European Cells and Materials Vol. 23 2012 (pages 195-208) DOI: 10.22203/eCM.v023a15 ISSN 1473-2262

DISCRIMINATION OF MENISCAL CELL PHENOTYPES USING GENE EXPRESSION PROFILES

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Abstract

The lack of quantitative and objective metrics to assess cartilage and meniscus cell phenotypes contributes to the challenges in fibrocartilage tissue engineering. Although functional assessment of the final resulting tissue is essential, initial characterization of cell sources and quantitative description of their progression towards the natural, desired cell phenotype would provide an effective tool in optimizing cell-based tissue engineering strategies. The purpose of this study was to identify quantifiable characteristics of meniscal cells and thereby find phenotypical markers that could effectively categorize cells based on their tissue of origin (cartilage, inner, middle, and outer meniscus). The combination of gene expression ratios collagen VI/collagen II, ADAMTS-5/collagen II, and collagen I/collagen II was the most effective indicator of variation among different tissue regions. We additionally demonstrate a possible application of these quantifiable metrics in evaluating the use of serially passaged chondrocytes as a possible cell source in fibrocartilage engineering. Comparing the ratios of the passaged chondrocytes and the native meniscal cells may provide direction to optimize towards the desired cell phenotype. We have thus shown that measurable markers defining the characteristics of the native meniscus can establish a standard by which different tissue engineering strategies can be objectively assessed. Such metrics could additionally be useful in exploring the different stages of meniscal degradation in osteoarthritis and provide some insight in the disease progression.

Keywords: Meniscus, cartilage, fibrocartilage, relative gene expression, tissue engineering, chondrocyte passaging, dedifferentiation.

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Introduction

The menisci are wedge-shaped tissues situated between the femur and tibia that play critical roles in the biomechanical function of the knee. These fibrocartilaginous tissues distribute and transmit load through the joint while acting as a shock absorber (Messner and Gao, 1998). Without normally functioning menisci, the femoral condyle and tibial plateau cartilage experience abnormal stress distributions. Full or partial meniscectomies to treat traumatic meniscal injuries or non-traumatic degenerative tears significantly impact the joint biomechanics and accelerate the onset of osteoarthritis (Allen *et al.*, 1984; Jorgensen *et al.*, 1987; Roos *et al.*, 1998). While both cartilage and meniscus are macroscopically exposed to compression, the structure of the meniscus and interactions with other tissues lead to the generation of tensile (or "hoop") stresses carried by the circumferentially oriented fibers in its outer region (Messner and Gao, 1998). Heterogeneity in the functional demands on different regions of the meniscus is reflected by the considerable spatial variation in cell phenotypes and extracellular matrix composition and structure.

Previous studies have identified four distinct cell morphologies in the meniscus, including cells in the inner, middle, outer regions and in the surface layer (Hellio Le Graverand *et al.*, 2001). The inner region of the meniscus is generally described as more similar to cartilage, with rounded cells resembling articular chondrocytes. On the other hand, the outer region of the meniscus is fibrous and vascularized with cells described as fibroblast-like (McDevitt *et al.*, 2002). Similar patterns of cell morphology are seen in tissues from various species, including those from immature bovines (Fig. 1). In addition to the differences in cell morphology, the extracellular matrix composition has also been shown to vary depending on the regions of the meniscus. Although the meniscus is primarily composed of collagen I, the inner region shows a higher content of collagen II, the major component and indicator of hyaline cartilage (Cheung, 1987; Kambic and McDevitt, 2005). Additionally, aggrecan, the large, aggregating proteoglycan that plays a major role in resisting compression in cartilage through interactions with interstitial fluid, has a higher density in the inner meniscus than its periphery (Valiyaveettil *et al.*, 2005). Similar patterns of meniscal matrix composition have been observed in multiple species (Chevrier *et al.*, 2009). Like other species, immature bovine menisci demonstrate substantially higher proteoglycan contents in the inner region (Fig. 2).

