Metabolomic Profiling and Characterization of a Novel 3D Culture System for Studying Chondrocyte Mechanotransduction

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Abstract

Articular chondrocytes synthesize and maintain the avascular and aneural articular cartilage. *In vivo* these cells are surrounded by a 3D pericellular matrix (PCM) containing predominantly collagen VI. The PCM protects chondrocytes and facilitates mechanotransduction, and PCM stiffness is critical in transmitting biomechanical signals to chondrocytes. Various culture systems with different hydrogels have been used to encapsulate chondrocytes for 3D culture, but many lack either the PCM or the *in vivo* stiffness of the cartilage matrix. Here, we demonstrate that primary chondrocytes cultured in alginate will form a pericellular matrix and display a phenotype similar to *in vivo* conditions. We found that primary human and bovine chondrocytes, when cultured in alginate beads with addition of sodium L-ascorbate for 7 days, had a pronounced PCM, retained their phenotype, and synthesized both collagens VI and II. This novel culture system enables alginate-encapsulated chondrocytes to develop a robust PCM thereby creating a model system to study mechanotransduction. We also observed distinct compression-induced changes in metabolomic profiles between the monolayer-agarose and alginate-released agarose-embedded chondrocytes indicating physiological changes in cell metabolism. Our data suggest that 3D preculture of chondrocytes in alginate before encapsulation in physiologically-stiff agarose leads to a pronounced development of pericellular matrix that is sustained in the presence of ascorbate. This novel model can be useful in studying the mechanism by which chondrocytes respond to cyclical compression and other types of loading simulating *in vivo* physiological conditions.

Keywords:

Chondrocytes, alginate hydrogel, pericellular matrix, mechanotransduction, metabolomics, osteoarthritis.

Introduction

Osteoarthritis (OA), the most common form of arthritis, is a chronic degenerative joint condition affecting more than 37% of people over the age of 60. It is a leading cause of pain and disability resulting in considerable productivity loss and healthcare expenditure [1]. Pathological changes in OA include progressive degradation and loss of articular cartilage, thickening of the subchondral bone, inflammation of the synovium, degeneration of ligaments and hypertrophy of the joint capsule [2]. What was once considered a simple “wear and tear” condition, is now known to be a disease of the whole joint with low-grade inflammation and other biological mechanisms playing a role in disease progression [3, 4].

The articular cartilage that lines diarthrodial joints is primarily composed of the extracellular matrix (ECM) and the pericellular matrix (PCM). Specialized cells of the cartilage, the chondrocytes, occupy 3-5% of tissue volume in adult human cartilage. They synthesize and maintain the avascular and aneural cartilage and are surrounded by a 3D pericellular matrix that facilitates mechanotransduction. These cells also maintain a functional extracellular matrix by synthesis and assembly of the collagen fibers, proteoglycans, and other matrix molecules [5, 6]. During regular joint loading *in vivo*, cartilage is subjected to mechanical stresses that can exceed 10 megapascals [7]. However, the chondrocytes in healthy cartilage are protected from these mechanical loads by the PCM in contrast to OA chondrocytes, which have a softer PCM. It is known that chondrocytes can sense and respond to mechanical stimuli, but the mechanisms by which this mechanotransduction occurs are not fully understood [8, 9]. Understanding the basic processes involved in chondrocyte response to mechanical loads will provide valuable insights for the treatment of osteoarthritis.

Various culture systems with different hydrogels have been developed to encapsulate chondrocytes and study chondrocyte mechanotransduction, but many lack either the PCM or the *in vivo* stiffness of the cartilage matrix [10-12]. These have stiffness values ranging from 5-10 kPa which is lower than the *in vivo* PCM stiffness surrounding the chondrocyte [13]. Physiological stiffness is important since it directly

transmits mechanical information to chondrocytes that drives biological responses via cellular mechanotransduction. Studies show that cyclical compression can upregulate transcription of ECM genes depending on the compression amplitude and frequency [14, 15]. Similarly, mechanical forces also play a role in gene expression of aggrecan and several other matrix proteins [16, 17]. PCM stiffness in healthy adult chondrocytes is in the range of 25-200 kPa, which significantly decreases in OA [18, 19]. Several studies find that chondrocytes when cultured in alginate beads will form a pericellular matrix and display a phenotype similar like *in vivo* conditions [20]. The alginate gel can also be solubilized using chelating agents to release the resulting chondrons, making it a useful system to study extracted chondrocytes with an endogenously produced matrix. Thus, the objective of this study was to develop a culture system for alginate-encapsulated chondrocytes to synthesize a robust PCM and study mechanotransduction via metabolic changes induced in the chondrocyte when subjected to mechanical loading.

We found that primary human and bovine chondrocytes, when cultured in alginate beads with addition of sodium L-ascorbate for 7 days, had a pronounced PCM, retained their phenotype, and synthesized collagens VI and II. In contrast, chondrocytes cultured as monolayers for 7 days did not form a distinct PCM. Our results indicate that 3D culture of chondrocytes in complete media with ascorbate leads to increased production of collagens VI and II. We observed that both primary osteoarthritic human and normal bovine chondrocytes, when cultured as a monolayer in ascorbate showed a pronounced pattern of secreted collagens VI and II resembling a PCM. Encapsulating the chondrocytes in alginate beads, and culturing in ascorbate resulted in chondrons (*i.e.* chondrocytes with PCM) that synthesized substantially more collagen VI and II near the perimeter of the cell. These cells display the three-dimensional round phenotype associated with chondrocytes *in vivo*. This suggests that formation and accumulation of a matrix through simulating 3D *in vivo* conditions is a crucial step that occurs in alginate encapsulation. Similarly, cells isolated from alginate gels and re-embedded in agarose displayed a robust collagen VI and II staining in the outer matrix when cultured in ascorbate. Metabolomic pathway analysis showed an upregulation in urea cycle and amino acid group metabolism pathways in compressed alginate-encapsulated

chondrocytes. Distinct compression-induced changes in metabolomic profiles between the monolayer-agarose and alginate-released agarose-embedded chondrocytes indicated physiological changes in cell metabolism.

Materials and Methods

Chondrocyte Harvest and Culture

Primary human chondrocytes were harvested from Stage IV osteoarthritis patients under IRB approval using previously established methods [21]. Bovine chondrocytes were harvested from intact ankle joints of 18-22 month old cattle obtained from a local abattoir. Chondrocytes were isolated by overnight digestion with Collagenase Type I (Gibco) at 2 mg/mL for 14 h at 37ºC. Isolated chondrocytes were cultured for 10 days in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS, Bio-Techne) and 10,000 I.U./mL penicillin and 10,000 µg/mL streptomycin (Sigma, hereby referred to as Complete media) in 5% CO2 at 37ºC. The chondrocytes were passaged at 90% confluency (~10 days) and used between passage 1-3. For monolayer studies, primary chondrocytes were seeded at a density of 160 cells/mm^2 onto 25mm x 25mm Fisherbrand microscope coverslips and allowed to grow into a monolayer. The coverslips with the attached cells were placed in a 60mm x 15mm tissue culture dish containing Complete media and incubated for 0-7 days with 50µg/mL sodium L-ascorbate (Sigma) with the media changed every other day. Monolayers grown in Complete media lacking sodium L-ascorbate for 7 days at 37ºC were used as control. Cells were analyzed at day 0, day 3, and day 7 for collagen production. Similarly, bovine chondrocytes were seeded at 160 cells/mm^2 on microscope coverslips and cultured in Complete media with 50µg/mL sodium L-ascorbate for 7 days in 5% CO_2 at 37ºC and analyzed at days 0, 3, and 7. Control cultures lacked sodium L-ascorbate in the media.

Alginate Encapsulation of Chondrocytes

Cells harvested from monolayers were encapsulated in alginate. For alginate encapsulation, cells were resuspended at a density of 4x10^6 cells/mL in sterile filtered

130 1.2%, w/v, sodium alginate (Sigma). This solution was then slowly dispensed through a 22 gauge needle in a dropwise fashion into a 102mM $CaCl_2$ solution. After near-instantaneous gelation, the alginate beads were allowed to crosslink for 15 min. The beads were then washed 4 times with 150mM NaCl and once with Complete media, before being cultured in 60 mm culture plates with complete media. The beads were
135 maintained in complete media containing 50 μg/mL sodium L-ascorbate for day 0, day 3 and day 7, with media changed every other day for histochemical analysis. Control cultures did not have ascorbate in the media. One group of alginate beads was cultured for 7 days with 50 μg/mL sodium L-ascorbate for agarose encapsulation and mechanotransduction studies.

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Embedding in High-Stiffness Agarose

After 7 days in culture, the alginate beads were dissolved with an EDTA-citrate buffer (150mM NaCl in 55mM sodium citrate with 50mM EDTA, pH6.8). The recovered cells were washed with PBS before mixing with low melting temperature agarose
145 (Sigma Aldrich) at a final concentration of 4.5% w/w (Jutila 2015). Agarose gels were placed in a 24-well tissue culture plate (Falcon) with 2 mL complete media containing 50 μg/mL sodium L-ascorbate and cultured in 5% CO_2 at 37ºC for 3 or 7 days for metabolomic studies and histochemical analysis. The media was changed every other day. Monolayer cells embedded in agarose and cultured with ascorbate for 7 days
150 served as controls.

