Primary growth plate chondrocyte isolation, culture, and characterization from the modern broiler

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ABSTRACT Lameness is a leading cause of animal welfare and production concerns for the poultry industry as fast-growing, high-yielding broilers seem more susceptible to bone disease and infections. A major limitation to the study of these disorders is the lack of a chicken immortalized chondrocyte cell. Primary cell isolation is a valid and complex method for establishing a relevant in vitro model for diseases. In this study, isolation and high-density culturing of primary chondrocytes form 1-d old chicks was followed by confirmation of cell type, identification of optimal phenotypic expression, and evaluation of cells functionality. mRNA expression, as well as protein production and secretion, of COLI, COLII, Sox9, ACAN, and COLXA1 on day 3 (d3), d7, d11, d14, d18, and d21 in culture showed that avian growth plate chondrocytes under these conditions exhibit optimal phenotypes from d3 to d7. This is evident by a shift from COLII dominant expression in early-culture to COLI dominant expression by late-culture in conjunction with a loss of other chondrocyte markers Sox9, ACAN, and COLXA1. Additionally, morphological changes seen through live cell imaging coincide with the shift of phenotype in mid- to late-culture periods indicating a dedifferentiated phenotype. The functionality of the cultured cells was confirmed using Brefeldin-A treatment which significantly reduced secretion of COLII by d7 chondrocytes. These results provide a foundation for future research utilizing avian primary chondrocytes with optimal phenotypes for disease modeling or passaging.

Key words: broiler, primary chondrocyte, growth plate, chicken, characterize

INTRODUCTION

The growth plate is home to intense remodeling from embryonic to early development and beyond. Longitudinal bone growth is the direct result of endochondral ossification done by chondrocyte cells within the growth plate. The junction where the dynamic growth plate meets the harder articular cartilage cap is often the site of infection, disease, and stressors (Mackie et al., 2011; Yair et al., 2012). In addition to mechanical stress from weight bearing and movement, these locations in the skeletal system are under immense metabolic and conformational stress as cells progress through stages needed for bone growth to occur (de Crombrugghe et al., 2001). The growth plate of modern broilers is the site of 2 common causes of lameness, bacterial chondronecrosis with osteomyelitis (**BCO**) and tibial dyschondroplasia (**TD**) (Rath et al., 2000; Wideman and Prisby, 2012; Wideman, 2016). These disorders are the result of weak points within the growth plate being either exploited by opportunistic bacteria or crippled by hypoxia (Jiang et al., 2015; Mandal et al., 2016). Either way, detection of these 2 disorders is only possible through necropsy. Additionally, it has been shown that their presence does not always coincide with visible lameness (Wideman et al., 2012). Regardless of the clinical status, leg disorder and lameness in general, are a major animal welfare and production concern to the poultry industry (McNamee et al., 1998; Thorp et al., 1993). Studies have shown dsRNA accumulation and mitochondrial dysfunction to be implicated in BCO etiology by analyzing affected bone tissue and human osteoblast cell line (Greene et al., 2019; Ferver et al., 2021). However, a major limitation in the study of this disease is the lack of a chicken specific and growth-plate relevant in vitro model. To that end, this study was undertaken to isolate, culture, and characterize primary growth plate chondrocytes from modern broiler chicks.

In order to evaluate the success and effectiveness of primary cell cultures, it is important to document and understand the changes in cellular phenotype in culture. There are several key stages to the chondrocyte cell cycle, starting with condensation of the mesenchymal stem cells into chondroprogenitor cells (Florencio-Silva et al., 2015). Condensation is regulated by transcription factors such as Sox9 and results in increased COLII expression (Hardingham et al., 2006). Following condensation, chondroprogenitors go through proliferation and differentiation into chondrocytes exhibiting COLII and ACAN expression and secretion (Demoor et al., 2014). The final stage chondrocytes undergo in vivo is hypertrophy where COL10A1 is a specific marker (van Donkelaar and Wilson, 2012). Expression of COLI is seen before condensation and in dedifferentiated chondrocytes, therefore, COLI is commonly used as a negative marker for chondrocytes (Tchetina et al., 2007). These markers were evaluated at different time-points during primary chondrocyte cell culture in order to characterize the cells and determine the moment of optimal phenotype for utilizing primary chondrocytes as in vitro models for diseases.

