Investigating the role of culture conditions on hypertrophic differentiation in human cartilage endplate cells

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Title: Investigating the role of culture conditions on hypertrophic differentiation in human cartilage endplate cells

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Abstract:

Cartilage endplate degeneration/calcification has been linked to the onset and progression of intervertebral disc degeneration and there is a critical need to understand mechanisms, such as hypertrophic differentiation, of cartilage endplate degeneration/calcification to inform treatment strategies for discogenic back pain. *In vitro* cell culture conditions capable of inducing hypertrophic differentiation are used to study pathophysiological mechanisms in articular chondrocytes, but culture conditions capable of inducing a hypertrophic cartilage endplate cell phenotype have yet to be explored. The goal of this study was to investigate the role of culture conditions capable of inducing hypertrophic differentiation in articular chondrocytes on hypertrophic differentiation in human cartilage endplate cells. Isolated human cartilage endplate cells were cultured as pellets for 21 days at either 5% O₂ (physiologic for cartilage) or 20.7% O₂ (hyperoxic) and treated with 10% fetal bovine serum or Wnt agonist, two stimuli used to induce hypertrophic differentiation in articular chondrocytes. Cartilage endplate cells did not exhibit a hypertrophic cell morphology in response to fetal bovine serum or Wnt agonist but did display other hallmarks of chondrocyte hypertrophy and degeneration such as hypertrophic gene and protein expression, and a decrease in healthy proteoglycans and an increase in fibrous collagen accumulation. These findings demonstrate that cartilage endplate cells take on a degenerative phenotype in response to hypertrophic stimuli *in vitro*, but do not undergo classical changes in morphology associated with hypertrophic differentiation regardless of oxygen levels, highlighting potential differences in the response of cartilage endplate cells versus articular chondrocytes to the same stimuli.

Key Words: cartilage endplate, hypertrophic differentiation, calcification, intervertebral disc, low back pain

Introduction:

Low back pain (LBP) has remained one of the top three leading causes of non-fatal health loss over the last thirty years¹. It is the second most common reason for opioid prescription² and costs >\$100 billion yearly³ in the United States alone. Intervertebral disc (IVD) degeneration is highly associated with LBP but the underlying disease pathology and mechanisms of pain generation are poorly understood⁴.

IVDs are the main joints of the spinal column and are composed of the nucleus pulposus (NP) and annulus fibrosus (AF). The cartilage endplate (CEP) is a thin layer of cartilage between the IVD and the vertebral body⁵. The CEPs distribute intradiscal pressures and prevent bulging of the NP into the adjacent vertebral bodies, and function as a gateway for nutrients and metabolites to enter and exit the avascular, aneural IVD⁶. With disease, the CEPs undergo structural and biochemical alterations including thinning, loss of water, decrease in proteoglycan content and calcification^{7,8}. Changes in CEP biochemistry can decrease diffusivity and NP cell viability⁹ and CEP degeneration/calcification is implicated in the onset and progression of disc degeneration⁶. Recent studies have demonstrated alterations in CEP quality may associate with structural damage¹⁰, which appears to be an initiator of vertebral bone marrow lesions¹¹. These vertebral bone marrow lesions and CEP defects are more prevalent in cohorts of patients with chronic LBP^{12,13}.

Although pathological changes to the CEP have been implicated in the initiation and progression of IVD degeneration and LBP, few treatment strategies consider therapeutically targeting the CEP¹⁴. Therefore, there has been increased focus on mechanisms associated with CEP degeneration and identification of potential therapeutic targets. For example, endochondral ossification, the mechanism by which cartilage differentiates into bone during development, has

been identified as a potential mechanism for CEP calcification. Ectopic calcifications in the human CEP are associated with AGE methylglyoxal-hydroimidazolone-1 and osteogenic markers collagen-X (COLX) and osteopontin. Bovine CEP cells cultured with AGEs demonstrated an increase in ectopic calcifications and this effect was mitigated by inhibition of RAGE (AGE receptor) supporting a role for AGE/RAGE in CEP calcification via endochondral ossification¹⁵. Another study has shown that ovariectomized mice have osteoporotic vertebrae, degeneration of the IVD and features of endochondral ossification in the CEPs, further implicating endochondral ossification as a potential mechanism by which degeneration is induced in the CEP¹⁶. Additionally, calcified CEP tissue isolated from rats with IVD degeneration demonstrated high levels of leptin expression. Leptin increased expression of hypertrophy markers osteocalcin and runt-related transcription factor 2 via the ERK1/2 signaling pathways in a dose and time dependent manner in CEP cells *in vitro*¹⁷. Furthermore, intraperitoneal injection of Ligustrazine (a natural compound isolated from Chinese herbal medicine *Ligusticum wallichii*) into rats with IVD degeneration attenuated CEP hypertrophy via suppression of TGF- β ¹⁸. These studies highlight a potential role for endochondral ossification, specifically hypertrophic differentiation, in CEP calcification/degeneration.

