Title

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Effect of passaging on bovine chondrocyte gene expression and engineered cartilage production

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Abstract

Tissue engineering strategies show great potential for repairing osteochondral defects in osteoarthritic joints; however, these approaches often rely on passaging cells multiple times to obtain enough cells to produce functional tissue. Unfortunately, monolayer expansion culture causes chondrocyte dedifferentiation, which is accompanied by a phenotypical and morphological shift in chondrocyte properties that leads to a reduction in the quality of *de novo* cartilage produced. Thus, the objective of this study was to evaluate transcriptional variations during *in vitro* expansion culture and determine how differences in cell phenotype from monolayer expansion alters development of functional engineered cartilage. We used an unbiased approach to explore genome-wide transcriptional differences in chondrocyte phenotype at passage 1 (P1), P3, and P5, then seeded cells into hydrogel scaffolds at P3 and P5 to assess cells' ability to produce cartilaginous extracellular matrix in 3D. We identified distinct phenotypic differences, specifically for genes related to extracellular organization and cartilage development. Both P3 and P5 chondrocytes were able to produce chondrogenic tissue in 3D, with P3 cells producing matrix with greater compressive properties and P5 cells secreting matrix with higher glycosaminoglycan/DNA and collagen/DNA ratios. Furthermore, we identified 24 genes that were differentially expressed with passaging and enriched in human osteoarthritis genome-wide association studies (GWAS), thereby prioritizing them as functionally relevant targets to improve protocols that recapitulate functional healthy cartilage with cells from adult donors. Specifically, we identified novel genes, such as *TMEM190* and *RAB11FIP4* that were enriched with human hip OA and may play a role in chondrocyte dedifferentiation. This work lays the foundation for several pathways and genes that could be modulated to enhance the efficacy for chondrocyte culture for tissue regeneration, which could have transformative impacts for cell-based cartilage repair strategies.

Impact Statement

Biological repair strategies that rely on expansion culture of allogenic chondrocytes result in significant changes in cell behavior that impact tissue production. Findings from this study show how expansion culture causes chondrocytes from juvenile bovines, which are younger and healthier than human cell sources used in biological procedures such as MACI, to behave like cells from osteoarthritic cartilage. Furthermore, we prioritized several pathways and genes that could be modulated to improve the success of chondrocyte culture for tissue regeneration. These findings have important implications for the development of effective cell-based replacements for cartilage defects.

Keywords: Tissue engineering; cartilage regeneration; expansion culture; growth factor priming

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Competing interest statement: There are no competing interests to disclose.

Introduction

Osteoarthritis (OA) is the most prevalent joint disease, affecting over 530 million people globally.[1] In OA, articular cartilage in diarthrodial joints deteriorates, leading to joint stiffness and pain. The underlying cause of OA is a complex interplay of biomechanical, biochemical, and cellular factors among joint tissues, with age, obesity, sex, injury, and genetics among potential triggers and risk factors for the disease.[2,3]

Tissue engineering strategies show great potential for repairing osteochondral defects, with the aim of restoring joint function, reducing pain, and prolonging the need for total joint arthroplasty.[4] Matrix-induced autologous chondrocyte implantation (MACI) is a promising therapy that involves extracting autologous chondrocytes from a non-load bearing region of the knee and subsequently expanding cells *in vitro* to obtain enough cells for seeding onto a collagen scaffold. The scaffold is then placed into a cartilage defect to produce *de novo* tissue.[5,6] Expansion culture leads to chondrocyte dedifferentiation, characterized by a shift in cell behavior from producing predominately collagen type II and aggrecan to producing collagen type I and versican, respectively. Chondrocytes lose their normal rounded shape and acquire a flattened, fibroblastic morphology.[7] Furthermore, passaging affects the ability of chondrocytes to redifferentiate and reduces the quality of cartilage matrix produced, where dedifferentiated chondrocytes produce fibrocartilage, rather than the hyaline tissue of healthy, native cartilage.[8-10]

Similarities have been identified between OA chondrocytes and *in vitro* dedifferentiated chondrocytes.[11] For example, collagen type I and III, which are common in fibrous tissues, are markers for chondrocyte dedifferentiation and have greater deposition in OA tissue.[11-13] Additionally, chondrocytes in adult cartilage are typically in a quiescent state; however, chondrocytes in OA cartilage, have been shown to cluster, suggesting that the cells have re-

acquired the ability to proliferate.[14,15] The ability to proliferate is a shared property between chondrocytes in OA cartilage, dedifferentiated chondrocytes in monolayer expansion, and chondroprogenitor cells.[16] Lastly, the process of monolayer dedifferentiation is augmented by catabolic factors, including interleukin (IL)-1β, IL-6, and tumor necrosis factor-alpha (TNF-α), that also play a role in OA physiopathology.[11,17]

Pro-inflammatory cytokines alter chondrocyte gene expression during *in vivo* OA development and *in vitro* cell dedifferentiation. In addition to affecting chondrogenic phenotype, *in vitro* exposure to IL-1β and TNF-α increases cell spreading, expression of F-actin, and stress fiber formation.[18-20] These cytoskeletal changes also occur with chondrocyte dedifferentiation,[10,21] and our recent work showed priming chondrocytes with growth factors (transformation growth factor beta-1 (TGF-β1), basic fibroblast growth factor (bFGF) and platelet derived growth factor-ββ (PDGF-ββ) during monolayer expansion culture overrode these cytoskeletal/mechanobiological pathways at lower passages.[22] Based on these similarities, we hypothesized that cell signaling pathways between dedifferentiated chondrocytes from monolayer expansion culture overlap with behavior from chondrocytes from OA cartilage.

Thus, the objective of this study was to evaluate transcriptional variations during *in vitro* expansion culture and determine how differences in cell phenotype from monolayer expansion alters development of functional engineered cartilage. Additionally, this study aimed to identify transcriptional markers for chondrocyte dedifferentiation based on their role in human OA. To do so, we investigated differences in gene expression at passage 1, 3, and 5 (P1, P3, and P5, respectively) and cast cells following P3 and P5 to examine the capability of cells to redifferentiate when encapsulated in a hydrogel and produce chondrogenic matrix. We identified genes with functional roles in OA that may play a role in chondrocyte dedifferentiation. This was achieved

through unbiased computational prioritization of genes exhibiting differential expression with passaging and enriched in OA human genome-wide association studies (GWAS). Findings from this study provide valuable insights into how cellular phenotype influences cartilaginous tissue production and identifies potential markers for chondrocyte dedifferentiation, which may have important implications for developing effective *de novo* cartilage.

Materials and Methods

Articular cartilage was harvested from two juvenile bovine knee joints (n=1 cow; 3-6 weeks old). Tissue was digested overnight in 5% (v/v) fetal bovine serum (FBS) containing 0.15% type IV collagenase, as previously described.[22] Digested cells were filtered through a 70 μm filter and counted. Chondrocytes were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) FBS, 1% antibiotic- antimycotic, and 1% sodium bicarbonate to confluence (plating density = $13.3x10^6$). Media was changed 3 times/week and cells were cultured through P5.

Total RNA was isolated using TRIzol reagent, Qiagen RNeasy mini kit, and Qiagen DNase1 kit at P1, P3, and P5, according to the manufacturer's instructions (n=3 wells/group). RNA quantity was assessed using a Nanodrop Spectrophotometer and RNA quality was assessed using a Bioanalyzer (RNA integrity Number >9.7). Library preparation, RNA-sequencing, and data processing was done at UCSF Functional Genomics. 50-bp single end reads were sequenced using an Illumina HiSeq 4000 and sequencing provided 547 million total reads. Quality control of raw RNA-seq data was assessed using FastQC, reads containing adaptors and low-quality reads were removed, and reads were aligned to the Ensembl Cow ARS-UCD1.2.95 genome (STAR_2.4.2a). Differential expressed analysis was performed to determine changes in gene expression with passage number (DESeq2 package in R).[23] Adjusted p-values were calculated

using Benjamini-Hochberg false discovery fate (FDR) method and genes with FDR<0.01 were considered significant.