MATERIALS AND METHODS

Cell Isolation and Culture

All animal experiments were approved by the University of Arkansas (Fayetteville, AR) Animal Care and Use Committee (protocol number 21050) and were in accordance with recommendations in NIH's Guide for the Care and Use of Laboratory Animals. Primary chondrocytes were isolated and pooled from the proximal tibiotarsus heads of 10-day-old broiler chicks with one leg rendering approximately 1×10^6 cells. The method of isolation was modified from previous work (Rasaputra et al., 2013). Chicks were reared in standard industry conditions for lighting and temperature for ten days with ad libitum access to food and fresh water. Leg quarters were aseptically separated from culled chicks before careful isolation of shavings from the growth plate the proximal tibia head. Shavings were placed in serum-free media, DMEM containing 4.5 g glucose/L, glutamine, 25 mM HEPES, 1 mM sodium pyruvate, and 10 ng/mL added ascorbic acid. This process was repeated for all chicks. The shavings were then transferred to digestion media composing of all aforementioned media with additional 10% FBS, 1 mg/mL hyaluronidase (Sigma Aldrich, St. Louis, MO), and 10mg collagenase IV (Worthington Biochemical Corporation, Lakewood, NJ) and incubated overnight at 37°C. Cells were then separated from tissue debris using ficoll gradient of Histopaq-1077 density gradient medium and centrifugation separation before being plated at a density of 2×10^5 cells/cm^2 in complete media. Complete media contained all components previously mentioned, except for the hyaluronidase and collagenase. Media was changed every 48 h and the cells were maintained in 37°C and 5% CO_2.

Live Cell Imaging

Cells were imaged at day 3 (d3), d7, d11, d14, d18, and d21 in culture using the Cytation 3 Cel Imaging Multi-Mode Reader (Bio Tek, Winooski, VT) at a magnification of 20x.

RNA Isolation, Reverse Transcription, and Real-Time Quantitative PCR

Total RNA was isolated from primary chondrocytes on each time point (n = 3/group) in accordance with the protocol of previous work (Carter et al., 2012). Cellular RNA was isolated using Trizol reagent (Life Technologies, Carlsbad, CA), based on manufacturer's instructions. RNA concentrations were determined using Synergy HT multimode microplate reader and total RNA was reverse transcribed using qScript cDNA SuperMix (Quanta Biosciences, Gaithsburg, MD). Amplification was achieved using Power SYBRGreen Master Mix (Life Technologies, Carlsbad, CA) and real-time quantitative PCR (7500 Real Time System; Applied Biosystems, Foster City, CA). The sequences for oligonucleotide primers for *r18s* was previously published (Ferver et al., 2021). Additional primers for chicken *COLIA1, COLIA2, COLII, ACAN, Sox9,* and *COL10A1* are summarized in Table 1.

Real-time quantitative PCR cycling conditions were 50° C for 2 min, 95°C for 10 min and 40 cycles of a 2-step

Table 1. Oligonucleotide real-time qPCR primers.

Gene	Accession number[a]	Primer sequence (5' → 3')	Orientation	Product size (bp)
COLIA1	NM_001030728	GTCGCCATCCAACTGACCTT	Forward	97
COLIA2	AY786316	TGCAGTGGTAGGTGACGTTCTG	Reverse	64
COLII	NM_001031027	GCAACACAAGGAGTCTGCATGT	Forward	58
ACAN	NM_001007854	AAATCCGCGTATCCACAAAGC	Reverse	60
Sox9	AF173612	ACCTGCCGCGACATCAA	Forward	60
COL10A1	NM_205518	GTCAATCCAGTAATCTCCGCTCTT	Reverse	123
		CAGAGCCGTGGAGAATGATTTC	Forward	
		GGCCGCCAGTTCTCAAATT	Reverse	
		CCCCTGTGCCGCTTTCT	Forward	
		GTCCTGTTCTTCTGTCATTTTCATGA	Reverse	
		CAGCTGCCAAATTCAGAATCC	Forward	
		GGAAACCTGAGAAAGAAGAATGAACA	Reverse	

[a]Accession number refer to Genbank (NCBI).Adipo, adiponectin; ACTB, beta.

amplification (95°C for 15 s followed by 58°C for 1 min). Relative expression of target genes was determined using the $2^{-\Delta\Delta CT}$ method and healthy bone tissue or untreated cells were used as calibrators (Schmittgen and Livak, 2008).

Protein Isolation and Western Blot Visualization and Analysis

Media and cell lysate from each time point (d3, d7, d11, d14, d18, d21) were homogenized in lysis buffer (10 mmol/L Tris base, pH 7.4; 150 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L EGTA; 0.1% Triton X-100; 0.5% Nonidet P-40; and protease and phosphatase inhibitors) and sonicated. Total protein concentrations were determined using a Bradford assay kit (Bio-Rad, Hercules, CA), ran in 4 to 12% gradient Bis-Tris gels (Life Technologies, Carlsbad, CA) and then transferred to polyvinylidene difluoride (PVDF) membrane. Once transferred, membranes were processed according to methods previously published (Ramser et al., 2021). Primary antibodies used were rabbit anti-COLI (Origene, Rockville, MD), mouse anti-COLII (Novis Biologicals, Littleton, CO), rabbit anti-ACAN (ABClonal, Woburn, MA), rabbit anti-Sox9 (ABClonal, Woburn, MA), and rabbit anti-COL10A1 (ABClonal, Woburn, MA). The protein signals were visualized using chemiluminescence (ECL Plus; GE Healthcare, Pittsburg, PA) and images were captured using the FluorChem M MultiFluor System (ProteinSimple, San Jose, CA). Protein loading was assessed by immunoblotting using rabbit antieglyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology, Dallas, TX) or Ponceau S Stain (G-Biosciences, St. Louis, MO). Image acquisition and analysis were performed by AlphaView software (version 3.4.0, 1993−2011; ProteinSimple, San Jose, CA).

