mL of recombinant Wnt3a induced phosphorylation of GSK3B independent of *Col11a1* knockdown and inhibited β -catenin phosphorylation.

3. Discussion

This is the first demonstration of the link between the extracellular matrix protein collagen α 1(XI) chain and the activation of the AKT/GSK3 β / β -catenin pathway during skeletal development. This cell culture model system provides a means by which to ask questions relevant to chondrodysplasia *in vitro*. These results support the hypothesis that *Col11a1* expression is required for chondrocyte differentiation and later steps of chondrocyte maturation during endochondral ossification. Mutations in the human *COL11A1* gene cause severe chondrodysplasia, affecting bones that develop by endochondral ossification, including the craniofacial skeleton, long bones, and the vertebrae [4,5,6,34,35]. Although the shape and structure of skeletal tissues impacted by *COL11A1* mutations have been described radiologically and at the ultrastructural level, the response of the cells to the altered matrix have not been fully characterized [12]. Since the cells are ultimately responsible for both generating and maintaining tissues, understanding the consequences of the absence or reduction of *COL11A1* expression has important ramifications for skeletal development, disease progression and therapies. We hypothesize that *COL11A1* expression is required for mesenchymal, prechondrogenic cells to transition to mature chondrocytes.

In this study, we investigated the relationship between chondrocyte phenotype and the expression of *Col11a1* using a mouse cell culture model system. We inhibited the expression of *Col11a1* during chondrogenesis by transfecting prechondrocytes with siRNA targeting *Col11a1* mRNA prior to inducing differentiation with ITS and ascorbate 2-phosphate (A2P). The siRNA prevented the normal upregulation of *Col11a1* expression, thus creating a cell culture model system in which to investigate the effects of collagen α 1(XI) protein deficiency on chondroprogenitor differentiation during cartilage development. We found that cells adopt a non-chondrocytic morphology, reduce matrix production detected by Alcian blue, alter gene expression, and activate AKT/GSK3 β / β -catenin pathway when treated with siRNA for *Col11a1*. Based on these results, we propose that *Col11a1* expression attenuates signaling in chondroprogenitor cells to regulate chondrocyte maturation and endochondral ossification.

Collagen type XI's role in skeletal development has previously been described as a structural constituent regulating collagen nucleation and fibril diameter. Additionally, *Col11a1* expression in cell culture models, including ATDC5 cells, has been used to identify cells as having a chondrocyte phenotype [21,36]. This study supports the role for *Col11a1* expression as essential for skeletal development (Li et al. 1995). Further, the results of this study are consistent with the idea that *Col11a1* is an early mediator of chondrogenesis required to establish the overt chondrocyte phenotype from a mesenchymal prechondrocyte.

Mutations in the *COL11A1* gene cause the severe skeletal dysplasia, fibrochondrogenesis. Skeletal development is severely affected in fibrochondrogenesis, causing craniofacial defects such as micrognathia and long bone defects with flared metaphysis attributed to disorganization of the growth plate [6,37,38]. Additionally, the cartilage of the larynx is often weak and causes respiratory distress, which is ultimately lethal. These morphological descriptions support collagen $\alpha 1$ (XI)'s role to promote cell shape, behavior and cartilage growth. The results presented in this study suggest that the inability of cells to stabilize the cell shape and cortical actin, cell-cell interactions requiring N-cadherin and β -catenin promote cell shape, prechondrogenic cell condensations, and regulate Wnt signaling [39,40,41,42]. Down regulation of these cell adhesions in favor of matrix-cell interactions is an essential step in chondrogenesis. Therefore, the inability of cells to establish stable cell-cell adhesions or transition to cell-matrix interactions may cause changes at the cellular level consistent with *Col11a1* associated chondrodysplasia in mouse.

The observed increases in mineralization may be due to accelerated hypertrophic differentiation or cartilage calcification in the absence of intermediate steps. Phosphorylation of GSK3 β has previously been shown to promote hypertrophic differentiation [31]. Hypertrophic chondrocytes are a normal part of the endochondral ossification pathway and are required to initiate mineralization in the growth plate under normal conditions [43,44]. On the other hand, premature initiation of the hypertrophic differentiation program has detrimental effects on the cartilage growth and integrity [45,46]. Additionally, hypertrophic chondrocytes can potentially transdifferentiate into osteoblasts during normal endochondral ossification [47].

Interestingly, we found that increased expression of *Col2a1* and *Mmp13* occurred upon knockdown of *Col11a1*. These results indicate that the expression of *Col2a1* and *Mmp13* is linked to collagen $\alpha 1$ (XI) through an unidentified genetic network. The upregulation of *Mmp13* was previously described in the articular cartilage of chondrodysplasia (*cho*) mouse with heterozygous null mutation in *Col11a1* [48,49]. The researchers proposed that increased *Mmp13* expression degraded the pericellular matrix and exposed chondrocyte discoidin domain-2 receptor (DDR2) to interact with the surrounding matrix, mainly collagen type II. The changes in gene expression in this study agree with their hypothesis.