The idea of cartilage taking various forms in a continuous spectrum between dense fibrous connective tissue and hyaline cartilage has been explored in the

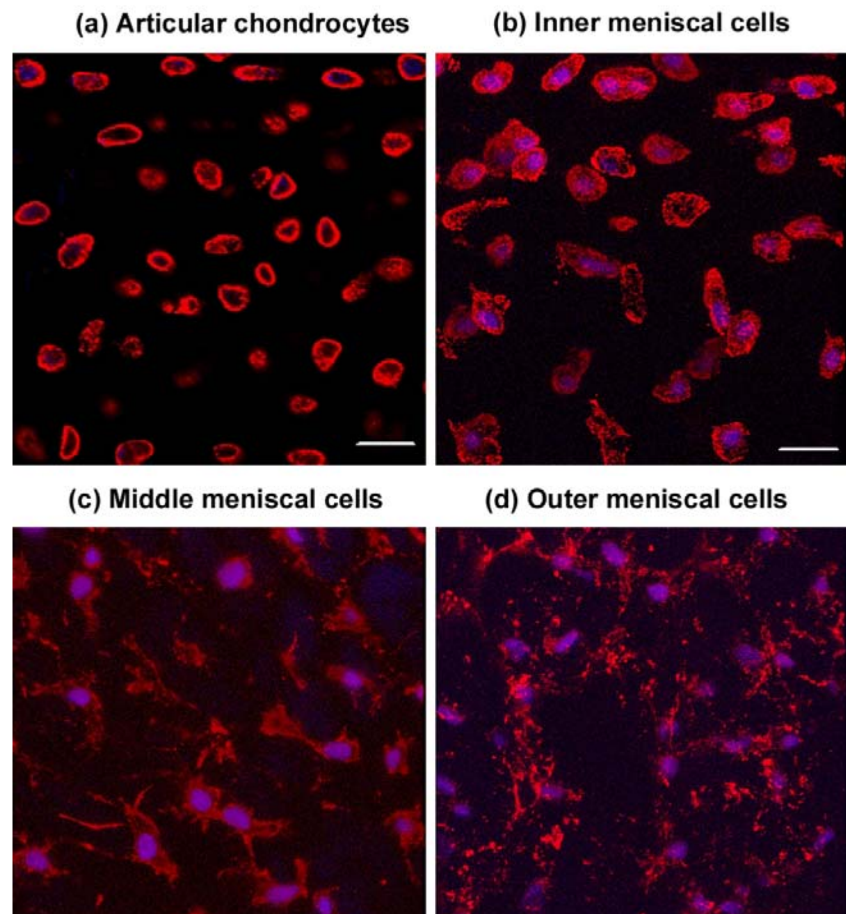

Fig. 1. Cell morphology of articular chondrocytes and meniscal cells from the inner, middle and outer regions of the meniscus. Images represent superimposed sections from ~20 µm z-stacks from confocal microscopy images. The meniscal cells transition from exhibiting a rounded morphology, resembling chondrocytes, in the inner zone to a stellate form in the outer zone. Red is F-actin, blue is DNA, and scale bar is 20 µm. Courtesy of Dr Eric Vanderploeg.

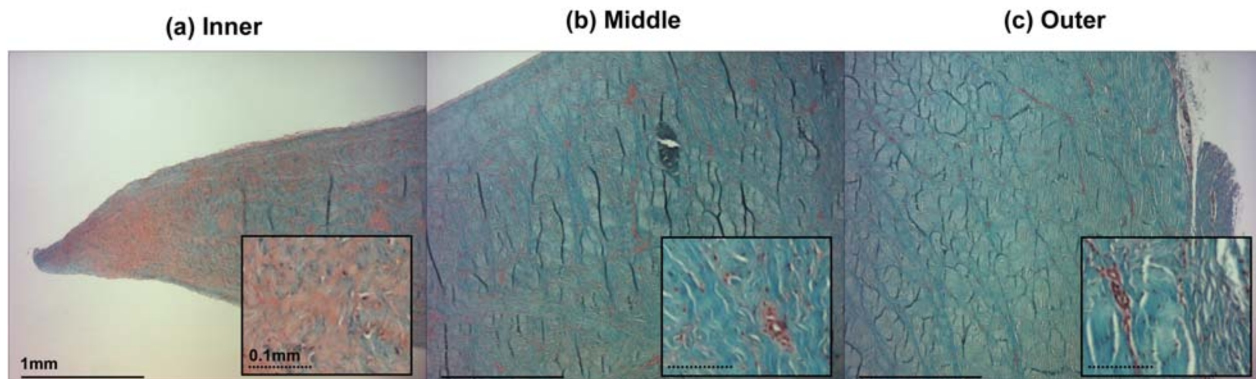

Fig. 2. Sulfated glycosaminoglycan distribution in the inner, middle, and outer regions of the immature bovine meniscus. Sections are stained with Safranin-O, showing sGAG in red, and are counterstained with Fast Green. Courtesy of Dr Christopher Wilson.