Immunofluorescence

Primary chondrocytes were plated on gelatin coated Nunc Lab-Tek II chamber slides (Thermo Fisher Scientific, Waltham, MA) until predetermined time-points in culture. Immunofluorescence staining was performed as previously described (Dridi et al., 2012). The signal was visualized using either DyLight 488- or 594-conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA). Vectashield with DAPI (Vector Laboratories, Burlingame, CA) was used as mounting media before slides were cover slipped. Images were obtained using Zeiss Imager M2 and AxioVision software version LE2019 (Carl Zeiss Microscopy, Oberkochen, Germany).

Brefeldin-A treatment

Primary chondrocyte cells were maintained until d7 in culture. Complete media was replaced with media either treated with 1 μg/mL of brefeldin-A (BFA, Sigma Aldrich, St. Louis, MO) or dimethyl sulfoxide (DMSO) as vehicle control (n = 3/group). Cells were maintained for another 24 h at 37°C and 5% CO_2 before both media and cell lysate were collected for protein isolation and analysis.

Statistical analysis

Data were analyzed by Student t-test or One-way ANOVA, as appropriate, using GraphPad version 7.03 (GraphPad Software, Inc., LaJolla, CA). Data are expressed as means ±SEM, with P-value < 0.05 set as statistically significant.

RESULTS

Cell morphology during culture period

Cytation 3 images, taken at 20x magnification, show cell size and shape change throughout the culture (Figure 1). Most notably, the change from smaller rounded cells to more geometric in shape, creating a "cobble-stone" appearance visible starting at d7 until d21. Aggregates of cells can also be seen more in midculture (d11 and d14) as compared to late-culture conditions (d18 and d21) (Figure 1).

Expression Profile of Chondrocyte Phenotype-Associated Markers

COLIA1 and COL1A2, which are markers for both condensation and dedifferentiation of chondrocytes, significantly increased during the culture period with and lowest expression on d3 and the highest expression at d18 (P < 0.05) (Figure 2A, B). Sox9, a marker for the condensation stage of chondrocyte cell cycle, was significantly upregulated in the early period of isolation and significantly decreased from d3 to d14, d18, and d21 (P < 0.05) (Figure 2C). COLII, a chondrocyte proliferation and dedifferentiation marker, showed the highest mRNA expression in the beginning of the culture period (d3, d7, and d11) and significant decreases in late-culture (d21) (P < 0.05, Figure 2D). Similarly, ACAN showed highest expression at d3 and d7 in culture and significantly dropped at d11 (P < 0.05, Figure 2E). COLX, a marker of hypertrophic chondrocytes, significantly decreased from d3 to d7 (P < 0.05) and then significantly decreased again at d11 (P < 0.05) and remained low throughout the duration of the culture period (Figure 2F).

At the protein levels, immunoblot blot analysis of the cell lysate showed significantly higher expression of COLI on d18 compared to d3, d7, and d11 (P < 0.05) (Figure 3A, B). Expression of Sox9 was also significantly higher on d3 compared to d14, d18, and d21 (P < 0.05) (Figure 3A, B). In media, COLII was significantly higher on d7 compared to d3, d14, d18, and d21 (P < 0.05) (Figure 3A, C). ACAN was significantly higher on d3 in media of cultured cells compared to all other time points (P < 0.01) (Figure 3 A, C).