To identify biological processes and cellular components impacted during expansion culture, gene ontology enrichment analysis was performed using DAVID Bioinformatics Database (https://david.ncifcrf.gov/). Significant differentially expressed genes (DEGs) were examined to determine which genes were up and down-regulation between P1, P3, and P5. The top ten most significant terms were identified. The number of DEGs belonging to that gene ontology term was divided by the total number of down or upregulated DEGs for that passage combination, multiplied by 100 (reported as a percent).

Gene-set enrichment analysis was performed between RNA-seq results and datasets for knee OA, hip OA, knee and/or hip OA (hip-knee), and OA at any site identified using the UK Biobank GWAS.[24,25] First human homologs were identified for all bovine genes by converting all bovine Ensembl gene IDs to human Ensembl IDs (R package babelgene). Next, MAGMA was used to generate gene-based scores from GWAS results, which were calculated using the most significant variant located within a ±50kb range of the gene region (total non-coding region = 100kb).[26] Adjustments were made for potential confounders, such as gene size, gene density, and linkage disequilibrium between variants, using an adaptive permutation procedure before calculating p-values, as gene-based empirical p-values may differ from GWAS reported p-values.

To determine whether human homologs of bovine DEGs were associated with human OA, a one-sided test was performed using MAGMA's gene set enrichment to determine whether gene-based scores in a specific gene set were greater than all gene-based scores, as described.[27] The significance of gene-based scores within a gene set was examined, and the Bonferroni method was used to control for family-wise error rate.

Following P3 and P5, cells were encapsulated in 2% (w/v) Type VII agarose at a concentration of $38.7\pm6.1x10^6$ cells/mL. A biopsy punch was used to create cylindrical constructs with a diameter of 4mm and a thickness of 2.44 ± 0.04mm. Constructs were cultured in serum-free DMEM media with 0.1μM dexamethasone, 40mg/mL L-proline, 50mg/mL L-ascorbic acid, 100mg/mL sodium pyruvate solution, 1X ITS$^+$ pre-mix, and 1% antibiotic- antimycotic (1mL media/construct) for 35 days. Media was changed three times per week and was supplemented with 10ng/mL TGF-β3 for the first 14 days.[28]

The production of engineered tissue was assessed by evaluating mechanical and biochemical properties of constructs on day 35. Young's modulus was determined from a stress-relaxation test in unconfined compression (loading rate = 0.05%/sec, 10% strain, 30-minute hold). After mechanical testing, sample wet weight (ww) was measured, and samples were lyophilized overnight to determine dry weight (dw). Water content was calculated as the difference between wet and dry weights normalized by the wet weight (*i.e.*, (ww-dw)/ww). DNA content was determined using the PicoGreen dsDNA Assay kit (Invitrogen), and results were used to calculate the number of cells per construct. GAG and collagen content were determined using the 1,9-dimethylmethylene blue assay[29] and hydroxyproline assay (OHP), respectively. For the OHP assay, the ratio of hydroxyproline to collagen was assumed to be 7.64.[30] GAG and collagen contents were normalized by DNA and wet weight. A two-way ANOVA, with factors of days in culture and passage number, was used to examine differences in DNA/construct (n=6-8 constructs /group). A Tukey multiple comparisons post hoc was performed whenever statistical significance was achieved. Differences in biochemical and mechanical properties of constructs created with cells from P3 and P5 were compared using a two-tailed unpaired Student's t-test (n=8 constructs/

group). Unless otherwise specified, for all statistical analyses, a p-value of ≤ 0.05 was considered statistically significant.

Results

Unbiased RNA-sequencing showed that samples were clustered by passage using the first two principal components (PCs; Figure 1A). P1 cells were clustered furthest from P3 and P5 along PC1, suggesting a convergence with higher passage number. Additionally, P3 was grouped closer to P5, indicating that differences between passages may decrease after P1. Along PC2, there was less variance for P1 cells than P3 or P5 cells. Changes in gene expression were evaluated based on passage number and a similar number of significant DEGs (FDR<0.01) were identified between when comparing P1 cells to P3 (P3/P1; 1,505 DEGs) or P5 (P5/P1; 1,389 DEGs; Figure 1B). 488 DEGs were identified as significantly different when comparing P3 and P5 cells (P5/P3), which was ~70% fewer than comparisons between P1 cells and the latter two passages. 127 DEGs were common between all three passages (Figure 1C). The detailed results of DEGs based on passage are available in Supplemental Table 1.

Three DEGs (*POSTN, COL9A1,* and *CSGALNACT1*) were identified as being among the most significantly downregulated genes between each passage comparison, suggesting gene expression of these genes progressively decreased with passaging (Figure 1D). Six genes associated with cartilage development were identified as being significantly downregulated with passaging: *COMP, CTSK, CCN3, CSGALNACT1, COL2A1,* and *COL11A1*. *MASP1* was among the most significant DEGs upregulated at each passage comparison and had the most significant expression of all genes at P3 (Figure 1E). Approximately 40% of the top ten most significant genes upregulated during passaging are linked to immune response, including *ADAM8, MASP1, MX1, APOA1, CTSH, CTSK, CFB,* and *IFI6* (Figure 1E). Lastly, *CTSK, GPNMP,* and *ACTA2,* were

identified among the most significantly upregulated DEGs between P3/P1, and the most downregulated DEGs between P5/P3, meaning they had the greatest expression at P3.

Gene ontology (GO) analysis assessed biological processes and cellular components associated with DEGs (Figure 2). DEGs downregulated with passaging were enriched for extracellular organization and skeletal system development for all passage combinations (Figure 2A). Additionally, DEGs downregulated between P5/P1 and P5/P3 were associated with connective tissue development (Figure 2A - blue line). Biological processes upregulated in P5/P1 and P5/P3 were mainly associated with cell migration (Figure 2B). DEGs upregulated between P3/P1 and downregulated between P5/P3 were associated with cell adhesion (Figure 2A-2B). When comparing cellular components associated with DEGs, DEGs downregulated between P3/P1 were enriched for nucleus and endoplasmic reticulum, and with the biological process, cholesterol biosynthesis (Figure 2C, green and orange lines). Interestingly, proteinaceous extracellular matrix was among the most significant cellular components for down- and upregulated DEGs between P5/P1 and P5/P3. Focal adhesions and cell substrate junctions were enriched at P3 and P5 compared to P1 (Figure 2D - purple line).

To gain insights into how transcriptional variations during monolayer expansion led to variations in tissue production, we examined how differential expression of genes related to extracellular matrix organization changed with passaging. 98 DEGs were identified as being significant in at least one passage combination (Figure 3). Next, we compared DEGs related to cartilage development that showed significant differences with passage. We identified 93 genes that were significantly different between at least one combination of passages (Figure 4). For both analyses, the greatest differences in gene expression were noted between P1 and P5. Since cells were cast for 3D culture at P3 and P5, we also separately examined significantly different gene

expression between only these passages, which noted 38 genes associated with extracellular matrix organization and 32 genes associated with cartilage development (Figure S1).