context of fibrocartilage formation in tendons and ligaments (Benjamin and Ralphs, 1998; Wren *et al.*, 2000). The placement in this spectrum is dependent on the extent of the fibrocartilage cell differentiation, which is influenced by the mechanical forces it experiences. While the majority of tendons and ligaments are composed of fibrous tissues, some regions such as the enthesis or contact region of wrap-around tendons, which are exposed to compression, have been found to naturally develop cartilage-like properties of avascularity and high contents of proteoglycan and collagen II (Milz *et al.*, 2008). The degree of this differentiation within the tendon varies according to the distance from the wrap-around surface against which the tissue is compressed, indicating that such transformation of tissue properties is in fact an adaptation to the mechanical response (Wren *et al.*, 2000). This concept can be extended directly to the meniscus, which similarly experiences a continuum of mechanical forces from compression to tension from the inner to the outer region.

The heterogeneous nature of the meniscus has been a challenge to replicate using tissue engineering strategies. Recent developments in biodegradable, porous scaffolds require a great amount of cells. Currently identified cell

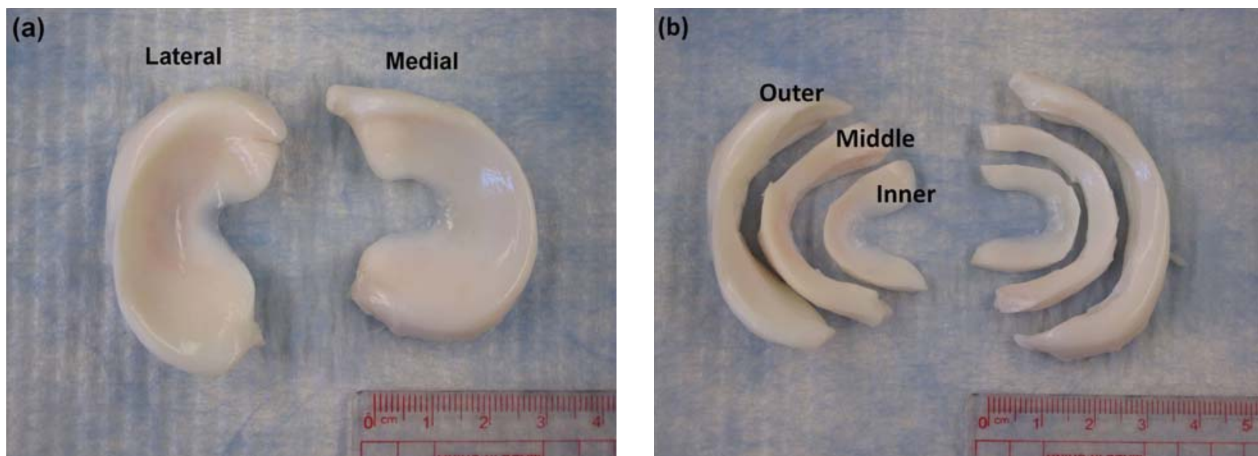

Fig. 3. (**a**) Lateral and medial immature bovine menisci. (**b**) Each meniscus was divided into inner, middle and outer zones.

sources such as primary chondrocytes and mesenchymal stem cells (MSCs) cannot be harvested in sufficient quantity and require expansion over multiple passages (Walsh *et al.*, 1999; Yamasaki *et al.*, 2005; Marsano *et al.*, 2007). Expanded chondrocytes, which tend to become more fibroblastic with passage, are also commonly used in cartilage repair techniques such as autologous chondrocyte implantation. Although this method has produced positive clinical results, complications included arthrofibrosis (Marlovits *et al.*, 2006). Some studies have suggested that phenotypic changes with serial passaging could make passaged chondrocytes more suitable for engineering fibrocartilage (Anderson and Athanasiou, 2008; Marsano *et al.*, 2007). However, the characteristics of these cells have not been quantitatively compared to the behaviors of meniscal cells from the native tissue, and an appropriate basis for doing so has not been established.