In vitro cell culture conditions capable of inducing hypertrophic differentiation are used to study pathophysiological mechanisms in articular chondrocytes, however, culture conditions capable of inducing or recapitulating CEP cell hypertrophy *in vitro* have yet to be identified. Our aim was to investigate the role of culture conditions capable of inducing hypertrophic differentiation in articular chondrocytes on hypertrophic differentiation in human cartilage endplate cells. Both Wnt agonist and 10% fetal bovine serum (FBS) have been used to induce hypertrophic differentiation in articular chondrocytes *in vitro*^{19–23}. As such, we explored the

effects of Wnt agonist and 10% FBS on CEP cells. Developing an *in vitro* cell culture model of hypertrophy using physiologically relevant osteogenic signaling pathways, such as the Wnt/ β -catenin pathway will give researchers the ability to control and explore aspects of CEP cell hypertrophy *in vitro*. In addition, both physiologic (5% O₂) oxygen levels experienced by cartilage and hyperoxic (20.7% O₂) oxygen levels were evaluated. Oxygen tension has been shown to play a role in regulation of IVD cell metabolism, gene expression and matrix production²⁴⁻²⁷. Lower oxygen levels experienced by cartilage and disc can inhibit hypertrophic differentiation in chondrocytes *in vitro*²⁸. Decreases in expression of hypoxia-inducible factor-1 α in the degenerated CEP suggests a less hypoxic microenvironment²⁹ likely due to neovascularization of the CEP with disease and these processes may augment hypertrophic differentiation of the CEP.

We hypothesized Wnt agonist and 10% FBS media would induce a degenerate phenotype via hypertrophic differentiation compared with Chondrogenic and Basal control media. Furthermore, we hypothesized that culturing CEP cells in 5% O₂ would limit hypertrophic differentiation induced by 10% FBS and Wnt agonist. Consequently, culturing CEP cells in 10% FBS or Wnt agonist media in 20.7% oxygen would enhance hypertrophic differentiation in CEP cells. The mechanisms of CEP degeneration/calcification are underexplored and identification of culture conditions capable of inducing hypertrophic differentiation *in vitro* could significantly contribute to elucidating the role of the CEP in IVD degeneration and LBP.

Methods:

Primary cell isolation and expansion

CEP cells were isolated from n=11 human cadaveric lumbar spines (Table 1) within 36 hours of death (Cooperative Human Tissue Network, Columbus, Ohio). Individual IVDs were isolated from

each spine. Macroscopic images of IVDs were taken, blinded and graded using the Thompson grading scale³⁰ by two individual expert spine researchers. Images of IVDs for CEP6 and CEP8 were not available and therefore Thompson grades are unavailable. Vertebral bodies were scraped to remove any remaining NP or AF tissue. The CEP tissue was carefully dissected from the vertebral bodies to ensure only shiny translucent hyaline cartilage was removed and cells were isolated using 0.2% protease (from *Streptomyces griseus* - Type XIV: P5147, Sigma-Aldrich, MO, USA) for 1 hour at 37°C, followed by digestion with 0.012% collagenase-II (from *Clostridium Histolyticum* – 17101015, Thermo Fisher Scientific, MA, USA) for 12 hours at 37°C³¹. Cells were expanded in disc cell media (Dulbecco's Modified Eagle Medium (DMEM, 4.5g/L glucose – 11995115, Thermo Fisher Scientific, MA, USA), 10% FBS (0437028, Gibco, MD, USA), 1% penicillin/streptomycin (P/S – 15140122, Gibco, MD, USA), 50 µg/mL ascorbic acid (AA – 97061-072, VWR, PA, USA), 0.5% Amphotericin B (FZ – 15290018, Gibco, MD, USA)) in 5% CO₂, 20.7% O₂ at 37°C, and fed every 3 days until 80% confluent. Cells were used at passage ≤p4.