Cryosectioning, Immunocytochemistry, and Confocal Imaging

Monolayer: Primary human and bovine chondrocytes cells grown as monolayers on microscope coverslips were fixed in 4% Paraformaldehyde/1X Phosphate Buffered
155 Saline (PBS) for 10 min at room temperature followed by three 5 min washes of 1X PBS. Cells were permeabilized with 0.1%Triton and blocked with 10% normal goat serum in 1X PBS for 30 min at room temperature. Cells were then incubated with primary antibodies to both Collagen VI (Rabbit Polyclonal Anti-Collagen VI antibody, ab6588 from Abcam) and Collagen II (Mouse Monoclonal 2B1.5 to collagen II,

160 ab186430, Abcam) in Triton X-100/1% Bovine Serum Albumin/1X PBS for 30 min at room temperature, followed by 0.1%Triton X-100/1% Bovine Serum Albumin/1X PBS for 1 h at room temperature. After three 5 min washes with 1X PBS, cells were incubated with a mixture of secondary antibodies donkey anti-Rabbit IgG H&L Alexa Fluor^{R}488 (Abcam), Goat anti-mouse IgG H&L Alexa Fluor^{R}568 (Abcam) and
165 Vibrant^{TM}DyeCycle^{TM} Violet Stain for Nuclei (Invitrogen) in 0.1%Triton X-100/1% Bovine Serum Albumin/1X PBS for 1 h at room temperature. Cells were washed three times with 1X PBS and the coverslip was mounted on a glass slide with ProLong^{TM} Diamond Antifade Mountant (Invitrogen). Digital images were acquired on a Leica TCS SP8 confocal microscope and images obtained with the Leica Application Suite Advanced
170 Fluorescence software.

Alginate-encapsulated chondrocytes: Alginate beads with encapsulated chondrocytes were spun down at 500 x g for 2 min, washed with 1X Phosphate Buffered Saline (PBS), and then fixed with 4% v/v Paraformaldehyde in 1X PBS for 15 min at room temperature, followed by a wash with 1X PBS. 100 µL of the alginate beads
175 were cytospun onto single frosted adhesive slides (Tanner Scientific) using a Thermo Scientific Cytospin^{TM} 4Cytocentrifuge. Immunocytochemistry was then performed as described earlier.

Agarose gels: Fixation and cryosectioning of agarose gels containing chondrocytes was performed using modified version [22]. Agarose gels were fixed in 2
180 mL 4% v/v Paraformaldehyde – 1XPBS for 30 min at room temperature followed by 3 washes in 1X PBS. The gels were equilibrated overnight in 30% sucrose/1X PBS at room temperature followed by incubation in 2 mL of 50/50 30%sucrose/OCT solution for 2 h at room temperature. The gels were then cryopreserved in Tissue Tek OCT cryo-compound (VWR Scientific Products) at -80ºC and 12 µm sections were mounted on
185 charged slides (Tanner Scientific) for immunocytochemistry.

Western Blot Analysis

Primary human and bovine chondrocytes were seeded at a density of ~31000 cells/cm^2 (2 mL media) in 6 well tissue culture plates (Falcon) and cultured in complete
190 media containing 50µg/mL L- sodium ascorbate for 7 days in 5% CO_2 at 37ºC. Cells were collected at days 0, 3, and 7. Control cultures lacked ascorbate in the media. Whole cell lysates were prepared from extracting proteins using a lysis buffer (0.1% Triton X-100/1XPBS containing a protease inhibitor). The protein concentration was measured with a Bicinchoninic acid (BCA) assay (Thermo Scientific Pierce™ Rapid
195 Gold BCA Assay Kit) and 8ug of total protein from each lysate was separated on a 4-15% SDS-polyacrylamide gel (Mini-PROTEAN® TGX™ Precast Protein Gels (BIO-RAD). After the proteins were transferred to nitrocellulose membrane (BIO-RAD), the membranes were blocked for 1 h in TBST containing 5% bovine serum albumin. Blocked membranes were then incubated with primary antibody (Rabbit Polyclonal Anti-
200 Collagen VI antibody, Abcam) diluted 1:2000 and incubated overnight at 4˚C, washed four times with TBST for a total of 20 min, followed by incubation with Donkey anti-Rabbit IgG H&L Alexa Fluor^{R}488 (Abcam) diluted 1:2000 for 1 h at room temperature. Loading controls were detected by running equal amounts of each lysate on a separate gel and probed with Rabbit Polyclonal Anti-Human, Anti-Bovine β actin (Rockland
205 Immunochemicals). Membranes were then imaged using a Typhoon Trio Imager.

Chondrocyte Cyclical Compression and Metabolite Extraction

Agarose gels in PBS were placed in a custom-built bioreactor in 5% CO_2 at 37ºC, loaded initially to 5% strain, followed by cyclical compressions with a 3.8% peak-to-peak
210 strain amplitude at 1.1Hz for 30 min (Jutila 2014). The agarose gels were flash frozen for 1 min in liquid nitrogen. Frozen gels were crushed and placed in -80ºC before extraction with 1 mL of HPLC-grade methanol:acetone (70:30) (Fisher Chemical). Pulverized gels with methanol:acetone were then vortexed every 5 min for 20 min and incubated overnight at -20ºC for macromolecule precipitation. Samples were then
215 centrifuged at 19000 x *g* for 10 min at 4ºC (Sorvall Legend X1R). The supernatants were transferred to fresh tubes and vacuum dried in a Savant SC110 speed-vac. The

resulting dried pellet was resuspended in 100 µl of 1:1 volume HPLC grade acetonitrile and water (Fisher Chemical) for further analysis by HPLC-MS

220 Untargeted Metabolomic Profiling and Statistical Analysis

Metabolites were detected via HPLC-MS (high performance liquid chromatography coupled to mass spectrometry) separation using previously established protocol on a Cogent Diamond Hydride HILIC 120 x 2.1 mm column and Agilent 1290 UPLC system [23]. Raw data were acquired in positive ion mode. Initially, we used MS-
225 Convert to re-format raw files, and XCMS Online for feature detection, retention time correction, and alignment [24]. Metaboanalyst was used to the normalize the data and to run the following statistical tests: principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), ANOVA (for multiple group comparisons), t-test (for two group comparisons), hierarchical cluster analysis (HCA), heatmap and
230 volcano plot analysis (for two group comparisons). Significant metabolomic features resulting from volcano plots and heatmap clusters were used to run the pathway analysis on MetaboAnalyst's "Functional Analysis" feature.

235 **Results**

Collagen VI and II expression is up-regulated after culture in ascorbate and increased through day 7

To study the expression of collagens VI and II in monolayers and alginate beads
240 we cultured primary human chondrocytes in 50µg/mL L-sodium ascorbate for 3 or 7 days in 5% CO_2 at 37ºC with no ascorbate media serving as a baseline control (day 0) (Fig. 1). Ascorbic acid is an essential cofactor for collagen synthesis which requires the hydroxylation of proline and lysine to form hydroxyproline and hydroxylysine (Alcain 1994). We assessed localization of collagen VI and II in monolayers, alginate-

245 encapsulated chondrocytes, and alginate-released agarose-embedded chondrocytes using immunofluorescence.

Using double immunofluorescence staining for collagens VI and II, we found that compared to day 0 (Fig. 1A), primary human chondrocytes when cultured as a monolayer in ascorbate showed a progressive increase in collagen staining at day 3
250 (Fig. 1B). This increase in collagen VI and II production was most noticeable at day 7 (Fig. 1D). Monolayer controls cultured without ascorbate showed less collagen production at day 7 (Fig. 1C). We hypothesized that culturing primary human and bovine chondrocytes in alginate would mimic *in vivo* physiological conditions, and in the presence of ascorbate would help synthesize a cell-directed matrix formation. Hence,
255 we encapsulated primary human chondrocytes in 1.2% sodium alginate and cultured them in ascorbate for varying lengths of time.

As seen with the monolayers, compared to day 0 (Fig. 1E), alginate-encapsulated chondrocytes synthesized higher amounts of collagen VI and to a lesser extent collagen II at day 3 (Fig. 1F) and day 7 (Fig. 1H). As expected, when cultured
260 without ascorbate, alginate-encapsulated chondrocytes produced less collagen (Fig. 1G). It appears that chondrocytes produced more collagen VI which is found in the pericellular matrix than collagen II when cultured with ascorbate. Supporting our hypothesis that culturing chondrocytes in alginate and ascorbate would mimic *in vivo* conditions, we noticed that the chondrocytes cultured in alginate beads had clearly
265 formed chondrons (*i.e.* chondrocytes with a shell-like PCM) that included more collagen VI near the perimeter of the cell in a rounded 3D morphology. This was in stark contrast to the diffuse pattern of secreted collagen VI produced by monolayer chondrocytes. We further wanted to investigate if these chondrons would retain collagen expression when released from alginate. Hence, chondrons released from alginate beads were re-
270 embedded in 4.5% physiologically stiff agarose and cultured for 7 days with ascorbate. As seen in (Fig. 1J), alginate released, agarose-embedded chondrocytes sustained collagen production in the presence of ascorbate when compared to the no ascorbate control (Fig. 1I) and also retained their 3D round phenotype.

