Characterization of Chondrocytes in Normoxic and Hypoxic Conditions: Proliferation and Metabolomic Profiles

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INTRODUCTION: Osteoarthritis (OA) is a multifactorial chronic degenerative joint condition affecting >37% of people over the age of 60. It is a leading cause of pain and disability accounting for considerable loss of productivity and healthcare expenditures up to $140 billion annually [1]. Since articular cartilage lacks vascularization, chondrocytes reside in a hypoxic microenvironment with oxygen concentration <5% and have adaptive mechanisms for chronic hypoxia [2]. Tissue engineering holds promise for cartilage repair and potential restoration of joint function in OA. Current cell-based cartilage repair therapies often involve monolayer expansion under normoxic conditions that may alter physiological and biochemical responses of chondrocytes. The aims of this study are (1) to explore the underlying mechanisms and pathways that are involved in hypoxic signaling and regulation in chondrocytes and (2) evaluate hypoxia as a culture platform for expression of cartilage-specific genes in retaining chondrocyte phenotype. Defining the key molecular players and optimal culture conditions may result in production of clinically viable tissue engineered chondrocytes.

METHODS: Primary human chondrocytes were harvested from discarded arthroplasty tissue and first passage populations were used for experiments. Bovine chondrocytes were harvested from knee joints of 18-22 month old cattle obtained, from a local abattoir. The chondrocytes were cultured either under normoxic (20% oxygen) or hypoxic (3% oxygen) conditions. For localization studies, chondrocytes were seeded on microscope coverslips to grow into a monolayer for 48h, followed by 12h of serum starvation. Cells were then pulsed with complete media for 6 h (DMEM with 10%FBS and 1% Penicillin-Streptomycin) containing 10 µM 5-bromo-2'-deoxyuridine (BrdU). Media was then replaced with complete media lacking BrdU and cells were allowed to grow for 72h. Cells were fixed with 4% PFA and immunocytochemistry was performed using monoclonal mouse anti-BrdU antibody and rabbit anti-Collagen VI antibody. For metabolomic analysis, bovine (N=3) and primary human (N= 3 male and 3 female donors) chondrocytes were seeded at a density of ~7900 cells/cm^2 in 24-well tissue culture plates and grown in complete media for 72h under normoxic or hypoxic culture conditions. After trypsinization, cells were resuspended in ice-cold methanol, vortexed, centrifuged and the supernatant containing metabolites was dried. Extracted metabolites were resuspended in 1:1 HPLC grade Acetonitrile:Water. All metabolite extracts were analyzed via liquid chromatography-mass spectrometry (LC-MS) (Waters Synapt XS, HILIC column). Raw LC-MS data were processed and converted via MSConvert and XCMS. For analysis, data were log transformed, standardized, and auto scaled. MetaboAnalyst was used to perform statistical analyses and facilitate pathway analysis using Mummichog.

RESULTS: Immunocytochemistry found that bovine chondrocytes cultured under hypoxic conditions had greater BrdU incorporation within the nucleus (Fig. 1A) than chondrocytes cultured under normoxic conditions (Fig. 1B). However, human osteoarthritic chondrocytes did not show BrdU incorporation within 72 h either under hypoxic (Fig. 1C) or normoxic (Fig. 1D) conditions. Interestingly, expression of Collagen VI was increased under hypoxic conditions in both bovine and primary human chondrocytes, indicating oxygen-dependent changes in protein expression. Unsupervised clustering of metabolomic profiles from bovine (Fig. 2A) or human (Fig. 2B) chondrocytes showed that clusters of metabolites separate according to hypoxic/normoxic group with moderate overlap in the 1st two dimensions. Volcano plot analysis found a total of 1570 oxygen-dependent metabolite features, of which 331 were higher in abundance in chondrocytes harvested from male donors (Fig. 2C) when cultured under hypoxia and these features mapped to Pantothenate and CoA biosynthesis and amino sugar and nucleotide sugar metabolism pathways. Metabolites with lower concentrations under hypoxia mapped to glycerophospholipid and sphingolipid metabolism, amino acid metabolism (alanine, aspartate, glutamate) and lysine and glycosoaminoglycan degradation. Similarly, 374 significant features were identified in female donors (Fig. 2D) using volcano plot and 87 metabolites were higher in chondrocytes cultured under hypoxic conditions that had significant hits to fatty acid metabolism and steroid hormone biosynthesis, while the TCA cycle was dysregulated in normoxia. Median intensity heat map analysis identified clusters of metabolite features (Fig. 3) with dysregulation across the groups. Several significant metabolic pathways from chondrocytes cultured from female donors under hypoxic conditions were upregulated and identified to amino acid (histidine, alanine, aspartate, glutamate, arginine and proline) and purine metabolism, fatty acid degradation, amino sugar and nucleotide sugar metabolism and glutathione metabolism.