Pellet Culture

Please refer to Table 1 and Figure 1 for study design. CEP cells were pelleted at 1x10⁶ cells/pellet in 15mL falcon tubes by centrifuging at 400xg for 5 minutes and cultured in 2mL of either **1) Chondrogenic** (DMEM, 4.5g/L glucose, 1% P/S, 1X ITS (I2771, Sigma Aldrich, MO, USA), 20µg/mL L-proline (P5607-25G, VWR, PA, USA), 50µg/mL AA, 2mM L-glutamine (25030081, Gibco, MD, USA), 1mM sodium pyruvate (11360070, Gibco, MD, USA), 10⁻⁷M dexamethasone (D4902-25MG, Millipore Sigma, MA, USA), 10ng/mL Recombinant TGF-β3 (from *E.coli* – 10778-940, VWR, PA, USA)³²), **2) 10% FBS** (10% FBS, DMEM, 4.5g/L glucose, 1% P/S, 10mM HEPES (15630080, Gibco, MD, USA), 20µg/mL L-proline, 50µg/mL AA, 2mM L-glutamine¹⁹), **3) Wnt agonist** (1X ITS, DMEM, 4.5g/L glucose, 1% P/S, 10mM HEPES, 20µg/mL L-proline,

50µg/mL AA, 2mM L-glutamine, 0.5µM Wnt Agonist (CHIR99021, Stemcell Technologies, Vancouver, CA)) added fresh²⁰) or **4) Basal** (1X ITS, DMEM, 4.5g/L glucose, 1% P/S, 10mM HEPES, 20µg/mL L-proline, 50µg/mL AA, 2mM L-glutamine) media (n=10 Chondrogenic, n=10 10% FBS, n=10 Wnt agonist, n=7 Basal) for 21 days in **5% O₂**, 5% CO₂ (n=10) or **20.7% O₂**, 5% CO₂ (n=5) at 37°C with media changes every 3-4 days. Cell viability was assessed by incubating pellets for 20 minutes in DMEM containing Calcein AM (2µM) (C1430, Thermo Fisher Scientific, MA, USA)/Ethidium homodimer-1 (1µM) (E1169, Thermo Fisher Scientific, MA, USA). A 20x fluorescent image of each pellet was taken using a Nikon TiE Inverted Microscope with a high-resolution DS-Qi2 camera to qualitatively determine live and dead cells.

Pellet Size, Matrix Composition and Cell Morphology

Pellets (Treatment: Chondrogenic (n=7), Basal (n=7), 10% FBS (n=6), Wnt agonist (n=7); Oxygen level: 20.7% O₂ (n=4), 5% O₂ (n=6)) were fixed in 10% neutral buffered formalin for 24 hours, dehydrated, then embedded in paraffin and cut into 4µm sections. Pellet size and cell morphology were evaluated using hematoxylin and eosin (H&E) as previously described³³. Pellet size was quantified using the manual measurements tool in Nikon NIS-Elements AR software and area measured in millimeters squared. Hypertrophic cell morphology was determined by qualitatively evaluating H&E stained pellets for enlarged/vacuolated hypertrophic cells¹⁹⁻²³. To determine matrix composition, the sections were stained using a picrosirius red (collagen)/alcian blue (proteoglycan) (PRAB) dual stain³³. Stained pellets were imaged using a Nikon TiE Inverted Microscope with a high-resolution DS-U3 camera at 10x or 20x magnification.

Quantification of Glycosaminoglycan content

Pellets (Treatment: Chondrogenic (n=9), Basal (n=10), 10% FBS (n=9), Wnt agonist (n=9); Oxygen level: 20.7% O₂ (n=6), 5% O₂ (n=5)) were digested in 400µL of papain digest (125µg/mL

papain (from Papaya latex – P4762, Sigma-Aldrich, MO, USA), 0.1M sodium phosphate monobasic monohydrate (71507-250G, Sigma-Aldrich, MO, USA), 5mM EDTA (E9884-1KG, Sigma-Aldrich, MO, USA), 5mM L-cysteine hydrochloride (2430-100GM, Millipore Sigma, MA, USA)) and incubated in 65°C overnight. Digested samples were used for dimethylmethylene blue (DMMB) assay to assess glycosaminoglycan (GAG) content³⁴. DNA content was assessed using a DNA Quantitation Kit (DNAQF, Sigma Aldrich, MO, USA) per manufacturer's instructions. GAG content was normalized to DNA content.

Quantification of Gene Expression

To determine relative gene expression, qRT-PCR was performed for all groups (Treatment: Chondrogenic (n=7-9), Basal (n=7), 10% FBS (n=7-9), Wnt agonist (n=7-10); Oxygen level: 20.7% O₂ (n=5), 5% O₂ (n=7)). TRIzol Plus RNA Purification Kit (12183555, Thermo Fisher Scientific, MA, USA) was used to isolate RNA and RNA was quantified using the Nanodrop 2000. Samples were reverse-transcribed to cDNA using qScript XLT cDNA SuperMix (95161, Quanta Biosciences, UT, USA) or Maxima H Minus cDNA Synthesis Master Mix (M1662, Thermo Fisher Scientific, MA, USA) and diluted to a final concentration of 15ng/reaction. QRT-PCR was completed in duplicate using Taqman Master Mix and primers (Table 2). Gene expression was determined using the $\Delta\Delta CT$ method relative to the housekeeping gene 18s³¹.