Human homologs were identified for genes differentially expressed during monolayer expansion culture of bovine chondrocytes for all passage combinations. Using MAGMA gene set enrichment analysis, all sets of differentially expressed genes were enriched for human hip and hip-knee OA (Table 1, Figure 5). However, none of the data sets were significantly enriched for knee or OA at any site (Figure 5C, D). Within the significantly enriched gene sets, the highest number of human homologs from the P3/P1 dataset were significantly associated with hip (13) and hip-knee OA (11; Table 1). All human homologs of bovine DEGs analyzed and the MAGMA results for hip and knee-hip OA can be found in Supplemental Table 2 and 3, respectively. Bovine genes associated with human hip and hip-knee OA were visualized with volcano plots for all passage combinations (Figure 6A-C). *COL11A1, FGFR3, NISCH, TWF2, PPM1M, MFHAS1, LMX1B* were common to both OA datasets (Figure 6A-C black text). A few DEGs were identified in multiple passage combinations, including *COL11A1, TMEM190, RAB11FIP4, COLGALT2, CDKAP1, GDF5, TNC, ARHGAP27, PPP1RB, RGL1.* In addition, all six bovine DEGs linked to human OA between P5/P3 were downregulated with passaging (Figure 6C). Heatmap analysis showed clear clustering between P1 compared to P3 and P5 for all DEGs (Figure 6D). *COL11A1 and RAB11FIP4* exhibited a distinct decrease in gene expression with passaging. Out the 24 DEGs identified to be associated with human OA, 10 genes are involved in the cell surface receptor signaling pathway (*APPL2, COL2A1, ECM1, FGFR3, GDF5, LTBP1, LTBP3, PPM1M, TGFB1, TSKU*), and these genes were also upregulated in P5 cells when compared to P3 cells (Figure 2B).

Despite efforts to maintain consistent seeding density between groups, P3 constructs had a lower initial seeding density compared to P5 constructs (day 1 DNA: P3= 8.14±1.36μg; P5=

10.11±0.73μg; t-test p=0.03; Figure 7A). P3 constructs showed a consistent increase in DNA content through day 14 and plateaued after TGFβ3 supplementation ended. In contrast, DNA content of P5 constructs continued to increase through day 35. At day 35, there were no differences in construct water content (p=0.056; Figure 7B). However, the swelling ratio of P5 constructs was 22% greater than P3 constructs (p<0.0001; Figure 7C), which we hypothesize to be due to greater dry weights for P5 constructs compared to P3 constructs (P3=6.0±0.2mg; P5=7.6±0.3mg). Greater GAG accumulation was observed in P5 constructs when normalized to DNA content (p=0.0056; Figure 7D), but GAG content normalized to wet weight was not significantly different (p=0.18; Figure 7E). In agreement with GAG production, P5 constructs had greater collagen content when normalized to DNA content (p=0.0016) but not wet weight (p=0.14; Figures 7F-G). The equilibrium modulus of P3 constructs was 26% greater than P5 constructs (p=0.0018; Figure 7H).

Discussion

Previous studies examined differences in gene expression during monolayer expansion; however, how changes in gene expression relate to differences in 3D tissue production are not well understood. Thus, we employed an unbiased approach to explore genome-wide transcriptional differences in chondrocyte phenotype at P1, P3, and P5. Subsequently, we embedded cells in agarose at P3 and P5 to assess cells' ability to produce extracellular matrix. Our analysis revealed distinct phenotypical differences, specifically for genes related to extracellular matrix organization and cartilage development. Both P3 and P5 chondrocytes demonstrated the ability to produce chondrogenic tissue in 3D culture. Notably, P3 cells producing matrix with greater compressive mechanical properties and P5 cells secreted matrix with higher GAG/DNA and collagen/DNA ratios. Furthermore, we identified 24 genes that were differentially expressed with passaging and enriched in human GWAS OA studies, which may prioritize them for additional functional studies

to improve protocols to recapitulate healthy functional cartilage with cells from adult donors for clinical procedures such as MACI.

Our findings revealed a significant increase in cell substrate adhesion and greater F-actin stress fiber formation between P1 and later passages, which are known markers of chondrocyte dedifferentiation during *in vitro* expansion culture.[10,21,22,31] Specifically, gene ontology analysis findings showed an upregulation of cell-substrate junction pathways in P3 and P5 cells compared to P1, which is consistent with dedifferentiated chondrocytes having greater adhesion to plastic. Pathways for actin cytoskeletal organization were significantly upregulated by P3 (*e.g.*, Figure 2B for P3), with no differences in actin formation between P3 and P5 cells. These results suggest that mechanosensitive cytoskeletal remodeling may have taken place by P3 as the cells respond to the stiff, 2D culture environment.

RNAseq results from 2D expansion culture provide some insight into molecular variations that may contribute to differences in construct behavior. Our findings revealed significant differences in the gene expression of collagens and proteoglycans, cartilage's primary structural components, between chondrocytes at P3 and P5. Consistent with previous literature,[8] we observed greater *COL2A1* expression at P3 than P5; however, no significant differences were observed in gene expression of fibrocartilage markers, *COL1A1* or *COL3A1*. Gene expression of *COL9A1*, which stabilizes cartilage matrix by connecting collagen fibrils, such as *COL2A1*, to other non-collagenic matrix components, such as cartilage oligomeric matrix protein (COMP),[32,33] was among the most significantly downregulated genes for each passage combination. Additionally, we identified significant differences in the expression of genes regulating the collagen metabolic processes, including *MMP2*, *MMP13*, *MMP28*, and *TNXB*. Expression of *MMP13* is of particular interest as it has a predominant role in OA, degrading both collagen and aggrecan.[34] Conflicting

results have been found regarding *MMP13* expression during expansion culture.[11,35] However, our findings show greater expression of *MMP13* at P3, which corresponded to the production of tissue with lower collagen/DNA content ratio when compared to P5 constructs. While these findings are interesting, future RNAseq studies on the constructs are needed to fully understand the relationship between the gene expression during 2D culture and the 3D tissue production.

Further analysis of differences in gene expression between P3 and P5, the passages at which cells were cast for 3D culture, revealed no significant differences in gene expression of aggrecan *(ACAN)* or versican *(VCAN)*, which are markers of differentiated and dedifferentiated chondrocytes, respectively. However, we found an upregulation of the proteoglycans, agrin *(AGRN)* and heparin sulfate proteoglycan 2 *(HSPG2)*, with greater expression at P5 compared to P3. *AGRN* is expressed in healthy cartilage and downregulated with OA,[36] while *HSPG2* is essential for cartilage development and plays an important role in collagen fibril and GAG organization.[37] P5 cells, which had greater expression of *AGRN* and *HSPG2*, produced greater GAG/DNA than P3 cells, but further investigation is needed to determine whether these genes played a role in the increased GAG accumulation in 3D culture.

In this study, chondrocytes passaged both to P3 and P5 demonstrated the ability to produce engineered tissue containing collagen and GAG in 3D culture; however, unexpected results were observed related to the passage number with greater tissue production. Difference in GAG and collagen content based on passage were found only when normalizing to DNA content, not wet weight. P5 constructs exhibited greater protein accumulation with normalized to DNA. Interestingly, despite the P5 constructs showing greater cartilaginous protein to DNA accumulation, P3 constructs had a greater compressive modulus. These results were surprising for a couple reasons. Firstly, given the understanding that water molecules are attracted to negative

charges on GAG molecules, it would be expected the passage with constructs containing greater GAG would also have increased compressive properties. An explanation for these inconsistencies may lie in the quality and type of proteoglycans or collagens produced, which would require more specific protein expression analysis. Secondly, prior research has yielded inconsistent findings regarding whether chondrocytes become irreversibly dedifferentiated during monolayer expansion, limiting the ability of chondrocytes at higher passages to produce *de novo* tissues. These variations in chondrocyte redifferentiation capabilities may be attributed to species variations. For example, human chondrocytes at higher passages (beyond P4) have exhibited a limited ability to generate *de novo* tissue.[38-42] Conflicting results also exist for chondrocytes sourced from juvenile animals, where some studies showed that chondrocytes were able to redifferentiate to produce cartilaginous tissue at higher passages,[43,44] while others have not. Our results support that bovine chondrocytes could produce engineered tissue at passage 5, even exhibiting greater GAG/DNA and collagen/DNA than constructs at P3. Future work is imperative to further understand the chondrogenic quality of the tissue and determine if these discrepancies observed between chondrocytes ability to produce engineered tissue at higher passages is species or age specific.