We next wanted to assess collagen expression in healthy chondrocytes cultured
275 with ascorbate and used a bovine model. When grown as monolayers, compared to the

day 0 baseline control (Fig. 2A) bovine chondrocytes showed a progressive increase in collagen VI and II production at day 3 (Fig. 2B) and day 7 (Fig. 2D). When cultured without ascorbate at day 7 (Fig. 2C), bovine chondrocytes still produced collagen albeit to a lesser extent than in the presence of ascorbate. We then proceeded to encapsulate the bovine chondrocytes in alginate and culture them in ascorbate. As expected for healthy cells, at day 0 alginate-encapsulated bovine chondrocytes expressed collagen VI and II (Fig. 2E) which substantially increased at day 3 (Fig. 2F) and day 7 (Fig. 2H) compared to the 7 day no ascorbate control (Fig. 2G). Interestingly, when cultured in ascorbate, bovine chondrocytes in alginate beads proceeded to form a shell-like pericellular matrix that surrounds the cell just like the primary human chondrocytes in alginate cultures. Similarly, alginate released, agarose-embedded bovine chondrocytes display a robust collagen expression in the pericellular matrix when cultured with ascorbate (Fig. 2I) and to a lesser extent in the absence of ascorbate (Fig. 2J).

Collagen VI protein expression is increased in monolayer cultured in ascorbate for 7 days

To assess collagen VI protein levels we used immunoblots. Both primary human chondrocytes and bovine chondrocytes were cultured as monolayers in the presence and absence of ascorbate for 7 days. Chondrocytes allowed to attach and grow for 24 h without ascorbate served as day 0 control. Analysis of protein expression using a rabbit anti-ColVI polyclonal antibody in cultured primary human chondrocytes showed that collagen VI protein levels increased from day 0 (Fig. 3A, lane 1) to day 7 with ascorbate (Fig. 3A, lane 3). When whole cell lysates were probed under reducing conditions, two major bands of ~ 240 kDa and ~ 120 kDa were detected.

Distinct metabolomic profiles are observed in primary human and bovine chondrocytes when subjected to cyclical compression

To investigate whether alginate pre-encapsulation influences cellular mechanotransduction, alginate-encapsulated chondrocytes were released and

305 embedded in physiologically stiff agarose and subjected to mechanical loading. Monolayer cultured chondrocytes embedded in agarose were treated similarly. Untargeted metabolomic profiling detected 2678 metabolite features in primary human chondrocytes and 3322 bovine chondrocytes (Supplemental Tables 1-2). Metabolite features identified as significant by ANOVA (FDR-adjusted p-values<0.05) were used
310 for further analyses. To assess overall variation between the different experimental groups, we used unsupervised Principal Components Analysis (PCA) and Hierarchical Clustering Analysis (HCA). PCA found moderate separation with more than 50% of the total variance associated with the first 3 components comparing alginate-encapsulated agarose-embedded and monolayer cultured agarose-embedded primary human
315 chondrocytes (Fig. 4A). There was moderate separation by HCA between alginate-encapsulated and monolayer-cultured agarose-embedded primary human chondrocytes under uncompressed conditions (Fig. 4B). PCA found slightly less separation between alginate-encapsulated and monolayer cultured agarose-embedded uncompressed bovine chondrocytes (Fig. 4C). Compared to primary human chondrocytes, bovine
320 chondrocytes had less distinct clustering of HCA identified metabolites (Fig. 4D).

We then evaluated the effects of cyclical compression on both alginate-released-agarose-embedded and monolayer-agarose-embedded chondrocytes. PCA captured 50% of the variance in the first 3 components (Fig. 5A). Cluster analysis of the top 25 metabolites did not find distinct clustering between the alginate and monolayer cultured
325 chondrocytes, suggesting donor dependent heterogeneity and differential regulation of pathways and metabolites in response to compression (Fig. 5B). Conversely, 44% of variation was seen in PCA for bovine chondrocytes (Fig. 5C) and HCA showed perfect clustering of metabolites with respect to alginate and monolayer cultured chondrocytes in response to compression (Fig. 5D).

330 We then compared the effects of compression on alginate-released-agarose-embedded primary chondrocytes and monolayer-agarose-embedded primary chondrocytes (Fig.6). PCA and clustering displayed minimal overlap between the compressed and uncompressed alginate-released-agarose-embedded chondrocytes. The variation in the data set for the first three components was higher than 50%,

335 demonstrating the importance of compression for driving changes in the chondrocyte metabolome. Similarly, heat map analysis revealed compression-induced differential changes in the metabolites obtained from both the groups. Hierarchical clustering also revealed that some of the clusters of metabolites were upregulated in the uncompressed alginate groups (light pink) and downregulated in the compressed
340 alginate group (light green) indicating the heterogeneity in the response of primary human chondrocytes to compression (Fig. 6A) This response can be attributed to sexual dimorphism of the donor cells as well as to the physiological condition of the harvested chondrocytes (Fig. 6A). Upon examining the effect of compression on monolayer-agarose-embedded chondrocytes we found moderate separation (44.5%)
345 between the first three components. HCA and heat map analysis further revealed differences in regulation of metabolites between the compressed and uncompressed monolayer cultured chondrocytes, validating their response to compression (Fig. 6B).

Unsupervised clustering showed less variability between compressed and uncompressed bovine chondrocytes cultured in alginate. HCA and heat map analysis
350 did not find distinct clustering of the metabolites in the alginate compressed and uncompressed group (Fig. 7A). Similarly, not much difference was observed in the compressed and uncompressed chondrocytes grown as monolayers and embedded in agarose. Distinct metabolomic profiles were not observed by HCA and heat map analysis (Fig. 7B). In primary human chondrocytes, hierarchical clustering of the top 50
355 metabolites displayed distinct clusters of metabolites that were upregulated in the compressed alginate cultured-agarose-embedded cells (Fig. 8A, red) and downregulated in the uncompressed (Fig. 8A, green). Interestingly, in the monolayer cultured-agarose-embedded chondrocytes hierarchical clustering of the top 50 metabolites revealed downregulation of metabolites in the compressed (Fig. 8B, red)
360 and an upregulation in the uncompressed groups (Fig. 8B, green).

We further utilized volcano plot analysis (Fig. 8C) to find metabolite features that differ between the alginate cultured and monolayer cultured chondrocytes encapsulated in agarose gels and subjected to cyclical compression. 120 significant metabolites were upregulated in the alginate cultured-agarose-embedded chondrocytes subjected to

365 compression with 354 metabolites downregulated. Pathways related to these upregulated metabolites mapped to branched chain amino acid degradation, urea cycle/amino group metabolism, steroid hormone biosynthesis, lysine metabolism and hormone metabolism. Downregulated pathways mapped to aminosugars and pyrimidine metabolism pathways in the alginate cultured cells.

370 192 significant metabolites were upregulated in monolayer cultured-agarose-embedded chondrocytes subjected to compression with 185 metabolites downregulated (Fig. 8D). Upregulated pathways included fatty acid metabolism, saturated fatty acid oxidation and tryptophan metabolism pathways. Downregulated metabolites represented pathways such as N-glycan biosynthesis, butanoate metabolism, and urea
375 cycle/amino group metabolism. Interestingly, some of the metabolites that were found in high abundance in the alginate cultured-agarose-embedded compressed group, such as hippuric acid were at lower concentration in the monolayer-cultured agarose-embedded compressed group. A study by Rushing *et al* showed that levels of hippuric acid were increased in urine samples of OA progressors, suggesting the importance of
380 hippuric acid as a metabolite in distinguishing OA progression [25].

<u>Distinct compression-induced metabolic pathways are associated with monolayer and alginate-cultured chondrocytes and vary between primary human and bovine chondrocytes</u>

Median heat map analysis of co regulated metabolites identified 9 different
385 cluster groups in primary human chondrocytes and 8 cluster groups in bovine chondrocytes. We detected many pathways that were upregulated in the uncompressed alginate cultured-agarose-embedded group including amino acid, urea cycle, Vitamin B6 and B3 metabolism, glycerophospholipid metabolism, and energy cycle metabolism (Fig. 9A, Clusters # 3 and 4). Glycine and Proline are major components of collagen
390 and we detected upregulation of several amino acids including arginine, proline, lysine, glutamate, histidine, tyrosine, aspartate and asparagine metabolism. Proline can be synthesized *de novo* from glutamate through the intermediate pyrroline-5-carboxylate (P5C), which is then converted to proline by P5C reductase [26]. Vitamin B6 is a critical

cofactor involved in diverse biochemical reactions that regulate cellular metabolism [27], whereas vitamin B3 (also known as niacin) plays a central role in energy metabolism [28]. Upregulation of beta oxidation of fatty acids in the peroxisomes was specific to uncompressed monolayer-cultured agarose-embedded chondrocytes (Fig. 9A, Cluster # 2). Pathways related to porphyrin and amino acid metabolism, as well as beta alanine, aspartate and asparagine metabolism were upregulated in compressed monolayer group (Fig. 9A, Cluster # 5). Downregulated metabolic pathways for the uncompressed alginate group were mainly associated with fatty acid oxidation, keratan sulfate biosynthesis, glycan biosynthesis, glycerophospholipid metabolism and purine metabolism (Fig. 9A, Cluster # 9).

Upregulated pathways in the uncompressed alginate cultured-agarose-embedded bovine chondrocytes included lysine degradation, amino acids glycine, threonine, serine and tryptophan metabolism, inositol phosphate metabolism, sphingolipid metabolism, and the phosphatidyl inositol signaling system. (Fig. 9B, Cluster # 1, 4). The energy related pathways such as starch and sucrose metabolism and ubiquinone biosynthesis were also upregulated in this cluster. Pathways that were upregulated in the uncompressed monolayer-cultured group included sugar metabolism for energy production, terpenoid backbone synthesis and electron transport and amino sugar metabolism (Fig. 9B, Cluster #5). Upregulated pathway in the alginate compressed cluster was related to an energy pathway, Panthotenate and CoA biosynthesis (Fig. 9B, Cluster # 8). Panthotenate is a key precursor to Coenzyme A biosynthesis, which is an essential cofactor and plays an important role in synthesis of phospholipids, fatty acids and the TCA cycle [29].