Immunohistochemical analysis for MMP-13 and COLX expression

Immunohistochemistry (IHC) was performed on formalin fixed, paraffin embedded and sectioned pellets (Treatment: Chondrogenic (n=7), Basal (n=7), 10% FBS (n=6), Wnt agonist (n=7); Oxygen level: 20.7% O₂ (n=4), 5% O₂ (n=6)). Slides were deparaffinized and rehydrated. Antigen retrieval was performed using 1X Dako Target Retrieval Solution (S169984-2, Agilent Technologies, CA, USA) preheated in a steamer. Slides were added to pre-heated solution and steamed for 15

(MMP13) or 20 minutes (COLX). Slides were cooled to room temperature and washed 2x5 minutes. Slides were treated with Dako Protein Block (X090930-2, Agilent Technologies, CA, USA) solution for 15 (MMP13) or 20 minutes (COLX). Slides were incubated with primary antibody for MMP-13 (LS-B3168-50, LifeSpan BioSciences Inc, WA, USA – recognizes proenzyme and cleaved fragments) diluted at a 1:1300 dilution overnight at 4°C, or antibody for COLX (LS-B14811, LifeSpan BioSciences Inc, WA, USA) diluted at 1:100 for 45 minutes in Dako Antibody Diluent (S302283-2, Agilent Technologies, CA, USA) at room temperature. A no primary antibody control was used for each round of staining. Slides were washed for 3x5 minutes. Endogenous peroxidases were blocked by incubating slides in 3% hydrogen peroxide (H1009-100ML, Millipore Sigma, MA, USA) in methanol (NC1270520, Thermo Fisher Scientific, MA, USA) for 10 minutes (MMP13) or 15 minutes (COLX). Slides were washed for 2x5 minutes and incubated for 30 minutes in Vector Biotinylated Goat Anti-Rabbit IgG Antibody (H+L) (BA-1000, Vector Laboratories, CA, USA) diluted 1:200 in Dako Protein Block at room temperature. Slides were washed for 3x5 minutes and incubated for 30 minutes in R.T.U. Horseradish Peroxidase Streptavidin (SA-5704, Vector Laboratories, CA, USA) at room temperature. Slides were washed for 3x5 minutes and treated with 3, 3'-Diaminobenzidine for 5 minutes using the ImmPACT DAB Peroxidase (HRP) Substrate kit (SK-4105, Vector Laboratories, CA, USA). Slides were rinsed with deionized water for 5 minutes, counterstained with Gills Hematoxylin No.2 (GHS232-1L, Millipore Sigma, MA, USA), rinsed with deionized water, and blued in bluing solution for 30 seconds (0.05% ammonium hydroxide (C993P14, Electron Microscopy Sciences, PA, USA) in water). Slides were dehydrated, cleared in xylene (HC-700-1GAL, Thermo Fisher Scientific, MA, USA), and mounted using Cytoseal Mounting Medium (23-244-257, Thermo Fisher Scientific,

MA, USA). Stained pellets were imaged using a Nikon TiE Inverted Microscope with a high-resolution DS-U3 camera at 20x magnification.

Quantification of MMP-13 and COLX

20X images (1-3 images depending on pellet size) were used to quantify staining. Images were taken from both the pellet center and periphery. Slides were blinded before imaging and remained blinded throughout image analysis. The IHC Profiler plugin for ImageJ was used to quantify staining. The IHC Profiler plugin³⁵ reports percent contribution of high positive, positive, low positive and negative cells. Based on this information, an H-score from 0-300 (300 being 100% high positivity and 0 being completely negative) was calculated for each image using Equation 1.

$$\text{H-score} = (\% \text{ high positive} \times 3) + (\% \text{ positive} \times 2) + (\% \text{ low positive} \times 1) \quad (1)$$

Statistics

All data was transformed using a Box Cox Transformation with optimal λ that minimizes the sum of squared error to achieve normality. A two-way ANOVA was used to assess the main effects of media treatment and oxygen condition, as well as the interaction between media treatment and oxygen condition. An Anderson-Darling test was performed on the data residuals to confirm normally distributed data. Post-hoc Tukey tests were used to determine which specific groups differed if the main effect of treatment or interaction was significant. All test were conducted using Minitab with $\alpha=0.05$ and $p<0.05$ was considered significant.