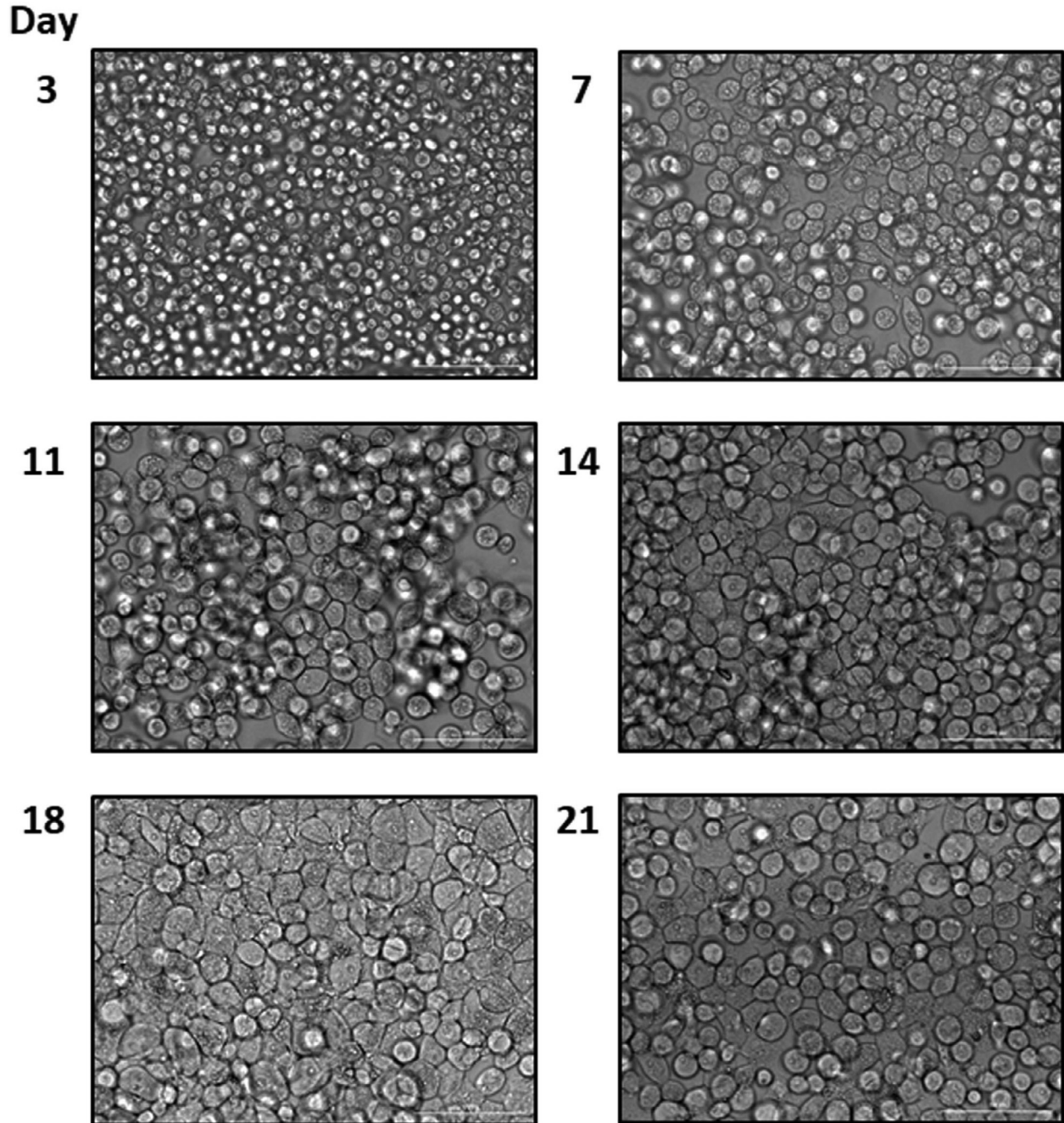In line with the immunoblot data, immunofluorescence staining in chondrocyte monolayer culture

Figure 1. Live-cell imaging of chondrocytes in culture. Live cells directly seeded after isolation at a density of 2×10^5 cells/cm^2 were imaged using Cytation 3 Cell Imaging Multi-Mode Reader at 6 different time points, d3, d7, d11, d14, d18, and d21 in culture at the same magnification (20x). The same wells were imaged at each time point.

detected spot-like shapes of COL I and COL II on d7 (arrowheads, Figure 3D) and network-like shapes on d18 (arrows, Figure 3D).

Next, chondrocytes were treated with BFA and showed significantly reduced secretion of COLII into the media, as evident by western blot analysis of the cell lysate and media from control and treated cells ($P < 0.05$) (Figure 4A, B).