In fact, despite a qualitative understanding of phenotypic variations within the meniscus and between meniscal cells and chondrocytes, no metrics have been identified to quantitatively distinguish cells from different regions of the meniscus, or distinguish meniscal cells from articular chondrocytes. Such information would be valuable for understanding phenotypic variations in disease and degeneration and would be particularly valuable as metrics for tissue engineering strategies. Some efforts have been undertaken to analyze the gene expression profile of chondrocytes through dedifferentiation using ratios such as collagen I (Col-1) to collagen II (Col-2), and aggrecan (AG) to versican (VS) (Martin *et al.*, 2001; Barlic *et al.*, 2008). While the two ratios showed significant difference between osteoarthritic and control cartilage tissues, only Col-1/Col-2 seemed indicate dedifferentiation of chondrocytes with passage. Another approach to capture gene expression trends in dedifferentiation has involved statistical methods like clustering and principal component analysis (Lin *et al.*, 2008). Although such approaches may be helpful in identifying groups of genes that behave similarly under certain conditions, they do not provide a clear quantitative marker that indicates cell phenotype. In addition, none of these studies examined the meniscus in this context. The goal of this study was to identify quantifiable regional markers in the meniscus and cartilage by employing statistical methods to analyze the gene expression profiles of articular chondrocytes and meniscal cells in native tissue isolated from different anatomical regions, with a focus on collagens, small and large proteoglycans, and aggrecanases. As the main components of meniscus and cartilage, the collagen and proteoglycan genes are responsible for the production of the extracellular matrix (ECM) while aggrecanase genes are critical to ECM regulation. In addition, aggrecanases may have a distinct role in the meniscus from that of cartilage and could be a potential differentiating factor between the two tissues. The phenotypic markers identified based on analysis of tissue gene expression were then used in a preliminary evaluation of serially passaged articular chondrocytes.

Materials and Methods

Tissue gene expression

Fresh menisci and femoral condylar cartilage were isolated from both stifles of six two-week old bovine calves (Research 87, Marlborough, MA, USA). Each medial and lateral meniscus was divided into outer, middle and inner regions and corresponding tissues from both legs were pooled for each animal (Fig. 3). Samples of cartilage, on the other hand, were obtained from the entire medial or lateral condyle. While the composition and gene expression profiles of articular cartilage would also be expected to vary depending on location, such changes would not be expected to have fully developed in two-week old calves. Additionally, the variation among regions of articular cartilage is likely to be smaller than the differences between cartilage and meniscus. About 100 mg of the articular cartilage (AC), inner meniscus (IM), middle meniscus (MM), and outer meniscus (OM) was minced and total RNA was isolated according to the manufacturer's instructions (RiboPure Kit, Applied Biosystems, Foster City, CA, USA). A NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) was employed to quantify the isolated RNA and verify its quality. The 260 nm/280 nm absorbance ratio was greater than 1.8 for all samples (1.97 ± 0.098, mean ±SD). For each RT-PCR reaction, 30 ng of the RNA was reverse-transcribed to cDNA (High

Table 1. Primer sequences.

Gene	GenBank Accession #	Primer Sequence 5'-3'	
18S rRNA	AF176811	Forward Reverse	TCG AGG CCC TGT AAT TGG AA GCT ATT GGA GCT GGA ATT ACC G
Collagen I	AB008683	Forward Reverse	AAT TCC AAG GCC AAG AAG CAT G GGT AGC CAT TTC CTT GGT GGT T
Collagen II	NM_001001135.2	Forward Reverse	CATCTGCTCAGCTGACCTCC GGGTCCTACGATGTCCTTGAT
Collagen VI	AF126375	Forward Reverse	CTG GAG AGC CTG GAC AGA AG GCC TTT GAA ACC AGG AAC AC
Aggrecan	U76615	Forward Reverse	CCT GAA CGA CAA GAC CAT CGA TGG CAA AGA AGT TGT CAG GCT
Versican	NM_181035.2	Forward Reverse	GAG ACA TGA TGG GGA AGG AA GTG GAA CAC ATC ACC ATC CA
Decorin	NM_173906	Forward Reverse	ACT GAA GGA ATT GCC AGA GAA CTA CGA CGA TCA TCT GGT TCA
Biglycan	S82652	Forward Reverse	GGT CCT CGT GAA CAA CAA GAT GGA TCT CAC ACA GGT GGT TCT
ADAMTS4	NM_181667.1	Forward Reverse	TGC CAG ACT AAG CAC TCA CC CTG TGG GAC ATT GAA AGC CT
ADAMTS5	AF192771	Forward Reverse	CTC CCA TGA CGA TTC CA AAT GCT GGT GAG GAT GGA AG