Results:

Viability

Viable cells (and some dead cells) were observed in all treatment groups and oxygen conditions (Figure 2). Due to the high cell density of the pellets, quantifiable viability data could not be

obtained and viability was assessed qualitatively. No qualitative differences were observed between groups.

Pellet Size, Matrix Composition and Cell Morphology

Pellet area was significantly reduced in the 10% FBS group compared to the Basal control (Figure 3a and b). Enlarged hypertrophic cell morphology was not observed in any of the groups (Figure 4). PRAB staining of cell pellets demonstrated a reduction in proteoglycan assessed by a decrease in alcian blue staining observed in the 10% FBS and Wnt agonist groups compared to the Basal group (Figure 2a). This was supported quantitatively by the results of the DMMB assay which revealed significant differences in proteoglycan (glycosaminoglycan (GAG)) content between treatment groups (Figure 5a-c). Significant decreases in GAG/DNA were measured in the 10% FBS and Wnt agonist groups compared to the Chondrogenic group. A significant reduction in GAG/DNA was observed in the 10% FBS group relative to the Basal (Figure 5a). There was no significant difference in GAG/DNA content measured between oxygen conditions (Figure 5a); however, there were differences in raw (not normalized) GAG and DNA between oxygen groups (Figure 5 b-c). Significant interactions between oxygen levels and treatment groups in GAG/DNA content and DNA content were observed and are shown in Figure 5 a and c.

Gene Expression

QRT-PCR demonstrated significant differences in matrix marker, hypertrophic marker and angiogenic/inflammatory marker gene expression between different oxygen conditions (Figure 6a). There was a significant up-regulation in COL2, SOX9, IHH, RUNX2 and IL6 in the 5% O₂ group compared to 20.7% O₂. There was a significant down-regulation in MMP-13 in the 5% O₂ group compared to 20.7% O₂ and no difference in VEGF, NGF or TAC1 expression between oxygen conditions (Figure 6a). Media treatments also had a significant effect on expression of

matrix marker, hypertrophic marker and inflammatory markers (Figure 6b). There was a significant up-regulation of COL2 in the Chondrogenic group and a significant down-regulation in COL2 in the 10% FBS group compared to the Basal control. There was a significant up-regulation of COLX and SPP1 and significant down-regulation of RUNX2 in the Chondrogenic group, a significant up-regulation in IHH, RUNX2, MMP13, and COLX in the 10% FBS group, and significant up-regulation of RUNX2 and MMP13 in with Wnt agonist group compared to the Basal control. There was a significant down-regulation in VEGF, NGF and IL6 in the Chondrogenic group, significant up-regulation of IL1 β in the 10% FBS group, and significant up-regulation of IL6 in the Wnt agonist group compared to the Basal control (Figure 6b). There were significant interactions between media treatment and oxygen conditions in SOX9, IHH and RUNX2 expression (Figure 6c).

Immunohistochemistry

Protein expression of MMP13 and COLX (both markers of hypertrophy) was evaluated due to large differences in MMP-13 and COLX gene expression between treatment groups. No effect of image location (center vs periphery) was observed for either protein. Interestingly, IHC for MMP-13 and COLX revealed no significant differences in protein expression between treatment or oxygen conditions as shown in Figure 7 and Figure 8.

Discussion:

The initiation and progression of IVD degeneration and LBP is highly associated with degenerative changes to the CEP, such as CEP calcification⁶, and hypertrophic differentiation of CEP cells has been identified as one potential mechanism of this process¹⁵. The goal of this study was to investigate the role of culture conditions on hypertrophic differentiation in human cartilage endplate cells. Both Wnt agonist and 10% fetal bovine serum (FBS) have been used to

induce hypertrophic differentiation in articular chondrocytes *in vitro*^{19–23}. As such, we explored the effects of Wnt agonist and 10% FBS on CEP cells. Interestingly, unlike articular chondrocytes, CEP cells did not exhibit a hypertrophic cell morphology in response to 10% FBS or Wnt agonist but displayed other hallmarks of chondrocyte hypertrophy and degeneration such as hypertrophic gene and protein expression, and a decrease in healthy proteoglycan and an increase in fibrous collagen accumulation.