DISCUSSION

Inflammatory bone diseases, especially those affecting the growth plates, are notoriously difficult to detect and treat due to the extensive layers of skin, muscle, and ligaments surrounding the general region of infection (Ciampolini and Harding, 2000; Dinev, 2009). In modern broilers, lameness is a serious and growing threat to both production and animal welfare. Common causes of lameness include BCO and TD, which both primarily affect the proximal growth plates of the long bones (Riddell, 1977; Pedersen et al., 2020). As no current immortalized chicken bone cell line exists, this study sought to isolate, culture, and characterize primary growth plate chondrocytes from modern broilers for their potential future use as in vitro models.
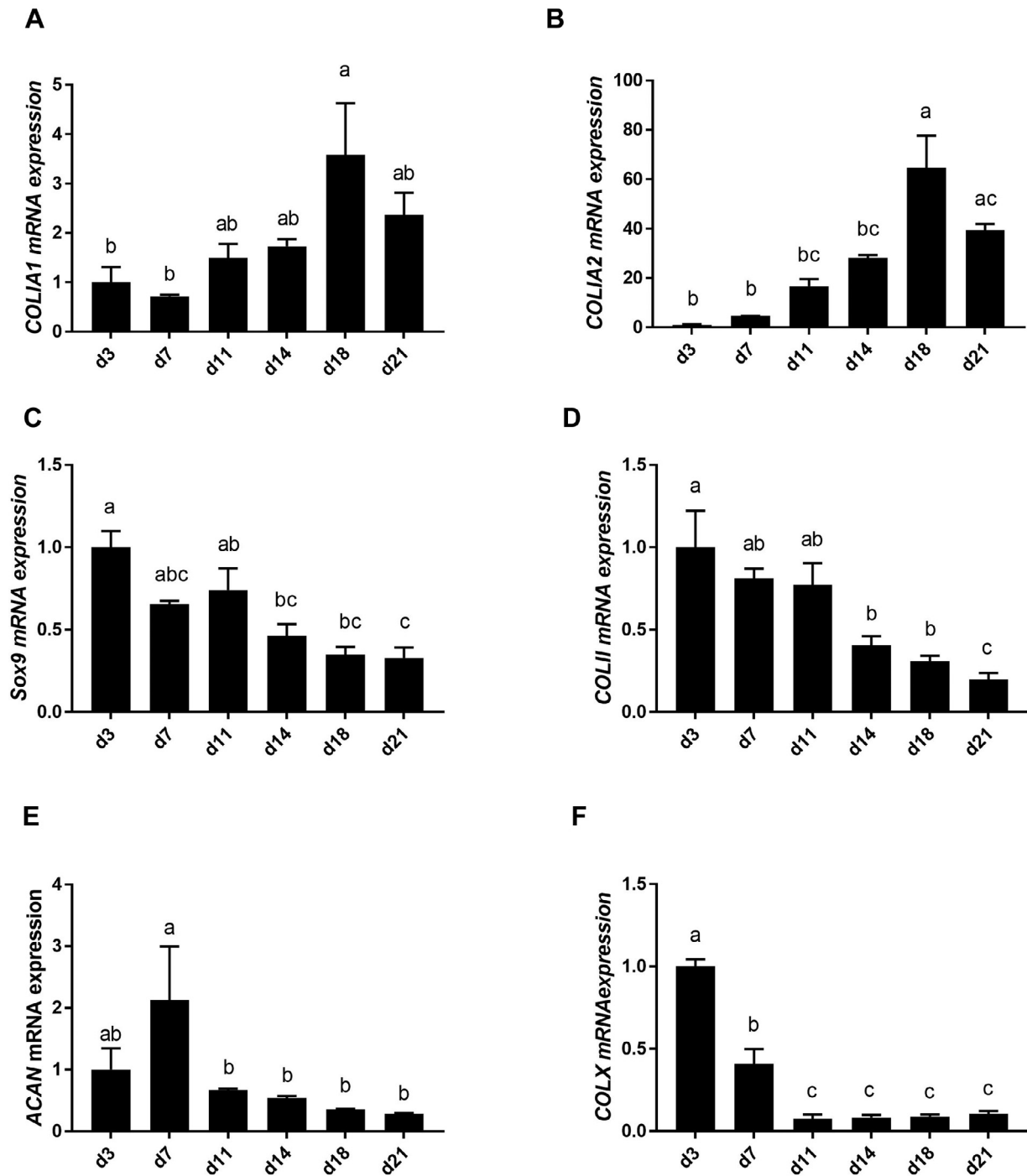
Figure 2. mRNA expression of chondrocyte markers within cultured primary cells throughout the culture period. Gene expression for *COLIA1* (A), *COLIA2* (B), *Sox9* (C), *COLII* (D), *ACAN* (E), and *COLX* (F) in chondrocyte cells at d3, d7, d11, d14, d18, and d21 in culture. Significance was determined using a one-way ANOVA with P-value < 0.05. Lowercase letters indicate significant difference between time points. ACAN, aggrecan; COL, collagen; Sox, SRY-Box.

Primary chondrocytes were seeded in accordance with high density plating methods for retaining chondrocyte phenotypic expression (Adolphe, 1992). This is believed to be done through the dense layers of chondrocyte cells mimicking a 3D culture environment which has been proven to aid in retention of proper chondrocyte phenotypes as opposed to monolayer culture techniques (Adolphe, 1992). Under this environment, the cells presented a commonly seen morphological change starting around d7 and becoming more prominent by d11 and onward.

The change in cell size and shape has been shown to coincide with shifts in expression of chondrocyte markers and ECM secretions (Liao et al., 2021; Rosenzweig et al., 2012).