Discussion

The local microenvironment governs structure-function relationships of chondrocytes in articular cartilage. Mechanical signals play an important role in maintaining homeostasis of the articular cartilage extracellular matrix and dysregulation can impair normal physiologic processes [30]. The objective of this work was to develop

a culture system for chondrocytes to produce a robust pericellular matrix for studying chondrocyte mechanotransduction.

Ascorbic acid is an essential cofactor for collagen formation and its requirement is mainly attributed to its role in prolyl hydroxylation. Hydroxyproline is important for the helical structure formation of collagen fibers and hydroxylysine plays a critical role in collagen crosslinking. Hence, we used ascorbate in our culture medium to ensure production of collagen. Previous studies show that ascorbic acid in culture medium enhances collagen production, and we also found this in the present study for both primary human and bovine chondrocytes. We observed that both primary human and bovine chondrocytes in monolayer culture with ascorbate showed a pronounced diffuse pattern of secreted collagens VI and II. On the contrary, when cultured in alginate, both primary human and bovine chondrocytes developed a three-dimensional round phenotype similar to *in vivo* chondrons and produced more collagen VI compared to monolayer. Corroborating the immunofluorescence data, western blot analysis revealed substantial production of collagen VI in the presence of ascorbate.

In healthy joints, primary articular chondrocytes maintain physiological and metabolic homeostasis which is altered in progression to osteoarthritis. This pathological shift includes cartilage remodeling characterized by degradation of the articular cartilage, disruption of the architecture, and a progressive deterioration of biomechanical properties. Changes within the chondrocytes include but are not limited to disturbed mitochondrial function, changes in energy requirements, altered cell signaling, and chondrosenescence [31].

We are beginning to understand some of the biochemical events that are involved in mechanotransduction and subsequent modulation of the chondrocyte phenotype. Cell-matrix interactions are crucial to mechanical sensing and this study shows that alginate-agarose constructs provide an improved model for studying chondrocyte mechanotransduction. Here, we identify conditions for chondrocytes to form an endogenous pericellular matrix and assessed biochemical pathways affected by cyclical compression.

Metabolomic profiling of primary human chondrocytes precultured in alginate showed differentially regulated pathways related to energy metabolism, lipid

metabolism, and amino acid metabolism compared to chondrocytes cultured as monolayer and embedded in agarose when subjected to cyclical compression. Volcano plot analysis found upregulated metabolite features mapped to branched chain amino acid (BCAA) degradation, urea cycle/amino group metabolism, steroid hormone biosynthesis, lysine metabolism, and hormone metabolism in alginate cultured agarose-embedded chondrocytes.

A previous study by Zhai *et al* has suggested the ratio of BCAA to histidine as a biomarker for osteoarthritis [32]. Catabolism of BCAA is tightly regulated, and its byproducts feed into the TCA cycle for energy production [33]. There also exists an inter-relationship between the TCA and urea cycle shunt through the intermediary fumarate, a breakdown product of arginosuccinate via arginosuccinate lyase in the urea cycle [34], that feeds into the TCA cycle for energy production. In cellular metabolism, the TCA cycle acts as a central hub providing a source of intermediary metabolites for precursors of biosynthetic pathways, cellular repair, and regeneration.

Interestingly, in monolayer cultured agarose-embedded chondrocytes, urea cycle/amino group metabolism was downregulated along with other pathways such as N-glycan biosynthesis and butanoate metabolism. Upregulated pathways were fatty acid metabolism, saturated fatty acid oxidation, and tryptophan metabolism. Fatty acids are a major component of lipids, and their metabolism through beta oxidation is essential for energy regulation [35]. This demonstrates the different energy sources that are used by chondrocytes upon applied cyclical compression when precultured in alginate versus culturing as monolayer.

Median intensity heat map analysis also found that different pathways were upregulated when chondrocytes were precultured in alginate and then subjected to compression. We observed that when primary chondrocytes were encapsulated in alginate and cultured in the presence of ascorbate, pathways related to amino acids were upregulated.

Of specific importance was the upregulation in the arginine, proline, lysine, and glutamate metabolism. Hydroxyproline and hydroxylysine derived from proline and lysine respectively are major amino acids found in Collagen VI (Col VI) in addition to glycine. Col VI is a prominent component of the PCM and plays a role in the

485 microenvironment of the chondrocytes. The PCM surrounds the chondrocytes and modulates the biomechanical, biophysical, and biochemical signals [18]. The PCM also directly transduces physical signals from the ECM to the cell possibly through an interaction of Col VI with integrins or other cell surface receptors. It is thought that the PCM plays a role in cell anchoring and matrix cell signaling and is most likely a contact
490 between the hard interterritorial cartilage matrix and the chondrocyte [36].

The observed upregulation of these amino acid pathways is consistent with our immunocytochemistry data that find enhanced production of cell-directed PCM synthesis. Downregulated metabolic pathways for the alginate group were mainly associated with fatty acid oxidation, keratan sulfate biosynthesis, glycan biosynthesis,
495 and metabolism of glycerophospholipids and glycosphingolipids. Lipids are essential components of biological membranes, with glycerophospholipids being the most abundant of the membrane lipids. Both glycosphingolipids and glycerophospholipids play important roles in cellular processes such as cell signaling and energy storage [37]. Interestingly, glycerophospholipid metabolism was upregulated in the alginate-
500 encapsulated chondrocytes, indicating that these membrane phospholipids may differentially modulate signaling cascades when cultured under different conditions.

Keratan sulfate is a widely distributed glycosoaminoglycan present in the ECM of the chondrocytes in articular cartilage and provides compressive resistance to cartilage [38]. In neurons, an additional key role for keratan sulfate lies in sensing ion fluxes
505 allowing them to respond dynamically to biochemical changes within the cell microenvironment [39].

Metabolism of inflammatory leukotrienes was down regulated in alginate-encapsulated agarose-embedded chondrocytes. Inflammatory mediators associated with OA often include cytokines, chemokines, prostaglandins, nitric oxide, and
510 leukotrienes [40]. Biosynthesis of coenzyme A (CoA) from its key precursor Pantothenate was also downregulated in the alginate encapsulated agarose-embedded chondrocyte group. Pathways detected in the monolayer cells embedded in agarose and subjected to cyclical compression included porphyrin, beta-alanine, aspartate and asparagine metabolism. Uncompressed pathways were phytanic acid and peroximal
515 oxidation which is involved in degradation of complex fatty acids.

Upregulated pathways in the uncompressed alginate cultured-agarose-embedded bovine chondrocytes included amino acid metabolism (lysine degradation, amino acids glycine, threonine, serine and tryptophan), lipid metabolism (sphingolipids), and the phosphatidyl inositol signaling system. Glycine is a primary amino acid found in collagen, and threonine and serine feed into the TCA cycle via pyruvate and acetyl CoA thereby regulating the energy cycle.

Tryptophan metabolism into its downstream metabolites like kyneurenine and derivatives modulate host inflammatory responses by binding to host transcription factor aryl hydrocarbon receptor (AHR) [41]. While AHR is the most described host receptor for tryptophan metabolites, there are other ligand activated transcription factors that play an important role in metabolism of xenobiotics, that can enter animal bodies through feed additives. Since xenobiotic metabolizing enzymes of the cytochrome P450 family are main targets of AHR induction, it validates the upregulation that we see in the metabolism of xenobiotics by cytochrome P450 in bovine chondrocytes [42].

Phosphatidylinositols are a group of phospholipid molecules that are present in cellular membranes and are key regulators of cell signaling and intracellular trafficking [43] whereas sphingolipids are lipid molecules that play critical roles in cell membrane biology to regulate cell function [44]. Energy-related pathways such as starch and sucrose metabolism and ubiquinone biosynthesis were also upregulated in this cluster. Other pathways that were upregulated included starch and sucrose metabolism. Non-structural carbohydrates such as sucrose and starch are metabolized for energy production [45]. Ubiquinone is an electron carrier in oxidative phosphorylation [46] and its biosynthesis was upregulated, contributing to the bioenergetic activity of ATP synthesis. Similarly the Panthotenate and CoA biosynthesis pathway upregulation in the alginate compressed cluster points to energy production through the TCA cycle. Pathways related to uncompresssed monolayer cultured chondrocytes identified primarily as carbohydrate and energy metabolism.

In summary, we present differences in morphology and mechanotransduction pathways when chondrocytes are cultured in monolayer (2D) versus alginate (3D).

Chondrocytes cultured in monolayer dedifferentiate and lose their characteristic phenotype over time, however, chondrocytes cultured in alginate maintain their phenotype and produce cartilage-specific ECM components. Similarly, our results indicate that primary human and bovine chondrocytes can form a robust pericellular matrix that mimics the *in vivo* phenotype when cultured in alginate in the presence of sodium L-ascorbate. Our study also shows substantial differences in metabolomic profiles of chondrocytes that are encapsulated in alginate compared to monolayer. Many pathways that were detected with the alginate-encapsulated chondrocytes were related to amino acid metabolism, fatty acid metabolism, O and N-glycan biosynthesis, phosphatidylinositol signaling, glycerophopholipid metabolism which is involved in membrane structural integrity and central energy metabolism. Pathways related to the monolayer cultured agarose-embedded chondrocytes were associated mainly with fatty acid oxidation, terpenoid backbone biosynthesis, porphyrin metabolism (seen also in bovine monolayer cultured agarose-embedded chondrocytes), amino sugar, and monosaccharide (fructose, mannose) metabolism and aspartate and asparagine metabolism. Taken together, imaging data, western blot and amino acid metabolism pathways suggest an increase in collagen production in the pericellular matrix when chondrocytes are cultured in alginate compared to monolayers. Future work will help in elucidating the different pathways that contribute to chondrocyte biology and provide important information not only for mechanotransduction studies but also for cartilage engineering.