Based on previous studies demonstrating 10% FBS induces hypertrophic differentiation in articular chondrocytes, we hypothesized addition of 10% FBS to CEP cells would also induce hypertrophic differentiation. Our histological data showed no morphological evidence of hypertrophic differentiation in CEP cells treated with 10% FBS after 21 days, whereas past studies found that articular chondrocytes take on a large, vacuolated hypertrophic morphology in pellet culture in response to 10% FBS at this timepoint^{19,21–23}. These differences in response to the same stimuli may be due to differences in cell phenotype between CEP cells and articular chondrocytes which we recently demonstrate on the gene level^{35,36}. However, we did observe a decrease in COL2, an increase in IL1- β , MMP13, RUNX2, IHH and COLX gene expression, and decrease in both pellet size and GAG content in the 10% FBS group compared to the Basal control. These findings are consistent with previous studies which found increased expression of COLX, MMP13, RUNX2 and decreased expression of COL2^{19,21}, GAG content and pellet size in articular chondrocytes³⁸ treated with 10% FBS.

We also hypothesized that addition of Wnt agonist to CEP cells would induce hypertrophic differentiation due to the role of the Wnt/ β -catenin pathway in modulating this process²⁰. We observed no characteristic morphological changes indicative of hypertrophic differentiation in the CEP cells treated with Wnt agonist after 21 days. We observed an increase

in RUNX2 and MMP13 gene expression with Wnt agonist compared to Basal, but saw no significant effects of Wnt agonist on any other dependent variables. Enochson et al. also found a significant increase in MMP13 gene and protein expression in chondrocyte pellets treated with Wnt agonist²⁰. However, when we examined MMP13 in CEP pellets on the protein level, we found no significant differences between groups, suggesting differing effects of Wnt agonist on the CEP compared to articular chondrocytes.

Furthermore, chondrogenic media containing TGF- β 3 has long been used to maintain a chondrogenic phenotype in articular chondrocyte cells in *in vitro* culture systems^{32,39,40} and we hypothesized that it would maintain a healthy CEP cell phenotype in our culture model. TGF- β 3 is known to play a role in cartilage development and maintenance and has been shown to inhibit expression of osteogenic and angiogenic markers such as RUNX2, MMP13, COLX and VEGF⁴¹. In alignment with previous findings, we observed a significant down-regulation of VEGF and NGF gene expression and increase in healthy proteoglycan accumulation (GAG) in the Chondrogenic group compared to the Basal, likely indicating a pro-anabolic/anti-angiogenic/neurogenic role for TGF- β in our culture system. On the contrary, we also observed a significant increase in gene expression of hypertrophic markers COLX and SPP1 in our Chondrogenic group compared to our Basal control, which was surprising but further highlights potential differences in the response of CEP versus articular chondrocyte cells to the same stimuli.

Hypertrophic markers MMP13 and COLX were examined on the protein level using IHC to validate our gene expression findings. Interestingly we found that cell pellets from all treatment groups express COLX and MMP13 protein with no significant differences between treatment groups. This did not correlate with our findings on the gene level, demonstrating a

potential disconnect between gene and protein expression in our culture system. The presence of COLX protein, a distinct hypertrophic marker, in CEP cells without a morphological hypertrophic phenotype was surprising. Though, COLX protein expression at the CEP-vertebral body interface in cadaveric human specimens ranging from very young fetuses to adults of 86 years of age has been previously demonstrated⁴², suggesting that CEP cells actually express baseline levels of COLX protein irrespective of a healthy or diseased state. This is supported by the expression of COLX in all treatment and control groups at the gene and protein level. Interestingly, studies to date examining hypertrophic differentiation in the CEP utilize common markers of articular chondrocyte hypertrophy as evidence of hypertrophic differentiation but have not examined CEP cell morphology^{15–18,43}. These studies together with our current work suggest that CEP cells may express hypertrophic markers without the characteristic changes in morphology seen in typical hypertrophic articular chondrocytes.

In addition, CEP cell pellets were cultured in both physiologic (5% O₂) oxygen levels experienced by cartilage and hyperoxic (20.7% O₂) oxygen levels. We hypothesized that 20.7% O₂ would result in greater hypertrophic differentiation of CEP cells in response to 10% FBS or Wnt agonist but saw no morphological evidence of hypertrophic CEP cells regardless of oxygen level. Findings related to oxygen level were variable, with increased gene expression of COL2 and decreased expression of MMP13 in 5% O₂ compared to 20.7% O₂. However, we also observed a significant increase in additional hypertrophic markers RUNX2 and IHH, and inflammatory marker IL6 in the 5% O₂ condition that further demonstrates differential responses of CEP cells and articular chondrocytes to the same culture conditions.