DISCUSSION: Our studies indicate that chondrocytes exhibit a different physiological response when cultured under normoxic and hypoxic conditions. When cultured under hypoxia even for short period of time, healthy chondrocytes have the capacity to proliferate and manipulate matrix gene expression. We observed that there were a greater number of cells that incorporated nuclear BrdU and stained for Collagen VI in healthy bovine chondrocytes when cultured under hypoxia than normoxia. Although we didn't observe BrdU incorporation in human osteoarthritic chondrocytes under hypoxic or normoxic conditions, these cells showed an increase in Collagen VI expression under hypoxia. Culturing chondrocytes under hypoxia appears to simulate endogenous conditions that support re-establishing the chondrocyte phenotype. Several distinct pathways were detected in chondrocytes obtained from male and female donors under hypoxic or normoxic conditions, indicating the activation of differentially regulated pathways under each condition. Here we demonstrate that hypoxia plays a major role in influencing chondrocyte metabolism and expression of matrix proteins. Elucidating chondrocyte responses to variations in oxygen can help in promoting and maintaining extracellular matrix production essential for tissue engineering and regeneration for eventual treatment of osteoarthritis.

SIGNIFICANCE: Understanding the molecular mechanisms underlying hypoxic signaling in chondrocyte proliferation and cell biology may provide viable treatment strategies for chondrocyte regeneration and improved cell based therapeutic interventions for osteoarthritis.

REFERENCES: [1]. Murphy et al. ACR 2017 28950426 [2]. Pattappa et al. Int J Mol Sci 2019 6387316

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IMAGES AND TABLES: Fig. 1: Expression of BrdU (red), Collagen VI (green), and nuclear marker VibrantTMDyeCycleTM Violet Stain in chondrocytes cultured under hypoxia and normoxia. Shown are 63x images of bovine hypoxic (1A), bovine normoxic (1B), human hypoxic (1C) human normoxic (1D). Scale bar = 25µm Fig. 2: Unsupervised PCA of metabolites from bovine (2A) and human chondrocytes (2B) under hypoxia (H, pink) and normoxia (N, blue) and Volcano plot analysis of metabolites identified in chondrocytes from male (2C) and female (2D) donors. Fig 3: Heatmap of metabolite clusters from chondrocytes across groups (HF=Hypoxic Female, NF=Normoxic Female, HM=Hypoxic Male, NM=Normoxic Male) Bar = Std Dev From Mean

Figure 1

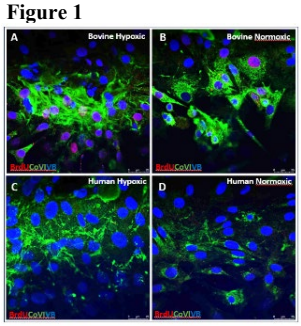

Figure 2

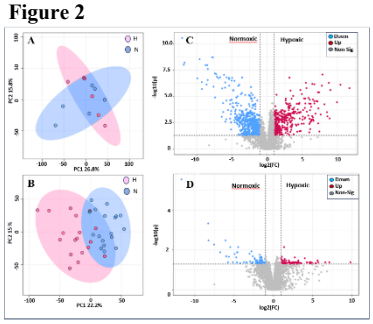

Figure 3