Capacity RNA-to-cDNA kit, Applied Biosystems) and amplified with the SYBR Green Master Mix (Applied Biosystems) and custom primers (Table 1) for 18S rRNA, collagen I, collagen II, collagen VI (Col-6), aggrecan, versican, decorin (DC), biglycan (BG), ADAMTS4 (aggrecanase-1, TS4) and ADAMTS5 (aggrecanase-2, TS5) in the Applied Biosystems 7500 Fast Real Time RT-PCR system. Primer sequences were obtained from literature (Dimicco *et al.*, 2007; Fitzgerald *et al.*, 2008) or engineered using Primer-BLAST. Amplification curves were analyzed using LinReg software v12.7 (Ramakers *et al.*, 2003) to correct for baseline values that produce much variability in amplification efficiency (Ruijter *et al.*, 2009). The amplification efficiency was assumed to be the same for all samples per amplicon and PCR run, as the observed variability has been shown to reflect random error rather than a true variation (Nordgard *et al.*, 2006). The relative gene expression (RGE) was determined by normalizing the efficiency-corrected expression levels of the target genes to that of 18S rRNA, a commonly-used reference gene in multiple studies involving cartilage and meniscus (Upton *et al.*, 2003; Hofstaetter *et al.*, 2004; Valiyaveettil *et al.*, 2005; Zielinska *et al.*, 2009). The stability of 18S rRNA has also been shown under standard cell culture conditions (Schmittgen and Zakrajsek, 2000). Data were log-transformed for statistical analysis. Medial-lateral and zonal differences in the tissue RGE were evaluated via a multifactor General Linear Model (GLM) with Bonferroni's test for pairwise comparisons and animal as a random variable (Minitab, Minitab Inc., State College, PA, USA). Significance was set at $p < 0.05$.

Linear discriminant analysis (LDA) and best subsets multinomial logistic regression (MLR) were performed to select robust predictors of tissue zone (AC, IM, MM and OM, with $n = 12$ in each zonal group) from all possible 36 ratios of target gene expressions (MATLAB, MathWorks, Natick, MA, USA; R, R Foundation for Statistical Computing, Vienna, Austria). For both LDA and MLR, the tissue zone was assigned as the response variable, and either two or three ratios were assigned as the predictor variables. All combinations were examined, and for each set of combinations, a model was constructed from the entire sample set and its output zonal group compared to the true group to assess its predictability. Cross-validation was additionally performed; one sample was iteratively omitted from the pool to construct the statistical model, with which its tissue origin was predicted. Combinations of ratios were ranked based on the fraction of correctly identified samples in the cross-validations, and the genes or gene ratios that appeared most frequently in the top 5 % of all combinations were identified.

Passaged chondrocytes gene expression

Chondrocytes were additionally isolated via collagenase digestion from the femoral condylar cartilage of a separate set of immature bovine stifles from one animal. Briefly, the tissue was minced into 1-3 mm^3 cubes and digested with 0.2 % collagenase overnight in serum-supplemented media. The resulting solution was filtered and centrifuged to obtain a pellet of chondrocytes. These isolated cells were subsequently plated in monolayer on six different plates at a seeding density of 8,000 cells/cm^2 in low glucose DMEM (Invitrogen/Life Technologies, Carlsbad, CA, USA) with 10 % fetal bovine serum (Invitrogen), antibiotic-antimycotic (Invitrogen), 10 mM nonessential amino acids (Invitrogen), and 50 μg/mL L-ascorbic acid-2-PO_4 (Sigma-Aldrich, St. Louis, MO, USA). The medium was changed every two days and the cells were passaged with 0.25 % trypsin (Invitrogen) once they reached confluence and reseeded at 8,000 cells/cm^2. Total RNA from 1-2 million cells was obtained at each passage from passage 0 to 4 (P0-P4). The 260 nm / 280 nm absorbance ratio was greater than 1.8 for all samples used (1.99 ± 0.058, mean ±SD). The absorbance ratio of the RNA obtained during P4 was