This study provides insight into regulation of CEP cell differentiation. Using biochemical techniques commonly used to induce hypertrophic differentiation in articular chondrocytes, we

were unable to observe a classical hypertrophic cell morphology in CEP cells. We observed increased gene expression of hypertrophic markers in response to 10% FBS and Wnt agonist however, these changes were not observed at the protein level for the markers MMP13 and COLX we evaluated using IHC. Since gene expression and hypertrophic differentiation are temporal in nature, this may indicate a missed timepoint in our culture model. The 21 day culture period we utilized is a common culture time used to induce hypertrophic differentiation in articular chondrocytes^{19,21-23}, although CEP cells may behave differently and undergo hypertrophic differentiation in a different time frame. Examining a single time point can be considered a potential limitation of the current study. Addition of an articular chondrocyte control to directly compare CEP and articular chondrocyte cell behavior *in vitro* and increasing sample size in order to further validate the effect of culture conditions and oxygen tension on sex and age-related differences in human CEP samples would enhance the rigor of the current study. Extreme care taken when separating CEP, AF and NP tissue during dissection, and previously observed differences in marker expression at the gene level between cells using the same isolation technique, suggest isolation of distinct cell populations^{36,37}. Nevertheless, cross-contamination with additional cell types, such as inflammatory cells, could still be a minimal concern. In addition, exploring the effects of the mechanical microenvironment on the CEP, which has been previously shown to modulate CEP cell phenotype⁴⁰, is an area of interest that warrants further investigation. Furthermore, one past study shows that exposing human CEP cells to free calcium impairs CEP homeostasis and promotes a catabolic phenotype, similar to that of our 10% FBS condition, with a decrease in secreted GAG and COL2⁴⁴. Unfortunately, hypertrophic differentiation of CEP cells in response to free calcium was not explored, but could be an alternative to the hypertrophic stimuli used in the current study due to its role in

hypertrophic differentiation of articular chondrocytes⁴⁵. These considerations, while important, were outside the scope of the current study but may be explored in future studies.

Conclusion:

This study evaluated the effects of culture conditions on hypertrophic differentiation in CEP cells *in vitro* in attempt to better understand mechanisms involved in CEP degeneration/calcification. We implemented common methods used to induce hypertrophic differentiation in articular chondrocytes, which included treatment with 10% FBS and Wnt agonist. Additionally, we examined two oxygen levels (5% O₂ and 20.7% O₂) to further evaluate culture conditions that may affect CEP cell hypertrophy. Surprisingly, we were unable to induce a classical hypertrophic cell morphology in CEP cells at either oxygen level which may indicate key differences in the physiology of CEP cells compared to articular chondrocytes. Additionally, the distinct differences in response between CEP cells and articular chondrocytes to hypertrophic stimuli highlighted by this work and the literature demonstrates a need to further understand mechanisms of how CEP cells contribute to calcification since it is known to be an important contributor to IVD degeneration.

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Table Captions:

Table 1: Demographics for cadaveric specimens used and breakdown of study design, including which specimens were utilized in each group and overall sample sizes.

Table 2: Genes and corresponding description of genes evaluated using qRT-PCR.

525 **Figure Legends:**

526 **Figure 1:** Overall study design with independent and dependent variables and methods used to
527 assess each dependent variable.

528 **Figure 2:** Representative images of cell viability where human cartilage endplate cells stained in
529 green are alive and cells stained in red are dead. Scale bar = 100 μ m

530 **Figure 3:** Picrosirius red (collagen) and alcian blue (proteoglycan) staining of CEP cell pellets
531 demonstrating differences in proteoglycan and collagen matrix accumulation between
532 Chondrogenic, Basal, 10% FBS and Wnt agonist treatment groups, Scale bar = 500 μ m (a).

533 Quantified pellet area in millimeters squared showing the main effects of oxygen levels and
534 treatment groups and the interaction between oxygen levels and treatment groups (b). Different
535 letters represent statistically significant differences ($p < 0.05$), error bars represented as SEM.

536 **Figure 4:** Representative images of hematoxylin and eosin stained CEP cells to evaluated
537 changes in cell morphology in different treatment groups and oxygen levels. Scale bar = 50 μ m.

538 **Figure 5:** Main effects of Chondrogenic, Basal, 10% FBS and Wnt agonist media treatments and
539 oxygen levels and interactions between media treatments and oxygen levels for normalized
540 GAG/DNA (a), raw GAG (b), and raw DNA (c) content in CEP pellets after 21 days. Different
541 letters represent statistically significant differences ($p < 0.05$), error bars represented as SEM.