Our results show that avian growth plate chondrocytes in high density culture exhibit shape changes accompanied by an early culture peak in *COLII* mRNA expression and protein secretion followed by a decline in COLII and increase in COLI production into late-culture when the shape change is most prominent. This
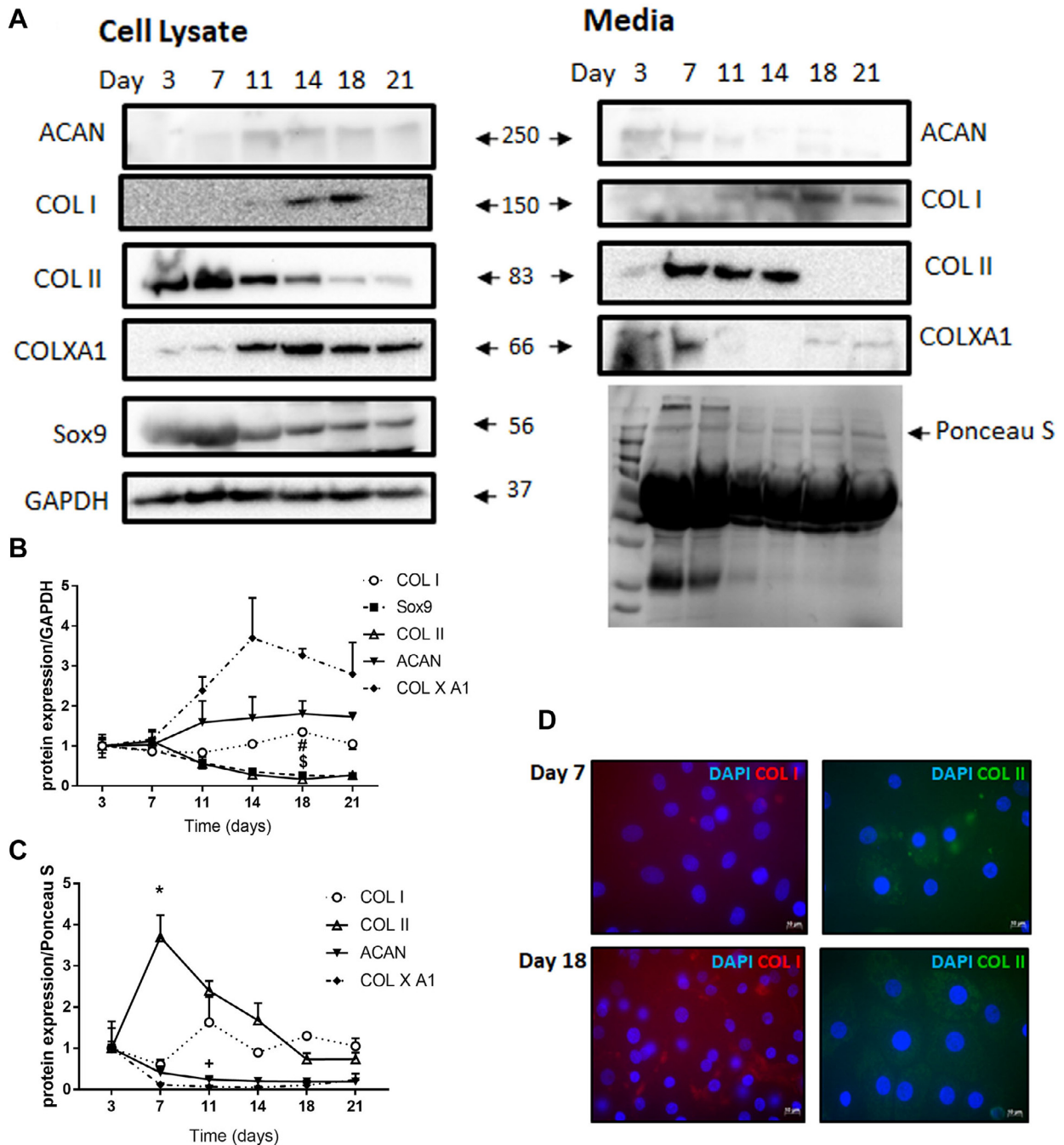
Figure 3. Evaluation of protein production and secretion by primary chondrocytes in culture. Protein expression for COLI, COLII, COLXA1, ACAN, Sox9, and GAPDH in cell lysate and media in addition to Ponceau S stain of the media blot and subsequent statistical analysis in culture (A, B, C). Significance was determined using a one-way ANOVA with P-value < 0.05. (B) $ indicates significant difference between Sox9 expression on d14, d18, and d21 compared to d3. # indicates significant difference between COLI expression on d18 compared to d3, d7, and d11. (C) + indicates significant difference between ACAN secretion on d11, d14, d18, and d21 compared to d3. * indicates significant difference in COLII secretion on d7 compared to d3, d14, d18, and d21. (D) Immunofluorescence staining of COLI (594-conjugated) and COLII (488-conjugated), along with DAPI nuclear dye, in chondrocytes d7 and d18 in culture. ACAN, aggrecan; COL, collagen; Sox, SRY-Box.

switch to COLI production is often seen in in vitro cultures as a monolayer results in dedifferentiation of chondrocytes. Indeed, live cell imaging shows decreased layering of cells by d18 and d21 indicating a loss of high-density, 3D-mimicking culture which is more conducive for retention of chondrocyte phenotype. This pattern of loss of COLII production is seen in other monolayer primary chondrocyte cultures. Human articular chondrocytes seeded at a density of 2.5×10^4 cells/