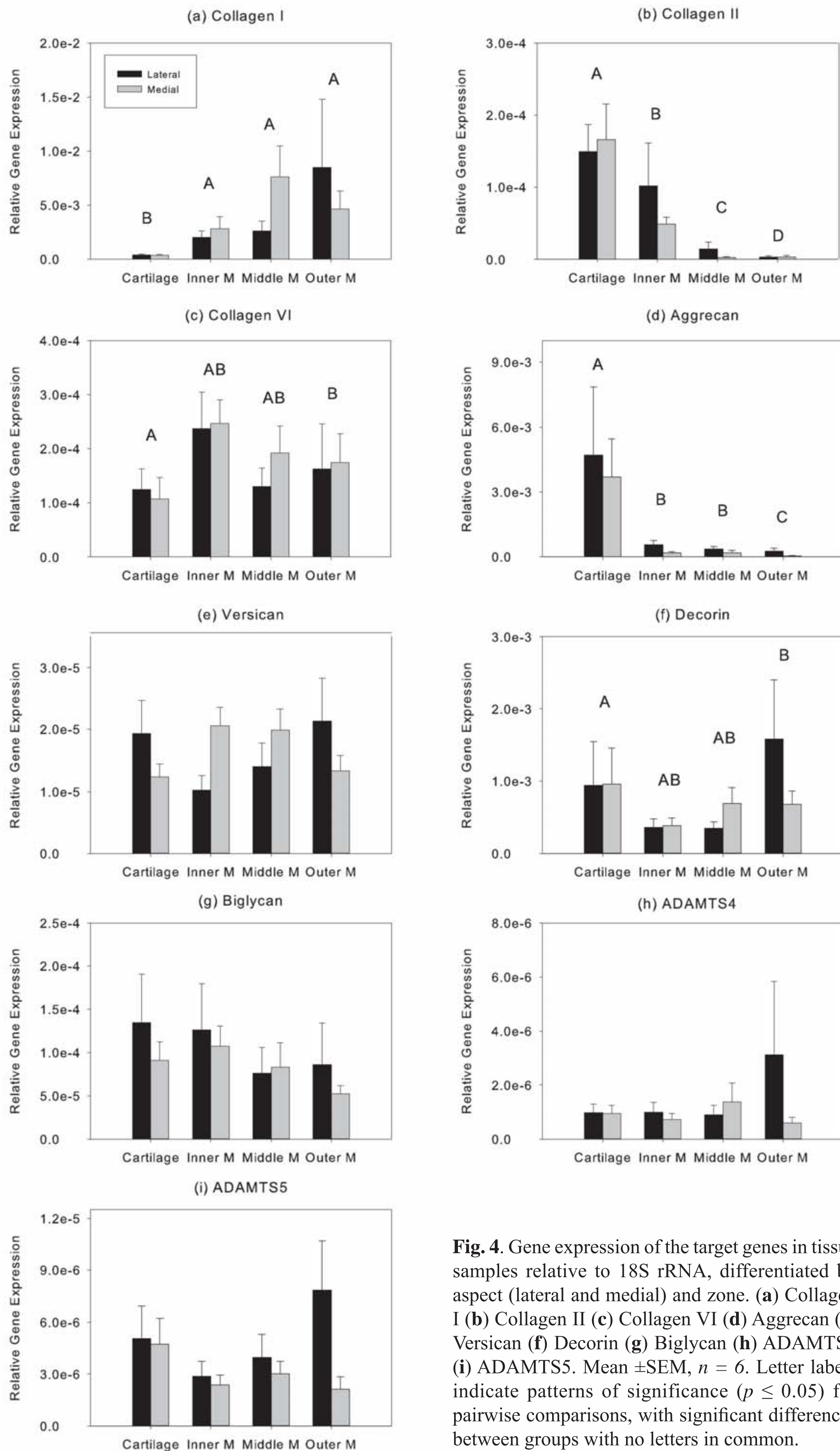

Fig. 4. Gene expression of the target genes in tissue samples relative to 18S rRNA, differentiated by aspect (lateral and medial) and zone. (**a**) Collagen I (**b**) Collagen II (**c**) Collagen VI (**d**) Aggrecan (**e**) Versican (**f**) Decorin (**g**) Biglycan (**h**) ADAMTS4 (**i**) ADAMTS5. Mean ±SEM, $n = 6$. Letter labels indicate patterns of significance ($p \leq 0.05$) for pairwise comparisons, with significant differences between groups with no letters in common.

Table 2. Frequency of genes and ratios in the top 5 % of 3-ratio predictors of zonal origin, using different statistical models, linear discriminant analysis (LDA), multnomial logistic regression (MLR), and ordinal logistic regression (OLR).