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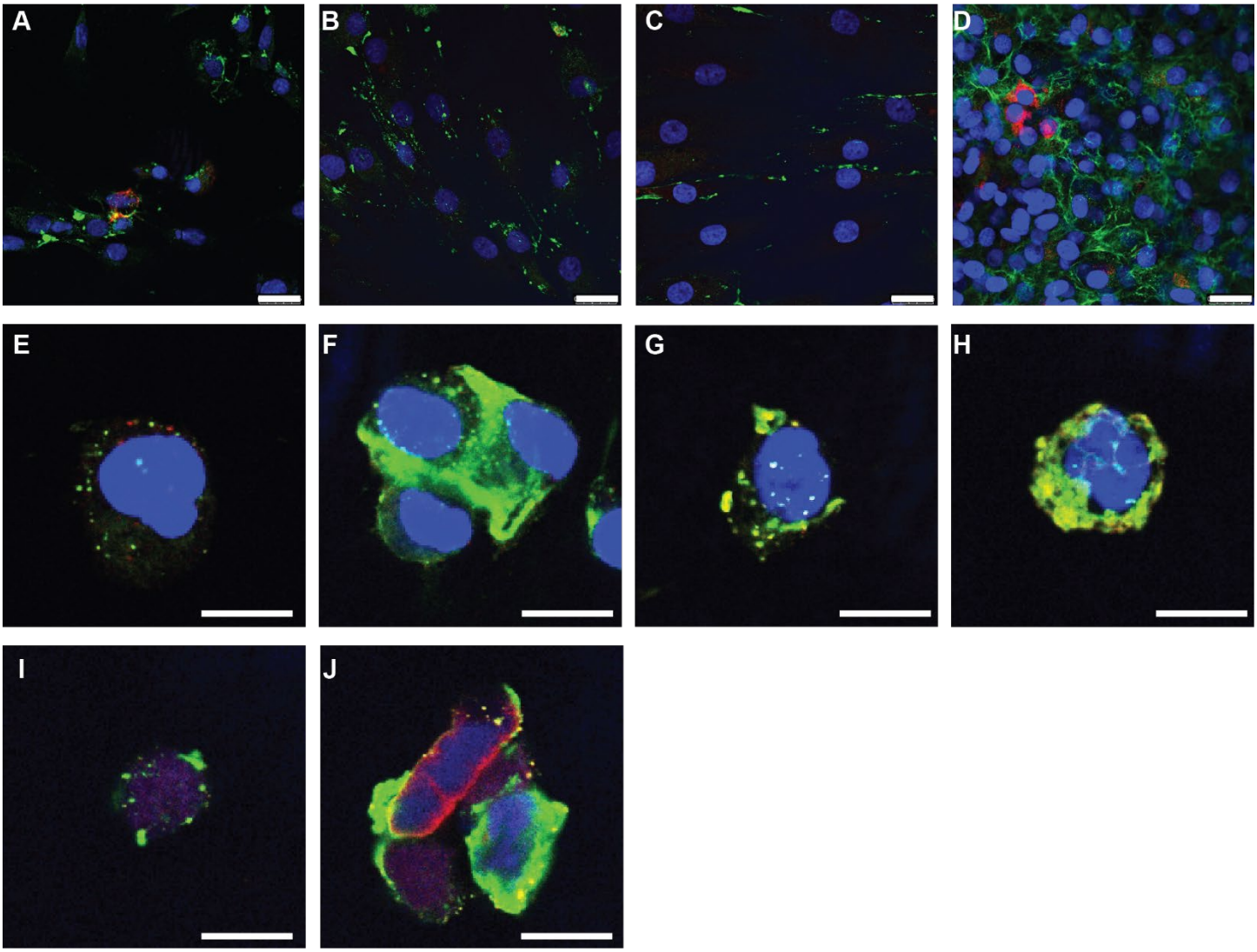

Figure 1

Collagen expression in primary human chondrocytes. Primary human chondrocytes were cultured and stained with antibodies to Collagen VI (green), Collagen II (red) and Nuclear Marker Vibrant™DyeCycle™ Violet Stain before imaging at 63X. Monolayer-expanded human primary chondrocytes showed a progressive increase in collagens VI and II when cultured (**A**) at day 0, (**B**) day 3 with ascorbate (**C**) day 7 no ascorbate control and (**D**) day 7 with ascorbate. (**E**) Alginate-encapsulated chondrocytes at day 0, (**F**) at day 3 with ascorbate, (**G**) day 7 without ascorbate and (**H**) day 7 with ascorbate in the medium. Sections of chondrocytes released from alginate and re-embedded in agarose stained for Collagens VI and II expression in the pericellular matrix (**I**) when cultured without ascorbate and (**J**) with ascorbate for 7 days. Scale bar represents 25 μm in panels A-D and 12.5 μm in E-J.

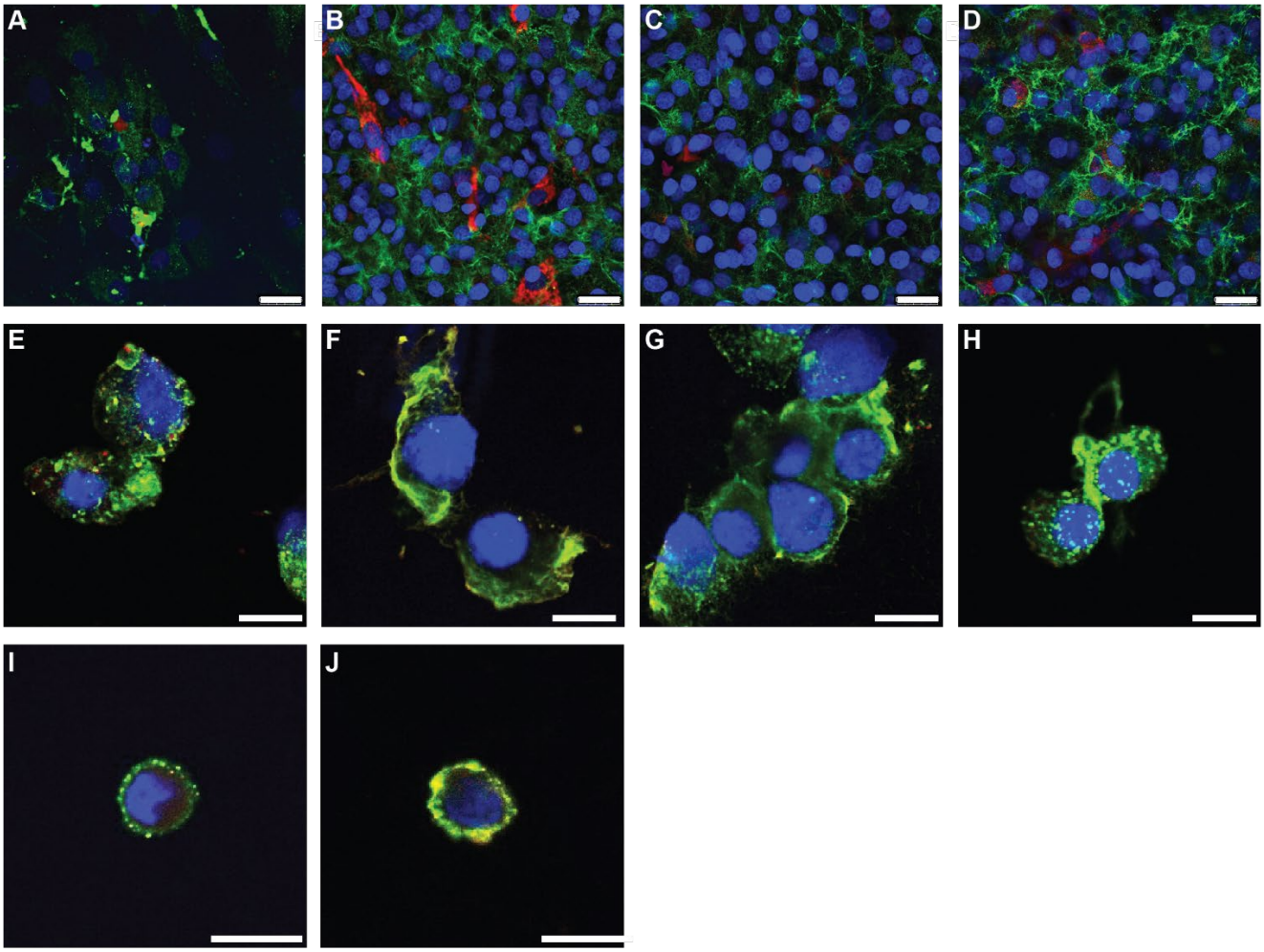

Figure 2

Expression of Collagens VI and II in bovine chondrocytes. Representative 63x images of bovine chondrocytes labeled with antibodies to Collagen VI (green), Collagen II (red) and Nuclear Marker Vibrant™DyeCycle™ Violet Stain. Panels (**A-D**) are monolayer expanded bovine chondrocytes from (**A**) day 0, (**B**) day 3 cultured with ascorbate, (**C**) day 7 no ascorbate control and (**D**) day 7 cultured with ascorbate. Panels (**E-H**) represent alginate beads cultured (**E**) at day 0, (**F**) day 3 with ascorbate, (**G**) day 7 no ascorbate culture and (**H**) day 7 ascorbate. Immunofluorescence staining of agarose sections depicting chondrocytes released from alginate beads and re-embedded in agarose and (**I**) cultured without ascorbate and (**J**) with ascorbate for 7 days. Scale bar represents 25 μm.