It is relevant to explain that the *Col2a1* mRNA transcripts detected in our assays were total *Col2a1* mRNA, and could not account for alternative splicing; therefore, it is possible that the increase in *Col2a1* mRNA is primarily *Col2a1* containing exon 2, which is highly expressed in mesenchymal cells. In contrast *Col2a1* transcripts lacking exon 2 are primarily found in differentiated chondrocytes [50,51,52,53]. Future investigation will include analysis of the *Col2a1* splicing pattern to further understand the cellular phenotype involved in *Col11a1* expression.

The genes *Hapln1*, *Ctgf*, and *Itgb3* each have essential roles in tissue morphogenesis and cartilage development and were upregulated by *Col11a1* knockdown. *Hapln1* is an essential constituent of the cartilage matrix whose expression stabilizes aggrecan and hyaluronan interaction [54]. *Hapln1* overexpression has recently been linked to mesenchymal stem cell phenotype associated with the expression of β -catenin [55]. *Ctgf* is involved in both insulin-mediated AKT/GSK3 β signaling and hypertrophic chondrocyte differentiation [56,57,58,59]. Additionally, the up regulation of *Itgb3*, which along with the *ItgaV* gene products, interact with the IGF-1 receptors enhances AKT signaling [60]. In agreement with the changes in gene expression, we observed that AKT and GSK3 β phosphorylation was increased in the absence of collagen $\alpha 1$ (XI) protein in ATDC5 cells and that TCF/LEF

activity was increased overall. A previous study found that GSK3 β inhibition in the cartilage induces cartilage degradation and decreases glycosaminoglycan detection [30]. Interestingly, inhibition of GSK3 β was recently shown to increase the volume of trabecular bone [61,62]. Additionally, GSK3 β inhibitors enhanced fracture healing by altering the mineralization pattern and skipping the formation of a cartilage template [63]. Consistent with these findings, we have observed in previous studies, that collagen α 1(XI) protein deficiency alters mineralization patterns, increase bone collar thickness and leads to wider trabecular structures, yet this is the first time we have implicated chondroprogenitor cells in the process of calcification [5,64].

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

This work suggests that it may be possible to rescue some aspects of chondrodysplasia caused by collagen α 1(XI) deficiency by modulating AKT, GSK3 β , or β -catenin activity in mouse. Future directions will improve upon the understanding of the relationship between human COL11A1 and AKT/GSK3 β / β -catenin, including activators and inhibitors of these cell signaling intermediates in cell culture and animal model systems. COL11A1 gene defects are also associated with intervertebral disc degeneration, nonsyndromic hearing loss [65], and osteoarthritis and may induce similar cellular responses in these conditions. Additionally, questions regarding the roles of the other collagen type XI and V alpha chains as well as alternative spliced forms of collagen α 1(XI) remain to be investigated. This information may lead to development of cell based and tissue regeneration therapies for surgical intervention or fracture healing.

The requirement for *Col11a1* gene expression to establish the chondrocyte phenotype during cartilage development is presented here. We show that although considered a structural protein, loss of *Col11a1* gene expression impacts cellular behavior and cell signaling pathways. Undoubtedly the mechanisms influencing the cell behavior are diverse and complex and therefore we are unlikely to isolate a single factor responsible for any deviations in behavior and function. Yet, understanding the cell phenotype at a particular stage provides the opportunity for investigation of cell targeted therapies.

4. Materials and Methods

4.1. Cell culture

Mouse chondrogenic cell line ATDC5 cells were cultured in DMEM/F12 containing 5% fetal bovine serum and 100 I.U./mL penicillin and 100 μ g/mL streptomycin. Cells were passaged at 80% confluence by dissociating with 0.25% trypsin/EDTA for 3-5 mins at 37°C. For differentiation experiments, the culture media was replaced with differentiation media after the cells reached 100% confluency. Differentiation media was DMEM:F12 supplemented with Insulin (10 μ g/mL), Transferrin (0.5 μ g/mL) Selenium (0.0067 μ g/mL) (ITS, Gibco) and 50 μ g/mL ascorbic acid 2-phosphate (A2P) (Wako Chemicals, USA). The differentiation media was replaced every other day for the duration of the experiment.