Tissue predictors				Passage predictors			
LDA		MLR		LDA		OLR	
Genes		**Genes**		**Genes**		**Genes**	
Col-2	476	Col-2	455	Agg	418	Agg	583
TS5	381	TS5	374	TS4	369	Col-1	557
Col-6	343	Col-6	361	Col-2	363	Col-2	144
Agg	298	Agg	280	Ver	253	TS4	144
Col-1	172	Col-1	188	Col-1	203	TS5	144
TS4	141	Dec	143	Col-6	154	Col-6	142
Dec	119	TS4	142	Big	132	Dec	142
Big	103	Ver	100	TS5	119	Ver	136
Ver	79	Big	99	Dec	101	Big	120
total	2112		2142		2112		2112
Ratios		**Ratios**		**Ratios**		**Ratios**	
log TS5/c2	151	Log TS5/c2	141	log agg/c1	352	log TS4/agg	138
log c6/c2	121	log c6/c2	127	log TS4/agg	33	log agg/c1	89
log TS5/c6	78	Log TS5/c6	78	log agg/c2	33	log TS4/c2	75
total	1056		1071		1056		1056

less than 1.8 for three of the six plates, and these samples were excluded from data analysis. Subsequent reverse transcription, RT-PCR and data analysis were performed as described above. The effect of passage number was compared with a one-factor GLM and Tukey's test for pairwise comparison. Similarly to the tissue samples, LDA and ordinal logistic regression (OLR) models were used to determine predictors of passage number, with the assumption that there was a progression with increasing number of passages. The ratios identified to best predict tissue origin from the tissue sample data set were separately tested using LDA.

Results

Regional discrimination

Medial-lateral differences were detected only for collagen II ($p = 0.036$) and aggrecan ($p = 0.013$) in a statistical model including both meniscus and cartilage tissue samples. For both genes, expression was higher for the lateral aspect. Zonal differences were exhibited in collagen I, II, VI, aggrecan, and decorin. As expected, aggrecan and collagen II levels were much higher in cartilage than in meniscus, while collagen I was lower. Within the meniscus, the inner region showed significantly higher expressions of aggrecan ($p = 0.0012$) and collagen II ($p < 0.001$) but significantly lower expression of decorin ($p = 0.0153$) than did the outer region. The means of the middle region were intermediate between the inner and outer regions for these genes, but were significantly different from both the inner ($p < 0.001$) and outer regions ($p = 0.0149$) only for collagen II, and significantly different from the outer region for aggrecan ($p = 0.0441$). No significant variation among zones were detected for versican ($p = 0.96$), biglycan ($p = 0.081$), TS4 ($p = 0.74$), or TS5 ($p = 0.23$) (Fig. 4). Interactions between aspect and zone were not significant for any gene, indicating comparable patterns for medial and lateral tissues.

Linear discriminant analysis and multinomial logistic regression identified the same set of ratio predictors, validating the robustness of the selection. The three genes that appeared most frequently in the top 5 % combinations

Table 3. Cross-validated predictions of (a) Tissue zonal origin and (b) chondrocyte passage number using the linear discriminant analysis models.

(a)	TRUE REGION			
PREDICTED REGION	**C**	**I**	**M**	**O**
C	12	0	1	0
I	0	12	1	1
M	0	0	7	2
O	0	0	3	9
Total N	12	12	12	12
Correct N	12	12	7	9

(b)	TRUE PASSAGE				
PREDICTED PASSAGE	**0**	**1**	**2**	**3**	**4**
0	6	0	0	0	0
1	0	5	1	0	0
2	0	1	3	2	0
3	0	0	2	4	0
4	0	0	0	0	3
Total N	6	6	6	6	3
Correct N	6	5	3	4	3