By analyzing both differences in gene expression during expansion culture and bulk differences in tissue production, we identified key genes that could potentially be modulated to improve success of chondrocyte culture for tissue regeneration. Our findings showed that secreted cell adhesion protein, periostin *(POSTN),* was among the most significant downregulated gene at each passage combination, suggesting the expression of *POSTN* decreases with chondrocyte dedifferentiation. *POSTN* has been identified as an anti-adhesion molecule, counteracting the adhesion functions of fibronectin and collagen type I[45] and is thought to promote cell proliferation

and migration.[46] Our previous work showed that *POSTN* was upregulated in cells primed with growth factors during expansion cultured compared to cells cultured in serum only media, and that primed cells exhibited reduced cell adhesion and greater cell proliferation.[22] To our knowledge, *POSTN* has not been previously linked to chondrocyte dedifferentiation and may play an important role in cell adhesion or proliferation during expansion culture.

Another notable gene identified that should be further investigated for its role in chondrocyte dedifferentiation and tissue regeneration is TMEM190, which was among the top ten most significant downregulated genes at P3 compared to P1 and was shown to be associated with human hip-knee OA. Previously, *TMEM190* was identified as one of the most significantly downregulated genes between adult primary human nucleus pulposus cells cultured on soft substrate (~0.5kPa) and cells cultured on stiffer polystyrene (~1GPa).[47] Many similarities exist between nucleus pulposus cells and chondrocytes, including a shift towards a fibroblastic-like morphology when cultured on stiff tissue culture plastic and maintenance of gene expression profiles when cultured on soft substrates.[48] Findings from our study showed that *TMEM190* expression increased with passaging, which was associated with increased adhesion, cell spreading, F-actin stress fiber development, and human hip OA. *TMEM190* was also identified in single cell-sequencing of cells from patients undergoing knee arthroplasty.[49] Taken together, these results suggest *TMEM190* may be associated with cellular response to matrix stiffness and play an important role in chondrocyte dedifferentiation or OA.

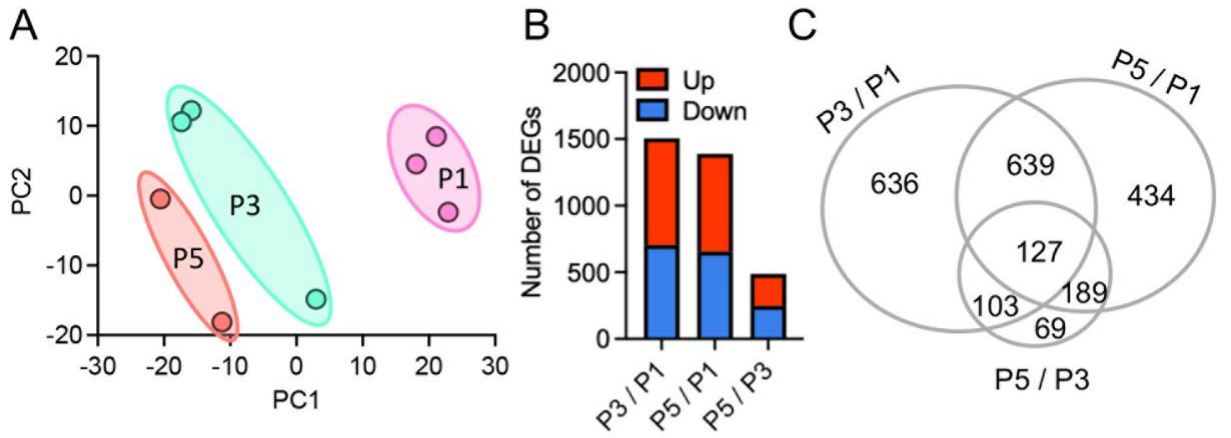
While analysis of the most up or downregulated genes, or of well-known genes in chondrocyte differentiation or OA is informative, our 'bovine to human' enrichment analysis additionally prioritized genes with both established and understudied functions in chondrocytes. For example, collagen type XI alpha 1 chain *(COL11A1)* and RAB11family interacting protein 4

(RAB11FIP4) are enriched with passaging and associated with hip-knee OA. *COL11A1* is associated with cartilage specific extracellular matrix, and several GWAS studies have linked SNPs in *COL11A1* to OA.[50,51] *RAB11FIP4* is a member of the Rab11 family interacting proteins and plays a crucial role in many physiological functions, including proliferation, differentiation, and immunity.[52,53] Furthermore, alterations in *RAB11FIP4* expression in chondrocytes were previously found in a study examining genome-wide DNA methylation profiles between human primary chondrocytes expanded in 2D monolayer culture and then cultured in 3D, compared to their respective autologous, macroscopically unaffected cartilage. Bomer et al. found transcriptionally active hyper methylation of a *RAB11FIP4* annotated differentially methylated region in cartilage grown from expanded primary human chondrocytes compared to the control cartilage.[54] Specifically, a negative correlation between DNA methylation and *RAB11FIP4* expression was observed. Consistently, our analysis identified decreased *RAB11FIP4* expression with culture, suggesting that it may be a transcriptional marker for chondrocyte dedifferentiation and should be further investigated to better understand its functional roles in chondrocytes and in OA.

In summary, this study investigated differences in gene expression of chondrocytes from juvenile bovines during expansion culture and engineered cartilage production of these cells in 3D scaffolds. Findings from this study suggest that many known genes associated with chondrocyte dedifferentiation and OA development were enriched during passaging; however, chondrocytes were still able to produce *de novo* engineered cartilaginous tissue. Furthermore, we prioritized genes that are differentially expressed with passaging, such as *TMEM190* and *RAB11FIP4*, that are enriched with hip-related OA and may play an important role in chondrocyte dedifferentiation.

Therefore, this work established the groundwork for investigating several pathways and genes that could be manipulated during expansion culture to enhance engineered cartilage tissue production.

Figures and Tables



D

Top 10 downregulated genes with passaging

Gene	P3 / P1 log2FC	P5 / P1 log2FC	P5 / P3 log2FC
COL2A1	-2.34	-3.29	
LIF	-3.49		
POSTN	-1.45	-2.67	-1.22
MELTF	-1.47		
COL9A1	-2.64	-4.86	-2.22
TMEM190	-3.64		
COL11A2	-3.04	-4.95	
COL8A1	-1.01		
CSGALNACT1	-1.53	-3.10	-1.56
COL9A2	-3.09		
F13A1		-5.11	-3.53
DES		-3.38	
LOXL4		-1.95	-1.08
SUSD5		-1.62	
COMP		-2.54	
CTSK			-1.29
THBS4			-2.78
ACTA2			-1.52
GPNMB			-2.04
CCN3			-1.21