cm^2 in monolayer showed decreased COLII and Sox9 mRNA expression after passaging (Ma et al., 2013). Additionally, the morphological changes observed in this study corroborate previous work. Embryonic chick chondrocytes seeded at 2.5×10^5 cells/cm^2 in comparable media showed attachment by d3 and a "polygonal" shape by d9 of culture, similar to the "cobblestone" shape observed in this study (Shrestha et al., 2015).
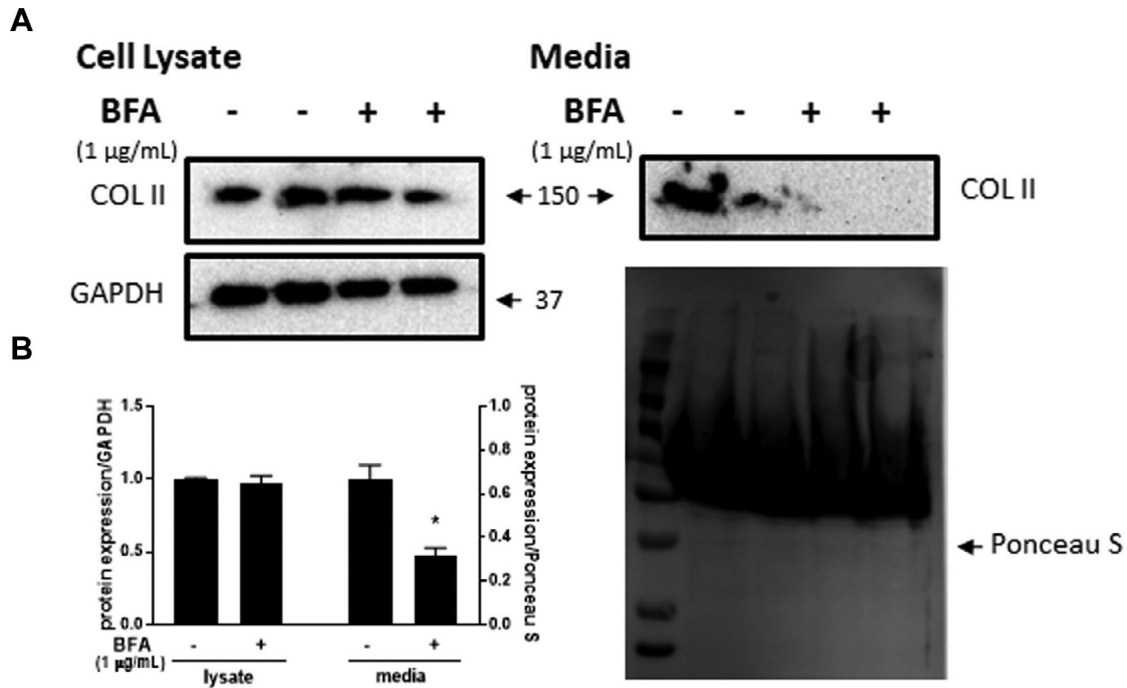
Figure 4. Effect of BFA treatment on d7 chondrocyte COLII secretion. Protein expression of cells and their media secretions treated with either BFA (1 μg/mL) or vehicle control at d7 in culture (A). Significance was determined using a student t-test with P-value < 0.05 (B). * indicates significant difference between control and treated cells.

Within early culture, Sox9 protein expression was highest compared to mid- and late-culture periods, which corroborated with mRNA expression as well. Sox9 is a key transcription factor in chondrogenesis, driving mesenchymal condensation into chondroprogenitors, and therefore was expectedly undetectable in media from cultured cells (Hussain et al., 2018). Specifically, Sox9 has been shown to regulate COLII, COL9A1, COL11A1, and ACAN gene expression in chondrocytes (Sekiya et al., 2000; Hardingham et al., 2006). Chondroprogenitor cells have been shown to express Sox9 and drive COLII production in mice (Shi et al., 2015). The early expression of Sox9 protein within the cells indicates the potential presence of chondroprogenitor cells and precedes increased expression of COLII in the cultured chondrocytes and media by d7. Murine growth plate chondrocytes showed high levels of Sox9, ACAN, COLII mRNA expression and significantly higher expression of COLXA1 on d3 in culture when compared to costal chondrocytes (Liao et al., 2021). COLXA1 mRNA expression was also significantly higher on d3 compared to all later culture time points. CCOLXA1 is a common product of hypertrophic chondrocytes in vivo and its presence in the earliest time point could be the result of sampling. The scraping of the growth plate results in sections deep enough to include layers of condensing, proliferating, and hypertrophic chondrocytes within the digestion and culture. The inclusion of these different staged chondrocytes into the culture could be the causative factor in *COLXA1* mRNA expression in early culture. The increased expression of *COLXA1* protein seen in later culture was not significant and could be

more likely related to transdifferentiation rather than hypertrophy as the cells also begin producing *COLI*. Furthermore, ACAN protein secretion was also highest on d3 which indicates the presence of proliferating chondrocytes within culture. This further supports the idea that multiple different cell stages are represented in the first few days of culture. However, the addition of ascorbic acid in the media is noteworthy because it has been shown that it induces chondrocyte proliferation in culture (Venezian et al., 1998; de Haart et al., 1999). Its inclusion is due to its ability to aid in chondrocyte phenotype retention and ECM secretion while decreasing oxidative stress (Farquharson et al., 1998; Chang et al., 2015).