542 **Figure 6:** Main effects of oxygen levels (a) and Chondrogenic, Basal, 10% FBS and Wnt agonist
543 media treatments (b) on matrix, bone and neurovascular & inflammatory marker gene expression
544 in CEP pellets after 21 days. Significant interactions between media treatments and oxygen
545 levels on SOX9, IHH and RUNX2 gene expression in CEP pellets after 21 days (c). Different
546 letters represent statistically significant differences ($p < 0.05$), error bars represented as SEM.

547 **Figure 7:** Representative images of MMP-13 IHC in CEP pellets and no primary control, Black
548 Scale = 100 μm , Red Scale = 50 μm . Main effects of Chondrogenic, Basal, 10% FBS and Wnt
549 agonist media treatments and oxygen levels and interactions between media treatments and
550 oxygen levels on quantified MMP-13 protein expression represented as H-Score. Different letters
551 represent statistically significant differences ($p < 0.05$), error bars represented as SEM.

552 **Figure 8:** Representative images of COLX IHC in CEP pellets and no primary control, Black
553 Scale = 100 μm , Red Scale = 50 μm . Main effects of Chondrogenic, Basal, 10% FBS and Wnt
554 agonist media treatments and oxygen levels and interactions between media treatments and
555 oxygen levels on quantified COLX protein expression represented as H-Score. Different letters
556 represent statistically significant differences ($p < 0.05$), error bars represented as SEM.

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Gene	Main Effect Oxygen	Main Effect Treatment	Interactions	Gene	Main Effect Oxygen	Main Effect Treatment	Interactions
COL2	p=0.001	p<0.001	p=0.142	SPP1	p=0.611	p=0.037	p=0.611
SOX9	p<0.001	p=0.025	p=0.015	VEGF	p=0.072	p<0.001	p=0.098
IHH	p<0.001	p=0.029	p<0.001	NGF	p<0.001	p<0.001	p=0.595
RUNX2	p=0.003	p<0.001	p=0.027	TAC1	p=0.631	p=0.095	p=0.038
MMP13	p=0.027	p<0.001	p=0.522	IL6	p<0.001	p<0.001	p=0.152
COLX	p=0.329	p=0.004	p=0.846	IL1b	p=0.067	p=0.007	p=0.707

Table 1. Demographics for cadaveric specimens used and breakdown of study design, including which specimens were utilized in each group and overall sample sizes.

ID	Sex	Age	Level	Thompson Grade
CEP1	Female	43	L1/L2	3
CEP2	Female	49	L3/L4	4-5
CEP3	Male	55	L1/L2	3
CEP4	Male	45	L1/L2 & L3/L4	Both 3
CEP5	Female	58	L3/L4 & L4/L5	Both 2-3
CEP6	Female	56	L4/L5	Unknown
CEP7	Male	46	L3/L4	2-3
CEP8	Female	31	L1/L2	Unknown
CEP9	Female	30	L2/L3 & L4/L4	Both 2-3
CEP10	Female	59	L3/L4	3
CEP11	Female	19	L3/L4	2
Total n=11 CEP	Basal (n=7)	Chondrogenic (n=10)	10% FBS (n=10)	Wnt Agonist (n=10)
20.7% O₂ (n=5)	CEP4 CEP7 CEP9 CEP10 CEP11	CEP4 CEP7 CEP9 CEP10 CEP11	CEP4 CEP7 CEP9 CEP10 CEP11	CEP4 CEP7 CEP9 CEP10 CEP11
5% O₂ (n=10)	CEP2 CEP4 CEP7 CEP8 CEP9 CEP10	CEP1 CEP2 CEP3 CEP4 CEP5 CEP6	CEP1 CEP2 CEP3 CEP4 CEP5 CEP6	CEP1 CEP2 CEP3 CEP4 CEP5 CEP6

Table 2. Genes and corresponding description of genes evaluated using qRT-PCR.

Target gene	Role
18s 4333760F	Housekeeping gene
COL2A1 Hs00264051_m1	Matrix marker; indicates chondrogenic phenotype
SOX9 Hs00165814_m1	Matrix marker; indicates chondrogenic phenotype
IHH Hs00745531_s1	Hypertrophic marker
RUNX2 Hs01047973_m1	Hypertrophic marker
MMP13 Hs00233992_m1	Matrix degrading enzyme; indicates hypertrophic phenotype
COL10	Hypertrophic marker
SPP1	Hypertrophic marker
VEGFA Hs00900055_m1	Angiogenic marker
NGF Hs00171458_m1	Neurogenic marker
TAC1 Hs00243225_m1	Substance P; indicates pain
IL6 Hs00174131_m1	Inflammatory marker
IL1 β Hs00174097_m1	Inflammatory marker