All FDR < 3×10^{-16}

E

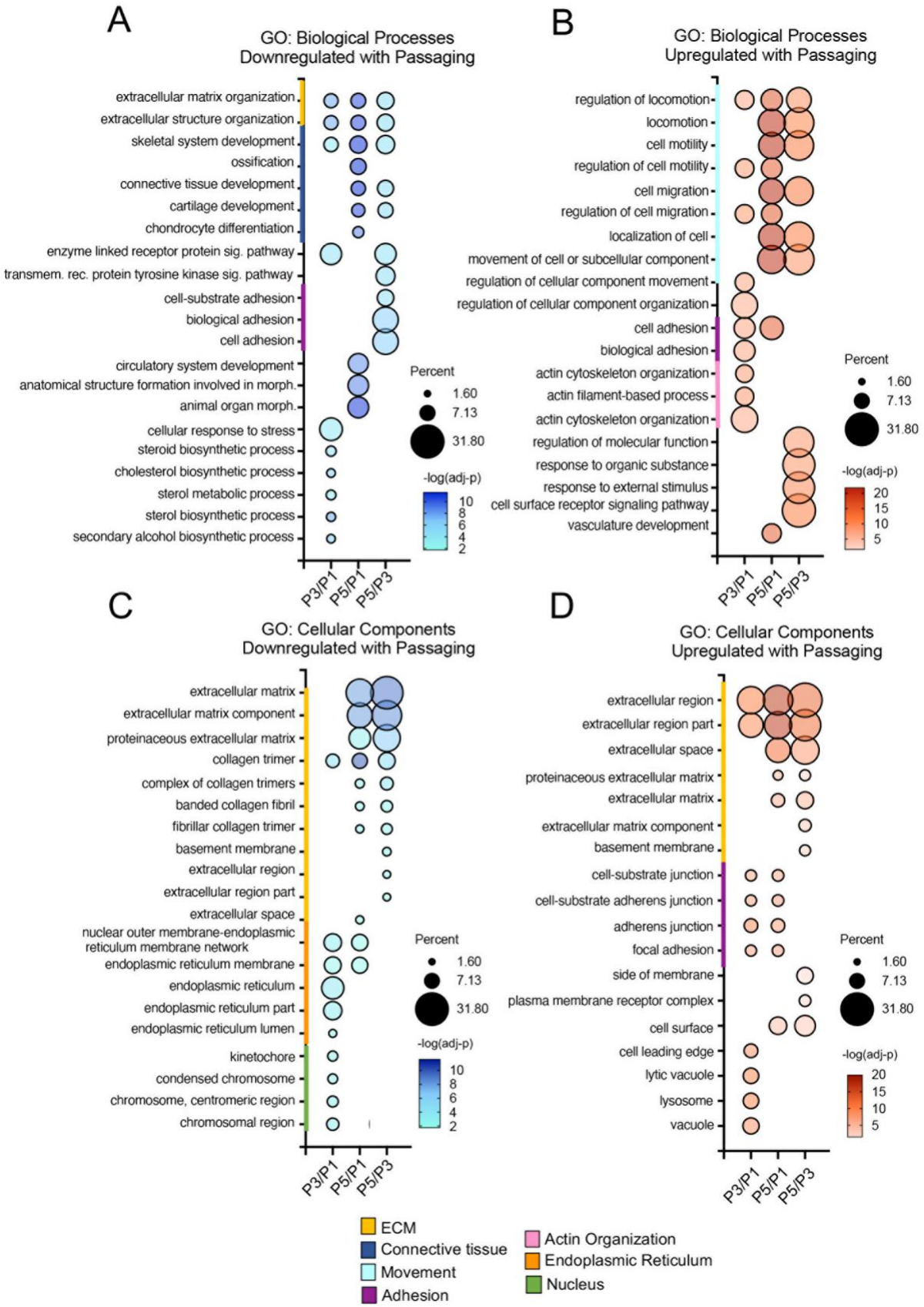
Top 10 upregulated genes with passaging

Gene	P3 / P1 log2FC	P5 / P1 log2FC	P5 / P3 log2FC
CTSK	1.97		
DKK3	1.90	2.01	
GPNMB	3.05		
CTSH	1.51		
ACTA2	2.13		
ACTG2	2.48		
MASP1	1.94	3.40	1.47
MMP2	1.02	1.41	
THBS3	1.55		
PODN	3.72		
F5		3.06	2.06
CFB		5.61	2.95
PAX3		3.80	2.55
ADGRB1		2.80	2.12
XDH		2.04	
IFI6		4.12	2.82
MX1		4.18	
SERPINE2			1.63
ADAM8			2.34
APOA1			1.41

All FDR < 2×10^{-26}

Figure 1: *Unbiased RNA-sequencing results for chondrocytes for passages 1, 3, and 5 (P1, P3, P5, respectively).* ***(A)*** *Principal component analysis shows separation based on passage number.* ***(B)*** *Significant DEGs downregulated (blue) and upregulated (red) for each passage combination (FDR<0.01).* ***(C)*** *Venn diagram showing the number of common and unique DEGs. Top ten most significantly* ***(D)***

*downregulated and **(E)** upregulated genes for each passage combination based on FDR value shown at the bottom of the table. Values in the columns denote log2(fold-change).*


A
GO: Biological Processes
Downregulated with Passaging
extracellular matrix organization
extracellular structure organization
skeletal system development
ossification
connective tissue development
cartilage development
chondrocyte differentiation
enzyme linked receptor protein sig. pathway
transmem. rec. protein tyrosine kinase sig. pathway
cell-substrate adhesion
biological adhesion
cell adhesion
circulatory system development
anatomical structure formation involved in morph.
animal organ morph.
cellular response to stress
steroid biosynthetic process
cholesterol biosynthetic process
sterol metabolic process
sterol biosynthetic process
secondary alcohol biosynthetic process
P3/P1
P5/P1
P5/P3
Percent
1.60
7.13
31.80
-log(adj-p)
10
8
6
4
2
B
GO: Biological Processes
Upregulated with Passaging
regulation of locomotion
locomotion
cell motility
regulation of cell motility
cell migration
regulation of cell migration
localization of cell
movement of cell or subcellular component
regulation of cellular component movement
regulation of cellular component organization
cell adhesion
biological adhesion
actin cytoskeleton organization
actin filament-based process
actin cytoskeleton organization
regulation of molecular function
response to organic substance
response to external stimulus
cell surface receptor signaling pathway
vasculature development
P3/P1
P5/P1
P5/P3
Percent
1.60
7.13
31.80
-log(adj-p)
20
15
10
5
C
GO: Cellular Components
Downregulated with Passaging
extracellular matrix
extracellular matrix component
proteinaceous extracellular matrix
collagen trimer
complex of collagen trimers
banded collagen fibril
fibrillar collagen trimer
basement membrane
extracellular region
extracellular region part
extracellular space
nuclear outer membrane-endoplasmic reticulum membrane network
endoplasmic reticulum membrane
endoplasmic reticulum
endoplasmic reticulum part
endoplasmic reticulum lumen
kinetochore
condensed chromosome
chromosome, centromeric region
chromosomal region
P3/P1
P5/P1
P5/P3
Percent
1.60
7.13
31.80
-log(adj-p)
10
8
6
4
2
D
GO: Cellular Components
Upregulated with Passaging
extracellular region
extracellular region part
extracellular space
proteinaceous extracellular matrix
extracellular matrix
extracellular matrix component
basement membrane
cell-substrate junction
cell-substrate adherens junction
adherens junction
focal adhesion
side of membrane
plasma membrane receptor complex
cell surface
cell leading edge
lytic vacuole
lysosome
vacuole
P3/P1
P5/P1
P5/P3
Percent
1.60
7.13
31.80
-log(adj-p)
20
15
10
5
ECM
Connective tissue
Movement
Adhesion
Actin Organization
Endoplasmic Reticulum
Nucleus

1

Figure 2: *Gene ontology (GO) enrichment analysis of DEGs based on passage number. Dot plots show top ten most significant biological processes and cellular components downregulated* ***(A, C)*** *and upregulated* ***(B, D)*** *based on passage number. Colored labels on y-axis show grouping for clarity. Dot size (percent)represents the ratio of genes in the gene ontology term divided by the total number of DEGs downregulated or upregulated for that passage combination, multiplied by 100. Sig. = signaling. Morph= morphogenesis. Transmem. rec. = transmembrane receptor.*