Once an established phenotype was determined, functional analysis of the cells was undertaken using BFA. BFA acts to inhibit protein secretion at a pre-Golgi apparatus stage through blocking guanine nucleotide exchange (Gruber, 1993). Chondrocytes at d7 in culture treated with BFA showed significantly decreased secretion of COLII compared to untreated controls. This indicates the active secretion of COLII by the cultured cells and further defines their functionality and phenotypic relevance for in vitro modeling of growth plate diseases and infections.

Taken together, these findings suggest that chondrocyte markers are at their most optimal between d3 and d7 in culture and that the shape change observed starting on d7 and fully present on d11 is likely due to dedifferentiation of chondrocytes. This is supported by a clear shift in collagen type secretion, from COLII dominant to COLI, in addition to loss of chondrocyte specific
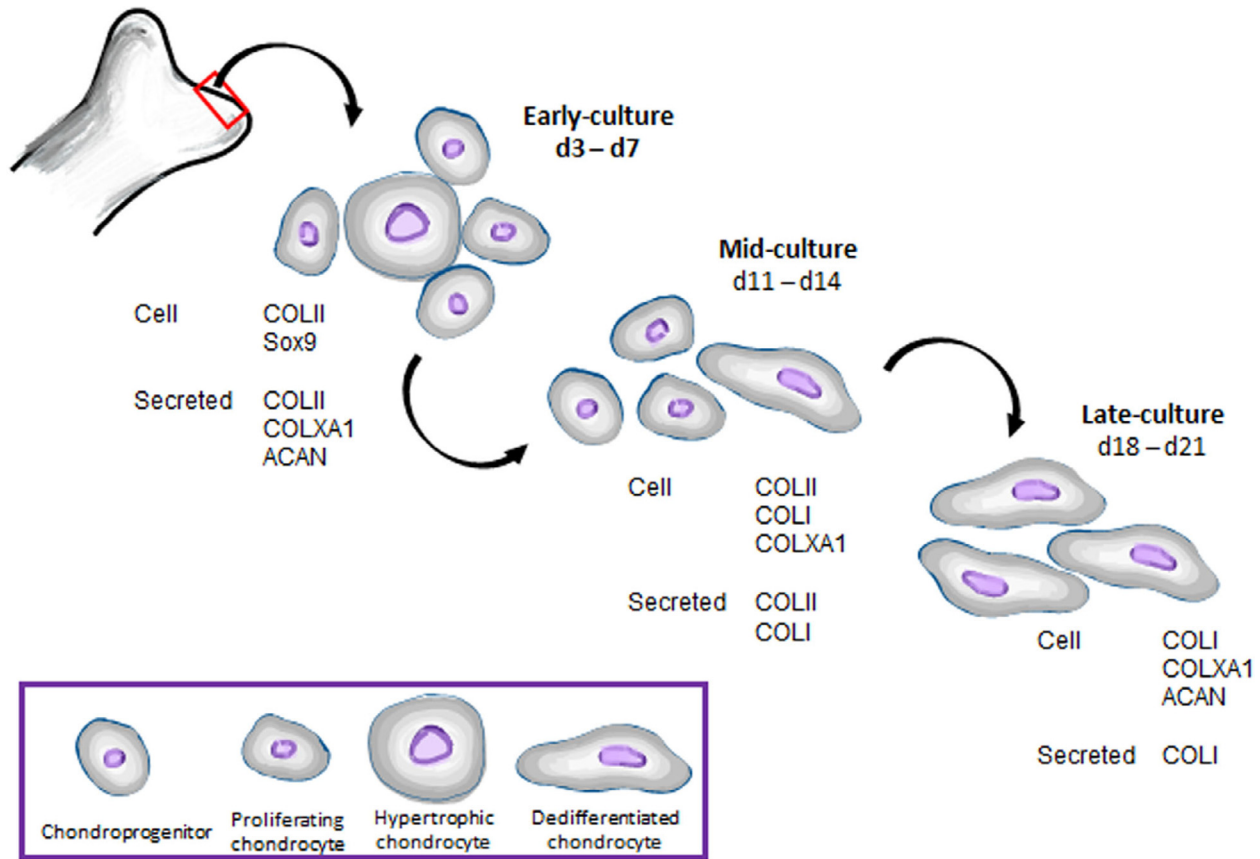
Figure 5. Summary of avian primary growth plate chondrocyte cell stages and phenotypes within culture. Chondrocytes isolated from 10-day-old chicks and cultured at a high-density (2×10^5 cells/cm^2) exhibit a transition of phenotype during early-, mid-, and late-culture indicative of changes in representative cell stages and a subsequent dedifferentiation of chondrocytes. These shifts can be visualized through the mRNA expression ad protein production and secretion of the cells. Chondrocyte markers are the most optimal for growth-plate in vitro models between d3 and d7 of culture.

ECM markers such as ACAN and COLXA1 (Figure 5). Further research is needed into whether this phenotype is maintainable through culture or if these cells could act as an in vitro model for already established molecular pathways involved in bone diseases and infections.

ACKNOWLEDGMENTS

DISCLOSURES

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