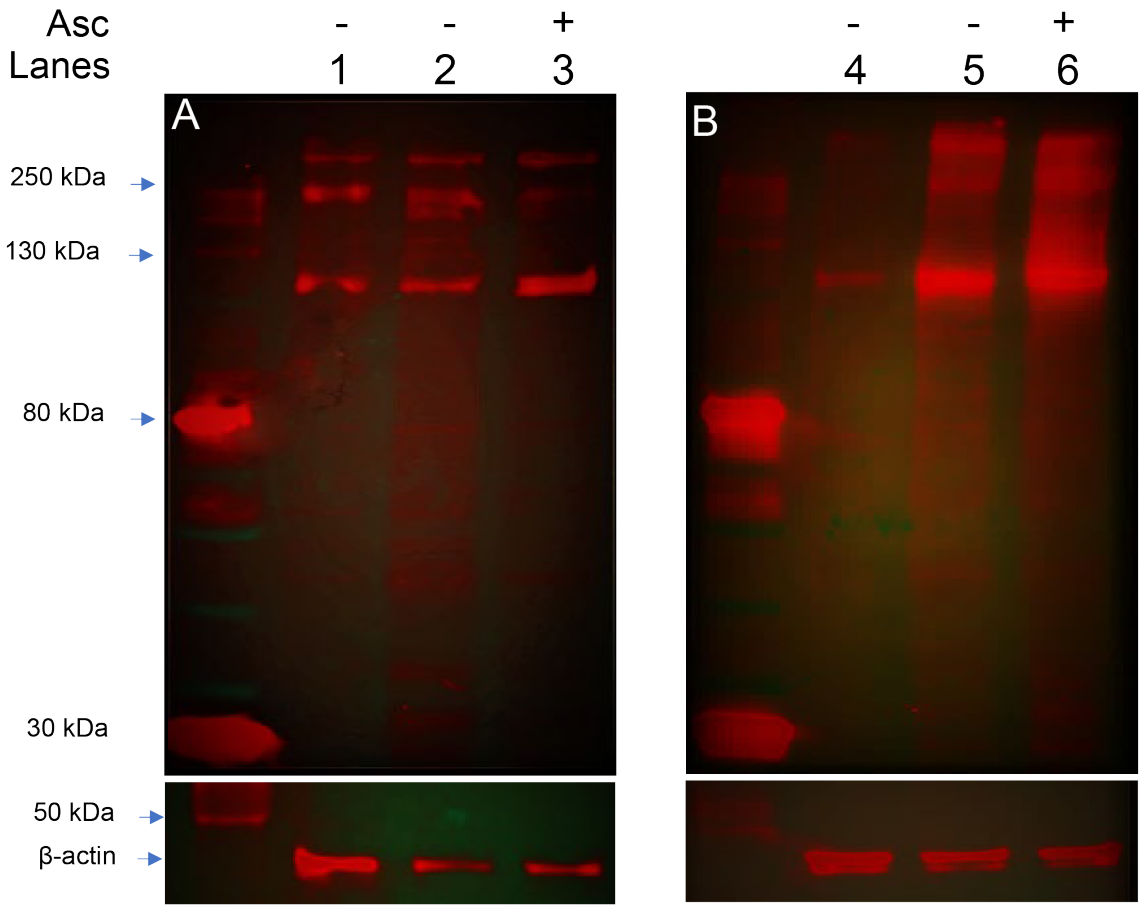

Figure 3

Ascorbate increases Collagen VI expression. Western blot analysis of primary human and bovine chondrocytes. Monolayer cell lysates from **(A)** primary human chondrocytes and **(B)** bovine chondrocytes were probed with antibody to Collagen VI. Lanes 1 and 4 represent cell lysates from day 0, lanes 2 and 5 represent cell lysates from monolayers cultured for 7 days without ascorbate (Asc). Cell lysates from monolayers cultured with ascorbate (Asc) for 7 days are shown in lanes 3 and 6. β-actin was used as loading control.

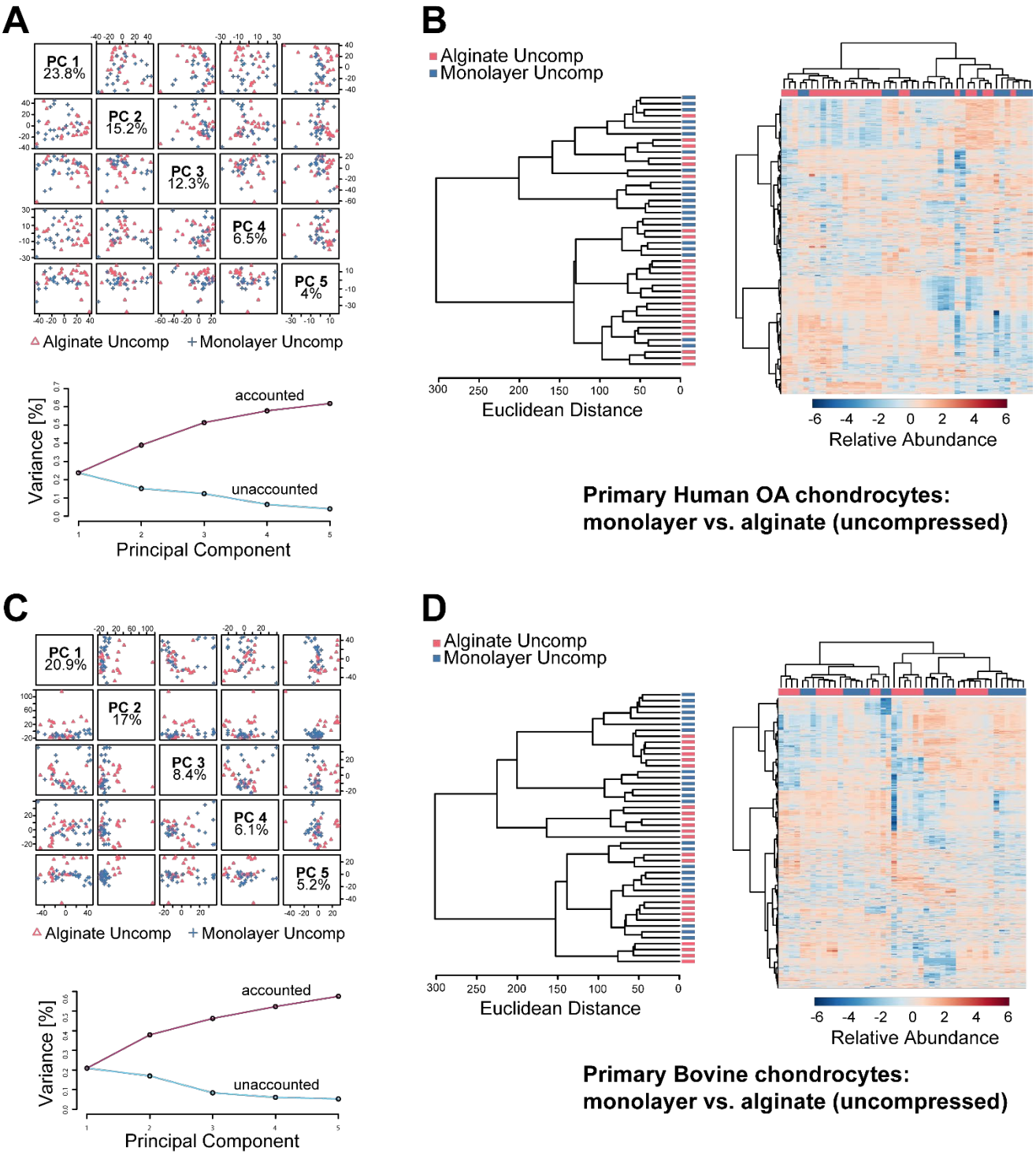

Figure 4

Alginate pre-culture drives differences in baseline metabolomic profiles in uncompressed chondrocytes. (A) For primary human OA chondrocytes principal components analysis finds substantial differences between monolayer controls and alginate pre-cultured samples with more than 50% of the overall variance associated with the first 3 components. (B) Clustering analysis finds good discrimination between monolayer and alginate pre-cultured samples of primary human chondrocytes. (C) For primary bovine chondrocytes, principal components analysis finds differences between monolayer and alginate pre-culture samples. (D) Hierarchical clustering finds minimal discrimination between monolayer and alginate pre-cultured bovine chondrocytes.