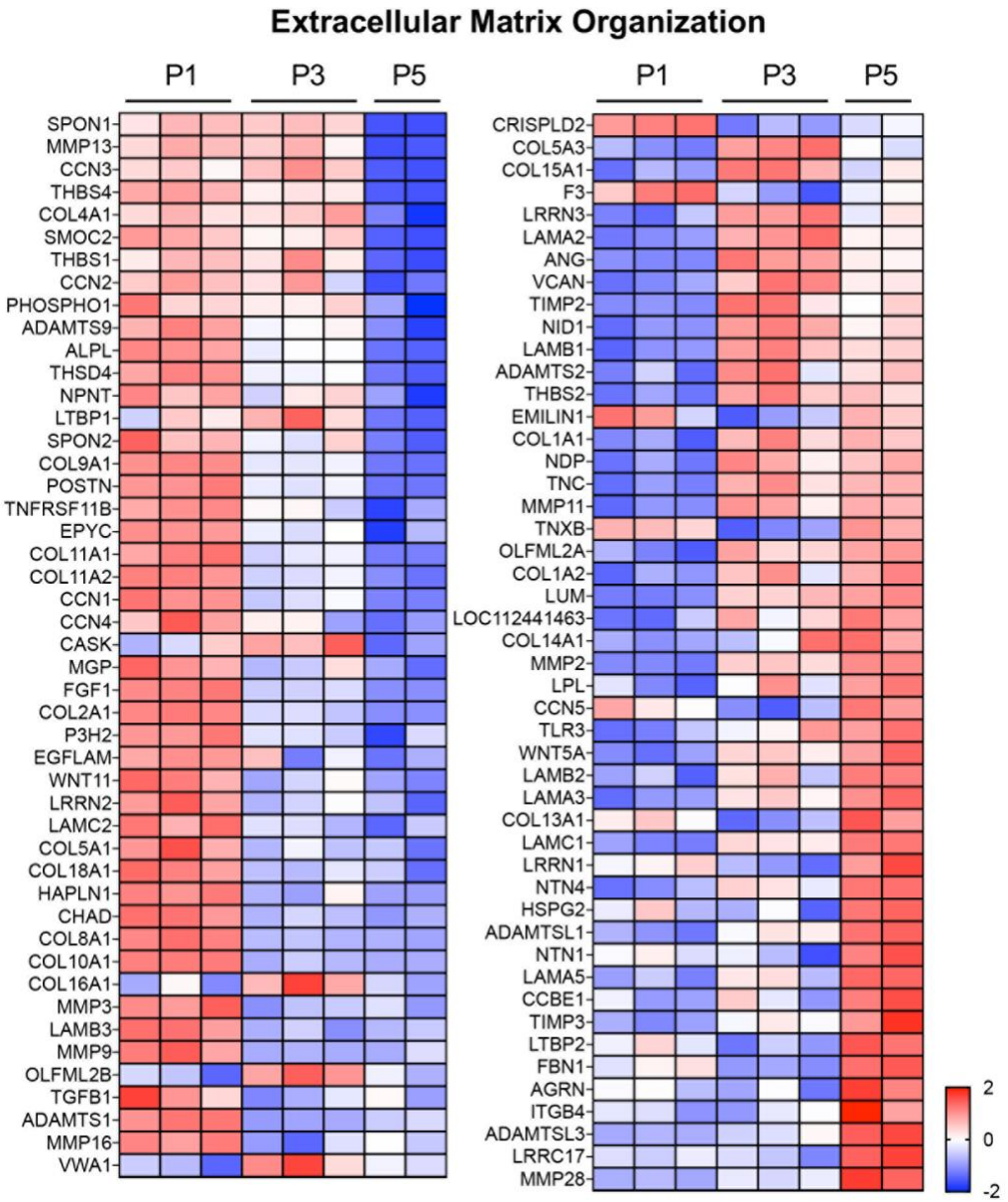


Figure 3: *Upregulated (red) and downregulated (blue) DEGs related to the gene ontology term "extracellular matrix organization" based on passage number. Heatmap illustrating DEGs significant in at least one passage combination. Heatmaps show the z-scores of gene expression patterns for each DEG compared to all samples in that row, rows represent DEGs, and columns represent samples.*

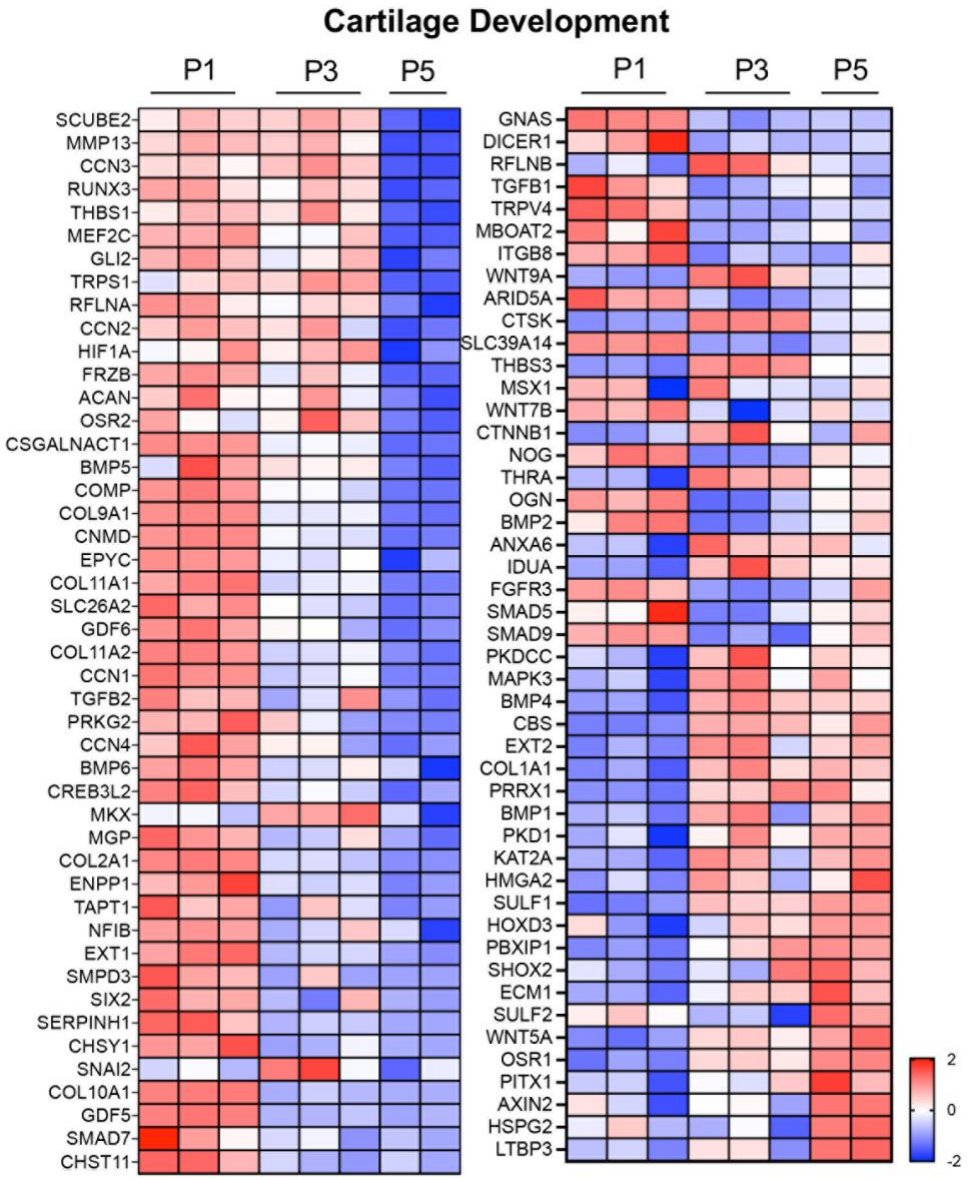


Figure 4. *Heatmaps illustrating upregulated (red) and downregulated (blue) DEGs related to the gene ontology term for "cartilage development" in at least one passage combination. Heatmaps show the z-scores of gene expression for each DEG compared to all samples in that row, rows represent DEGs, and columns represent individual samples.*