4.2. *Col11a1* knockdown by siRNA transfection

ATDC5 cells were seeded in 6-well plates with 2.5×10^6 cells per well in culture media and allowed to reach 80-90% confluency prior to transfection. siRNA targeting *Col11a1* was designed and purchased from Invitrogen. The 21-base pair double stranded siRNA was generated from interrogating RefSeq NM_007729.2 and targeting exon 2 at nucleotide location 590. Lipofectamine RNAiMAX was used for transfections following standard

protocols. Transfections were performed in triplicate with a siRNA-lipid complex master mix prepared in Opti-MEM media. The final master mix solution contained 1 mL Opti-MEM, 10 μ L of 10 μ M siRNA stock and 18 μ L of Lipofectamine RNAiMAX. Control wells were transfected with negative control siRNA and performed in triplicate with each experiment under the same conditions as the experimental siRNA. BLOCK-iT Alexa Fluor Red Fluorescent Oligo was used to determine and optimize the transfection efficiency. A transfection efficiency of 90-100% was achieved when determined by monitoring uptake of the fluorescent oligonucleotide. Cell cultures recovered for 24 hours before changing the media to differentiation medium.

4.3.3D Micromass cultures

Micromass cultures were generated by dissociating cells with 0.25% trypsin/EDTA from the wells and resuspending to 2.0×10^7 cells/mL in differentiation medium containing ITS and 50 μ g/mL ascorbate 2-phosphate. Ten microliters of the cell suspension was gently spotted in tissue culture wells. The cells were allowed to adhere for 2-3 hours in a tissue culture incubator at 37°C with 5% CO₂ before adding additional medium. The medium was changed every other day for the duration of the experiment.

4.4. Quantitative RT-PCR (qPCR) analysis

RNA was extracted and purified using the RNeasy minikit following manufacturer's instructions (Qiagen). Isolated RNA was analyzed by spectrophotometry for purity and quantity and used immediately for cDNA synthesis or alternatively, aliquoted and stored at -80°C. RNA was reverse transcribed into cDNA using the RT2 first strand kit. The cDNA template was analyzed by qRT-PCR reaction using Sybr Green and specific primers or directly in RT2 profiler PCR arrays in 96 well format. Reactions were carried using the Roche Lightcycler 96 target specific primers (Table 1). The relative amount of PCR product was normalized to the indicated reference genes and the change in threshold cycle (dCt) was compared using Student's t-test. The fold change was calculated using the 2- $\Delta\Delta$ Ct method [66].

Table 1. Primers used for RT-PCR.

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

4.5. Luciferase Assays

Signal 45-pathway reporter array was used to identify changes in transcription factor activity following manufactures instructions. Lipofectamine 2000 was used with Opti-Mem to simultaneously reverse transfect *Col11a1* siRNA and a mixture of transcription factor responsive firefly luciferase reporter and a constitutively expressing Renilla constructs. 5.0 x10³ cells per well were added to each well of a 96-well plate containing 100 µL of transfection media containing 10 µM *Col11a1* siRNA and 0.8 µL Lipofectamine 2000 per well. The cells were incubated at 37°C with 5% CO₂ for 24 hours prior to changing the media to the indicated treatment. Dual luciferase reporter assay system and Glomax multi detection system was used to measure the activity of the transcription factors following standard protocol.

4.6. Western blot analysis

Cell lysates were extracted using ice-cold radioimmunoprecipitation (RIPA) cell lysis buffer (ThermoFisher Scientific) supplemented with protease and phosphatase inhibitor cocktails added directly to the wells of six-well plates after washing with cold phosphate buffered saline (PBS). The cell lysates were collected and added to microcentrifuge tubes and the supernatant was cleared by centrifugation at 4°C, 14,000xg for 15 min and transferred to a new tube. Protein lysates were quantified using bicinchoninic (BCA) protein assay (Pierce). Proteins were separated by SDS PAGE and then transferred to nitrocellulose membranes for western blot analysis. SDS-PAGE gels were stained with coomassie blue to verified equal amounts of loading and transfer for each sample. Membranes were blocked using a 5% (w/v) non-fat milk solution in Tris-buffered saline with 0.1% Tween-20, pH 7.4 (TBST) buffer for 1 hour at room temperature prior to incubation with primary antibodies. Primary antibodies were diluted in 5% (w/v) bovine serum albumin (BSA) in TBST prior to overnight incubation of the membranes at 4°C with shaking. Immunoblots were washed and then incubated with horseradish peroxidase (HRP) conjugated secondary antibodies for 1 hour at room temperature. Signal was detected using Pierce enhanced chemiluminescence (ECL) western blotting substrate. Western blots were performed using the following rabbit primary antibodies from Cell Signaling Technology at a dilution of 1:1000 unless otherwise stated: phospho-β-catenin (Ser33/37/Thr41), β-catenin (6B3) mAb, AKT, phospho-AKT (1:2000) (Ser473) (D9E) XP mAb, Gsk-3beta, phospho-GSK3-β (Ser9) (5B3) mAb, β-Actin (13E5) mAb. Collagen type XI α1 chain specific rabbit polyclonal antibodies were designed to recognize epitopes within the amino propeptide (7990) or variable region 2 (1703) [36].

4.7. Alcian Blue and Alizarin Red staining

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

4.8. Immunocytochemistry

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

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