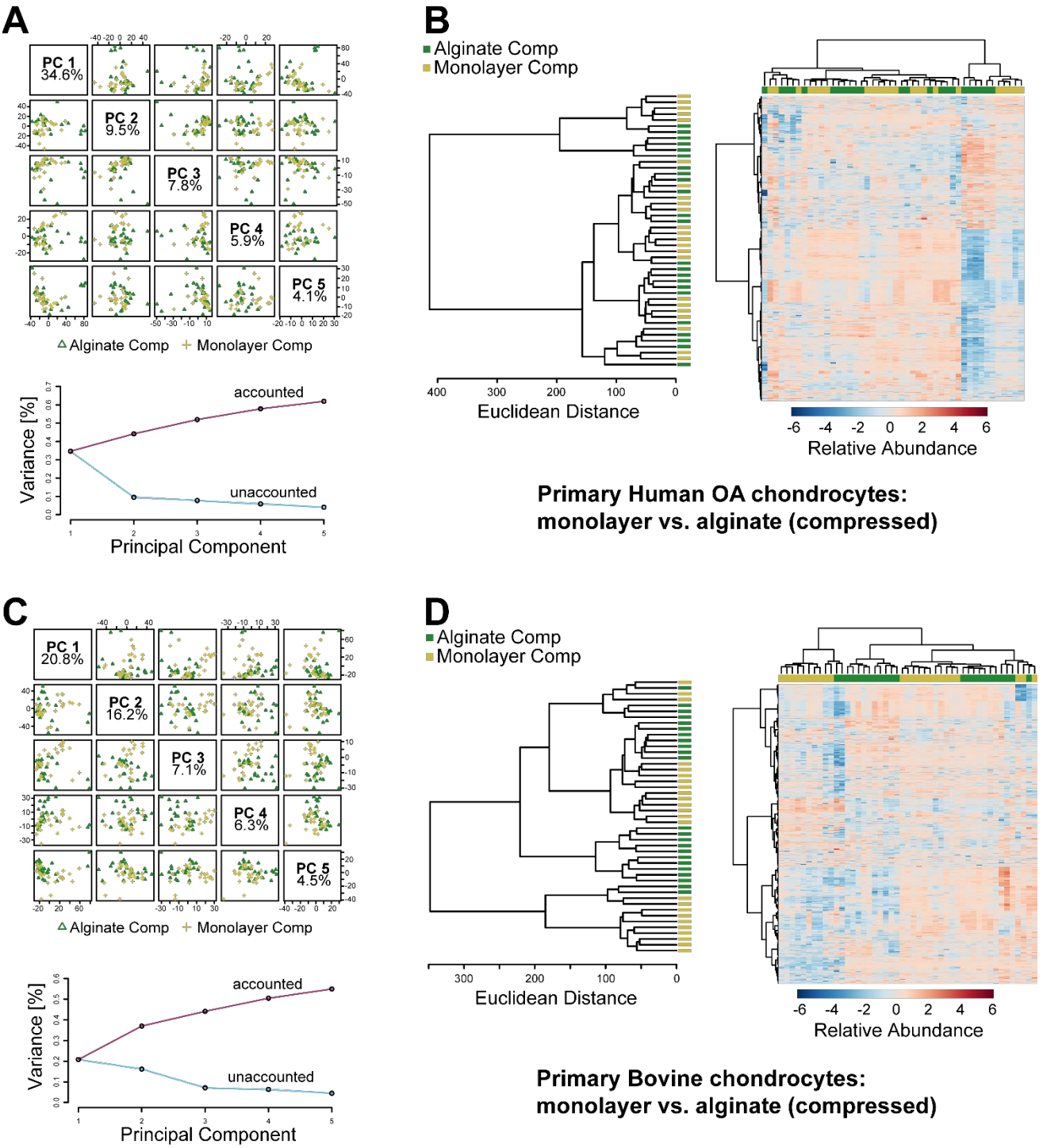

Figure 5 Alginate-encapsulated chondrocytes exhibit differences in compression-induced metabolomic profiles compared to monolayer-encapsulated chondrocytes. Metabolomic profiles from primary human chondrocytes shown in panels (A) and (B). Metabolomic profiles from primary bovine chondrocytes shown in panels (C) and (D).

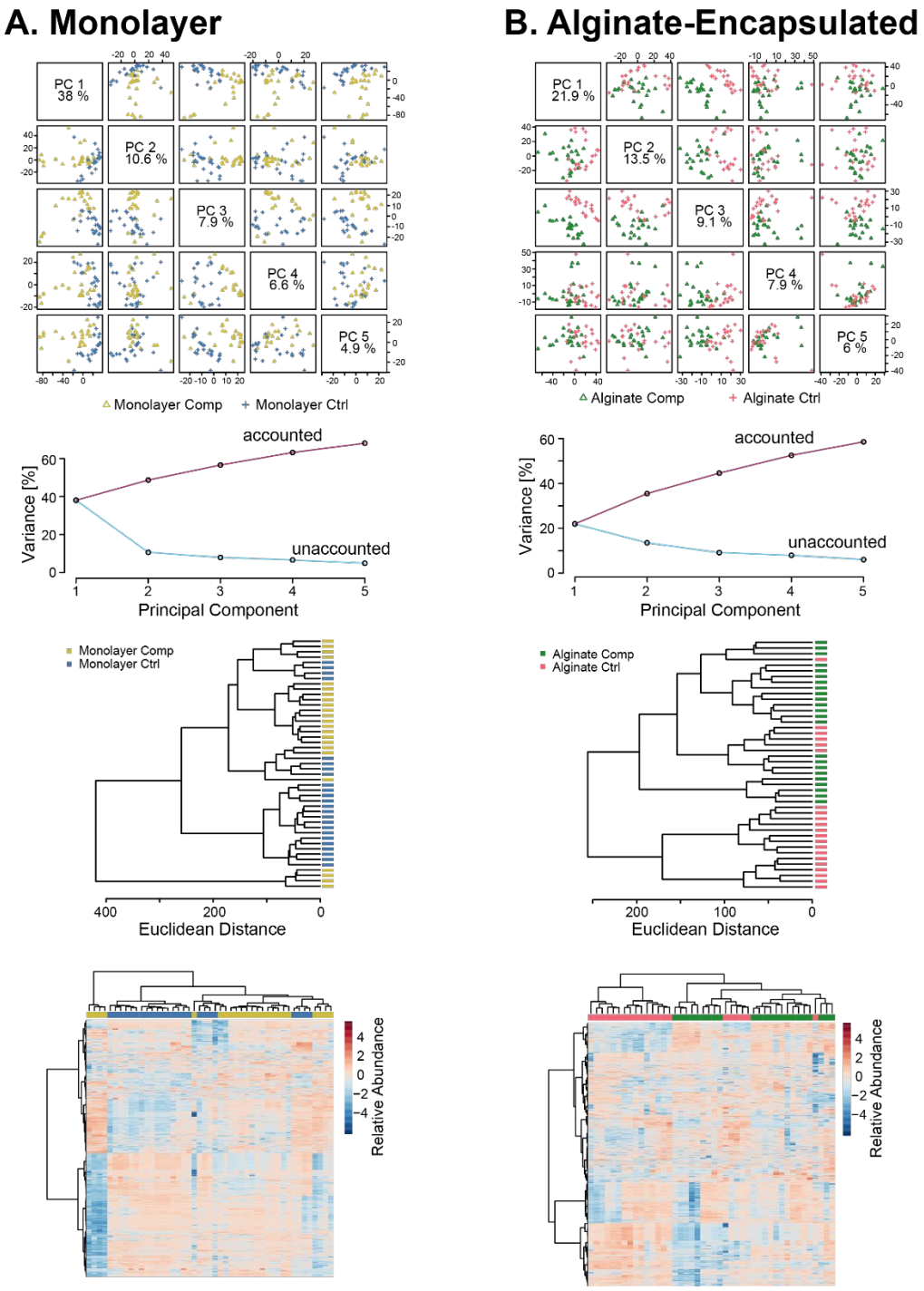

Figure 6 Comparison of compression-induced metabolomic profiles between monolayer (A) and alginate-encapsulated (B) primary human chondrocytes. Principal components analysis (top) finds that both systems exhibit differences between compression-induced changes in metabolomic profiles. Clustering and heatmaps show differences in compression-induced metabolomic profiles (middle and bottom).