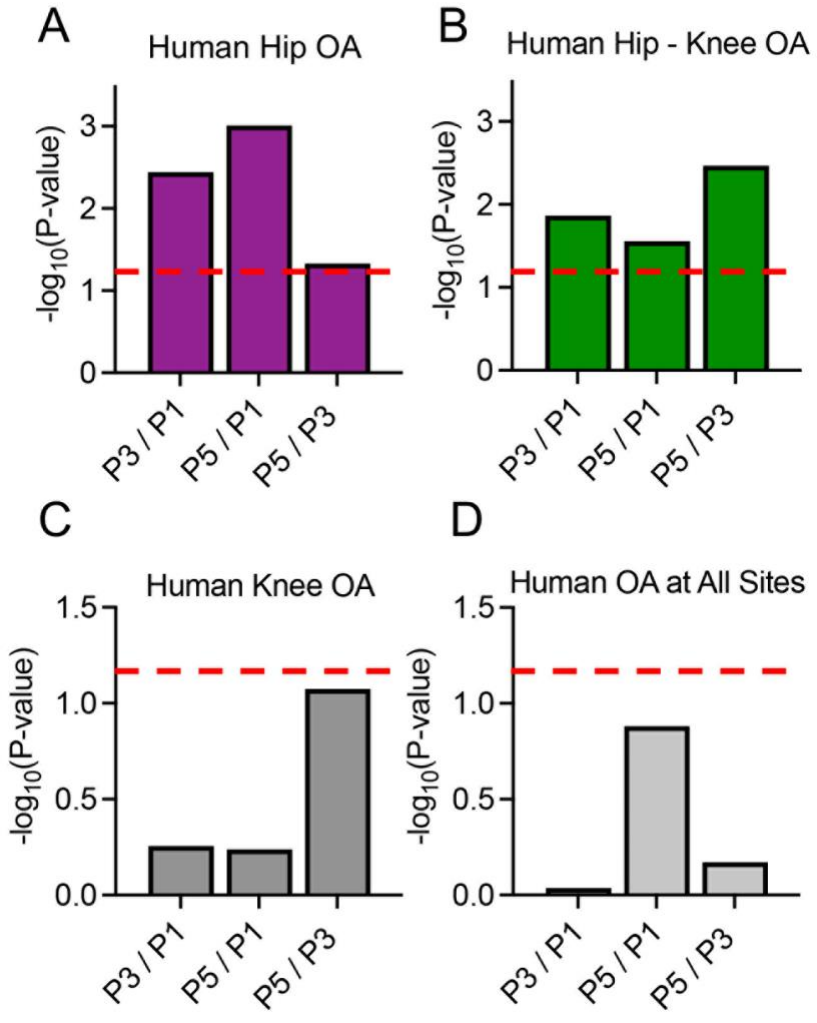


Figure 5: *Gene set enrichment analysis was performed using DEGs from bovine RNA-seq data along with data from UK Biobank and arcOGEN GWAS. The bovine DEGs (FDR<0.01) were enriched for human* ***(A)*** *hip OA and* ***(B)*** *hip-knee OA across all passage combinations. However, human* ***(C)*** *knee OA and* ***(D)*** *OA at all sites did not reach significance. The red dashed line represents the threshold for significance (p<0.05).*

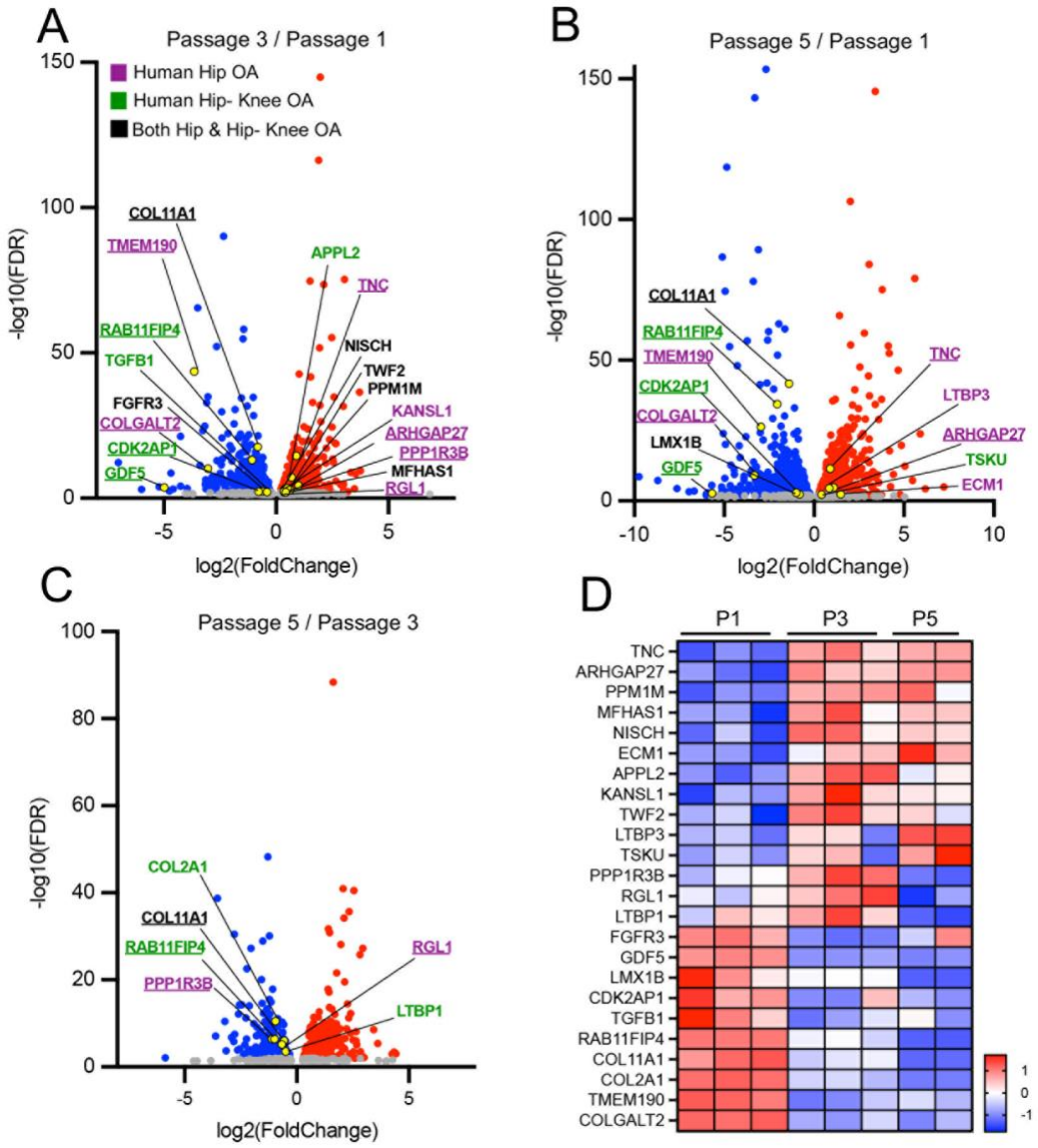


Figure 6: *Significant human OA associated genes from bovine dataset. Volcano plots show bovine homolog of significant upregulated (red) and downregulated (blue) OA-associated DEGs in* ***(A)*** *P3 compared to P1,* ***(B)*** *P5 compared to P1, and* ***(C)*** *P5 compared to P3. Grey dots represent non-significant DEGs (FDR<0.01). The color of the gene name represents whether the gene is enriched for human hip OA (purple), human hip-knee OA (green), or both hip and hip-knee OA (black). Underlined gene names represent genes that are significant in more than one passage combination.* ***(D)*** *Heatmap analysis of bovine DEGs that were enriched for at least one type of OA in at least one passage comparison. Z-scores for each*

DEG compared to all samples in that row (blue= downregulated; red= upregulated). Rows represent DEGs and columns represent samples.

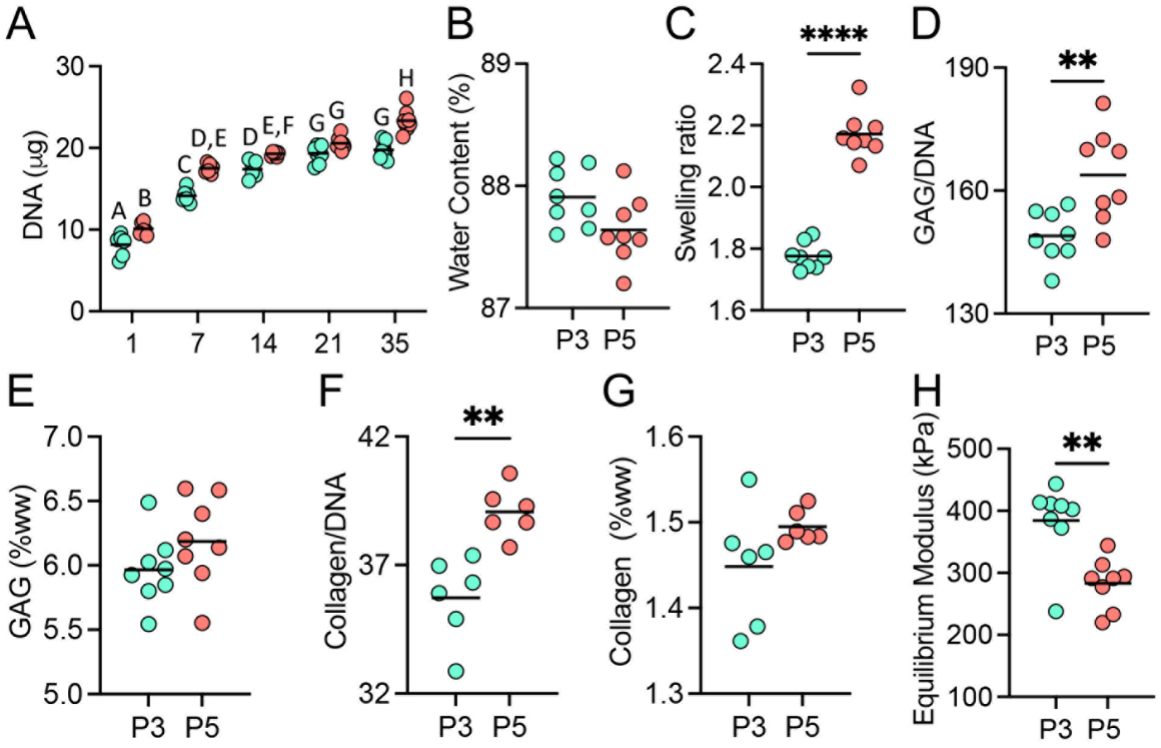
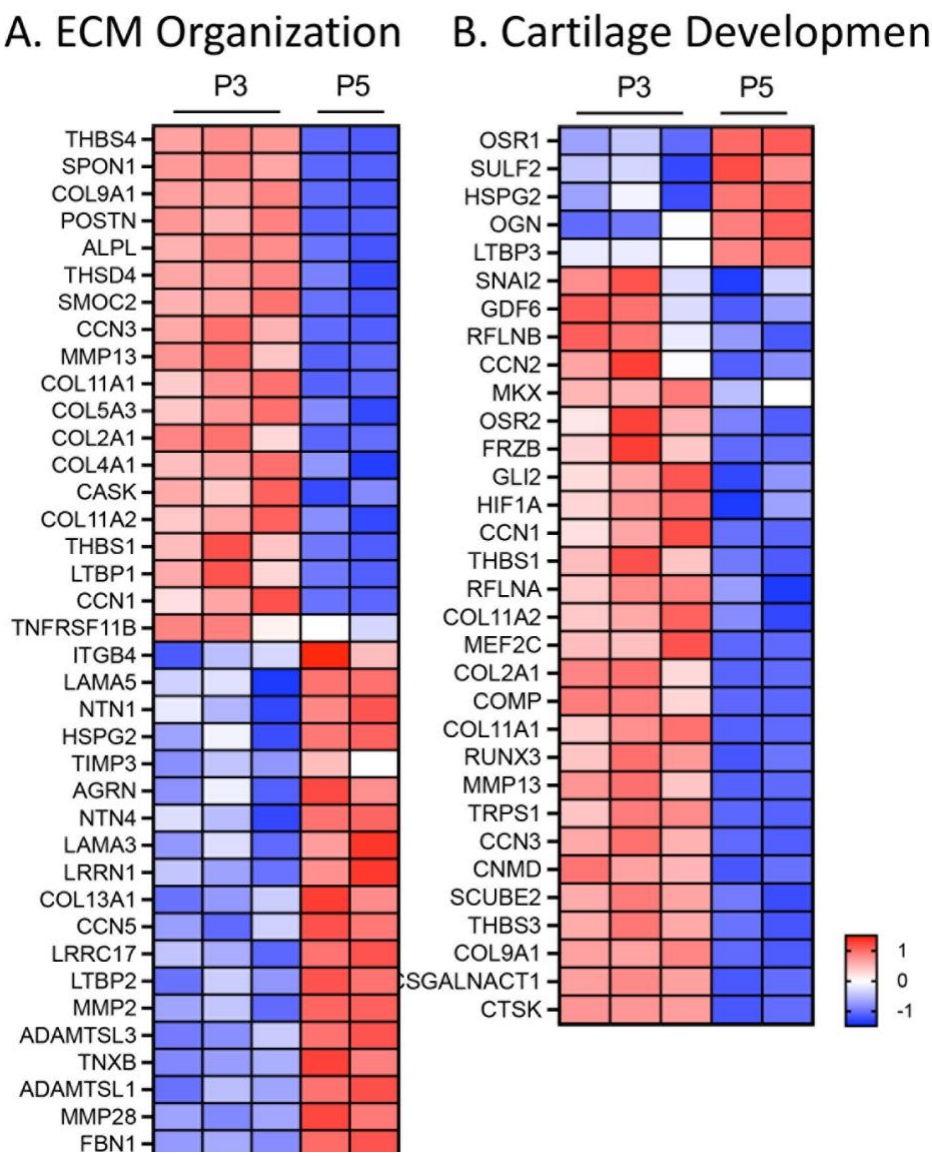


Figure 7: *Engineered tissue production of P3 (teal) and P5 (orange) constructs after 35 days of 3D culture.* ***(A)*** *DNA per construct was measured weekly until day 35. Groups connected by the same letter are not statistically different.* ***(B-H)*** *Biochemical content and mechanical properties measured after 35 days in culture. **p<0.01, ****p<0.0001.*



Supplemental Figure S1: *Heatmap illustrating significant DEGs between P3 and P5 for the gene ontology term* ***(A)*** *"extracellular matrix organization" and* ***(B)*** *"cartilage development". Heatmaps show the z-scores of gene expression patterns for each DEG compared to all samples in that row, rows represent DEGs, and columns represent samples.*

Passaging Gene Set	# Bovine DEGs	# Genes in MAGMA analysis	Hip OA		Hip- Knee OA	
			MAGMA enriched p-value	# MAGMA sig. genes	MAGMA enriched p- value	# MAGMA sig. genes
P3 / P1	1505	1470	3.6×10^{-3}	13	0.014	11
P5 /P1	1389	1354	9.8×10^{-4}	8	0.028	6
P5 / P3	488	480	0.046	3	3.4×10^{-3}	4

Table 1: *MAGMA UK Biobank GWAS enrichment results for human hip OA and hip-knee OA based on bovine gene expression data sets. Sig = significant*

Supplemental Table 1: *Bovine RNA-sequencing differentially expressed gene analysis for chondrocytes at three passage combinations, P1-P3, P1-P5, and P3-P5. Excel sheets are separated based on passage combinations and DEGs with FDR<0.05 are included.*

Supplemental Table 2: *MAGMA gene-based scores of human hip OA GWAS for each bovine DEG gene set (P1-P3, P1-P5, P3-P5). Results for each passaging gene set are presented in separate sheets. Yellow rows highlighted show the significant hip OA associated genes ($p \leq 0.05$).*

Supplemental Table 3: *MAGMA gene-based scores of human hip-knee OA GWAS for each bovine DEG gene set based on passage number. Yellow rows highlighted show the significant knee-hip OA associated genes ($p \leq 0.05$).*

1

2

3

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