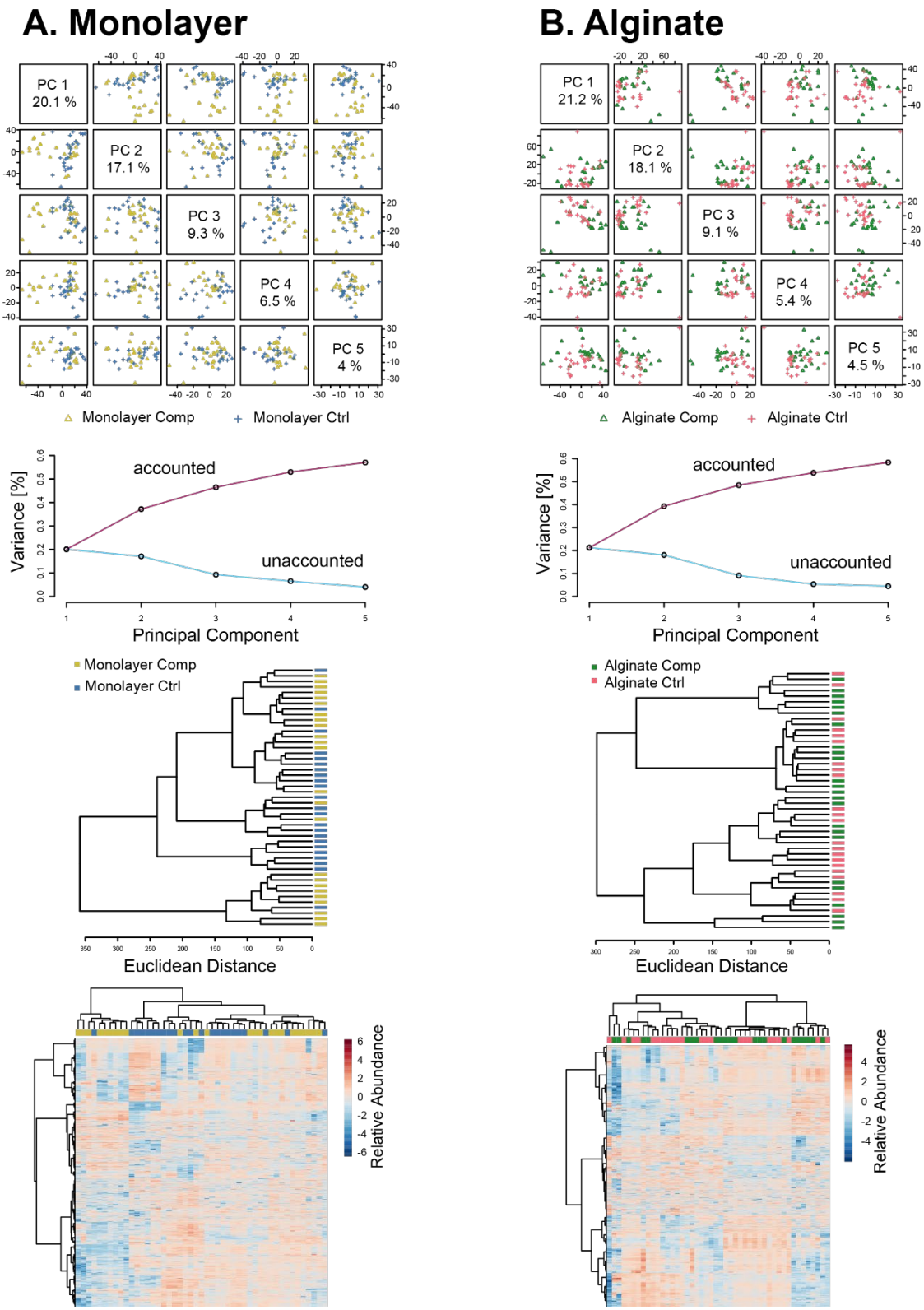

Figure 7 Comparison of compression-induced metabolomic profiles between monolayer (A) and alginate-encapsulated (B) primary human chondrocytes. Principal components analysis (top) finds that both systems exhibit differences between compression-induced changes in metabolomic profiles. Clustering and heatmaps show differences in compression-induced metabolomic profiles (middle and bottom).

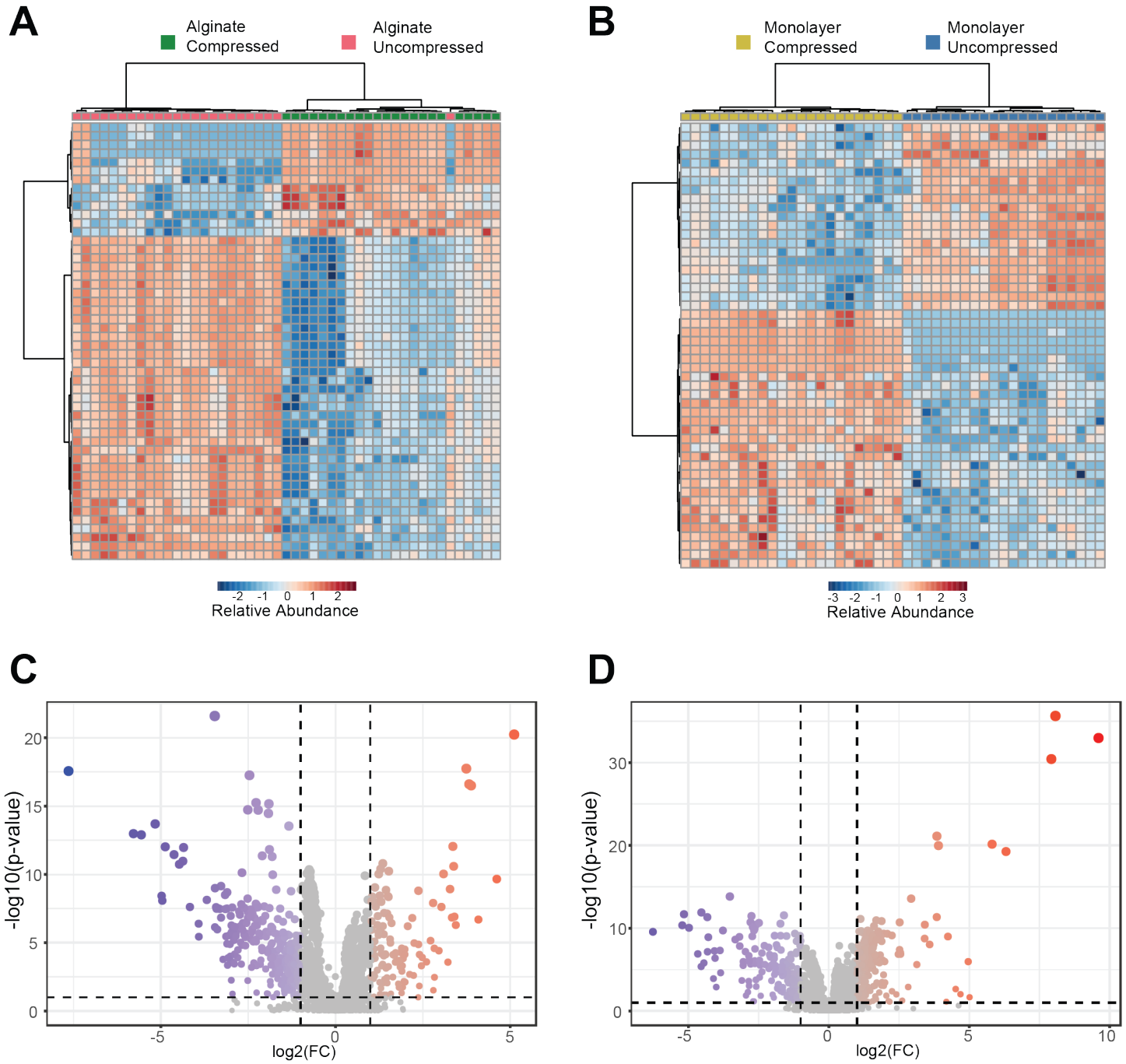

Figure 8

Comparison of mechanotransduction between alginate and monolayer systems. (A) Top 50 metabolites different between compressed and uncompressed controls in alginate system. (B) Top 50 metabolites different between compressed and uncompressed controls in monolayer system. (C) Volcano plot of compression-induced metabolites in alginate system. (D) Volcano plot of compression-induced metabolites in agarose system.

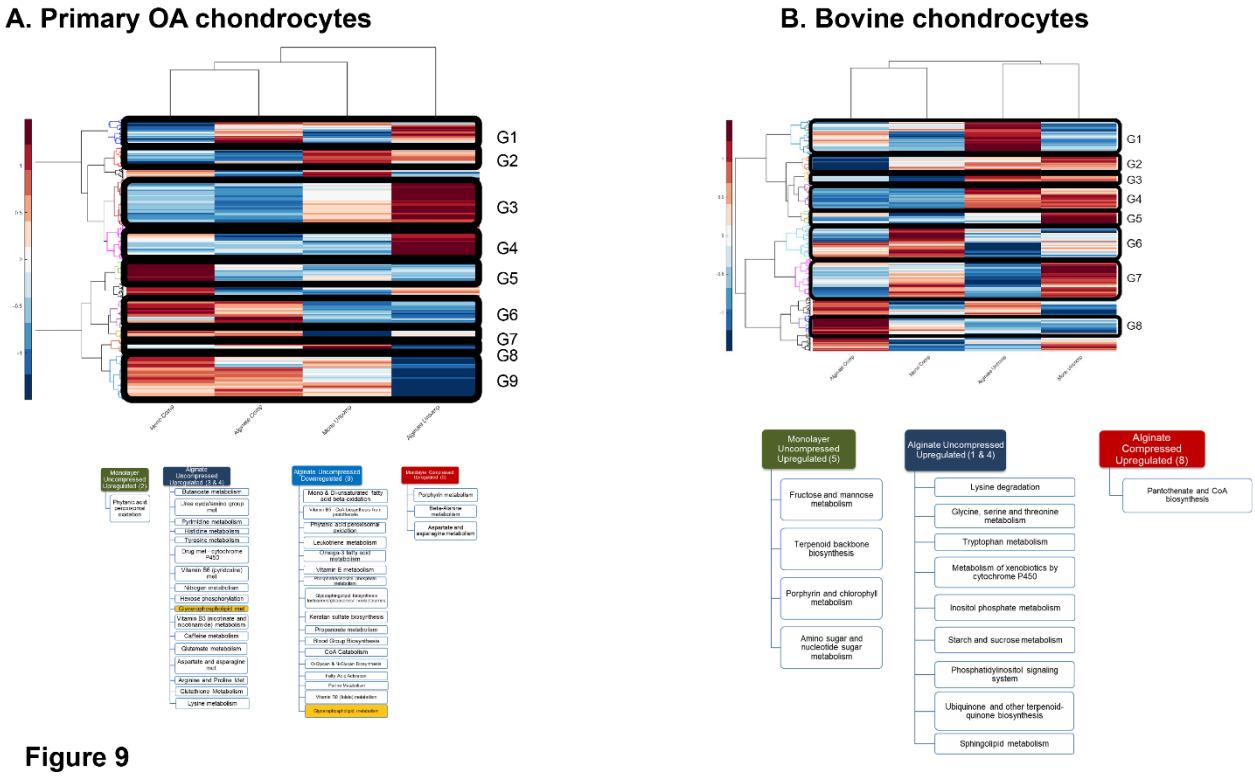

Figure 9

Figure 9 Summary of culture-related and compression-induced pathways for (A) primary OA chondrocytes and (B) bovine chondrocytes. Pathways determined from clusters of metabolites shown in top heatmaps.