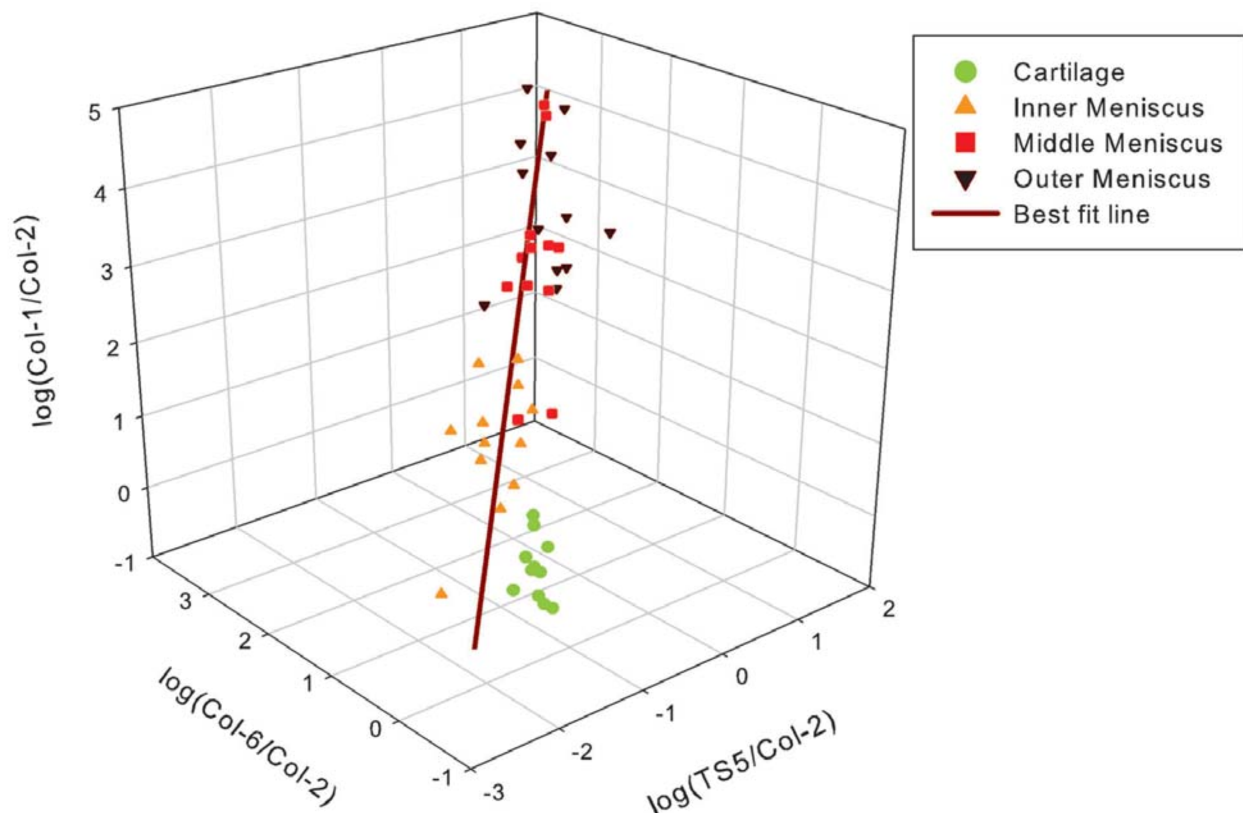

Fig. 5. Tissue samples plotted in the three-dimensional coordinate system defined by the best three ratio predictors of tissue origin: log(Col-6/Col-2), log(TS5/Col-2) and log(Col-1/Col-2). The line indicates the best fit regression through all sample data points. Note the clear clustering of data points for different regions of origin and the apparent progression from cartilage to inner, middle, and outer meniscus.

of two and three ratios were Col-2, Col-6, and TS5 (Table 2). With cross-validated discriminant analysis, the combination of these three genes correctly identified 81 % of the samples. The ratios appearing most frequently in the best (top 5 %) predicting three-ratio LDA models were Col-6/Col-2, TS5/Col-2, and TS5/Col-6. In the best two-ratio predictor models, the three ratios included Col-1/Col-2 instead of TS5/Col-6, which is simply a combination of Col-6/Col-2 and TS5/Col-2. Thus, the combination of Col-6/Col-2, TS5/Col-2, and Col-1/Col-2 were taken as the best combination set of ratios. This set of ratios correctly identified 83 % of the samples in cross-validated discriminant analysis (slightly better than the best set of individual genes), with the most common misidentification being between the middle and outer regions (Table 3a). Viewed on a 3D scatter plot of these three ratios, clear clustering and distinctions between the zones were evident (Fig. 5).

Passage discrimination

Aggrecan and collagen II both decreased dramatically (p = 0.0044 and p = 0.0026, respectively) past the freshly isolated stage of P0 and steadily declined with further passage (Fig. 6). On the other hand, collagen I increased until the second passage but significantly decreased with further passage. Similarly, decorin also showed an increasing and decreasing trend where it showed highest expression during P1. No significant changes were observed across passages for collagen VI, versican, or biglycan. The two aggrecanases showed dependence on passage number; however, no clear trend could be defined.

OLR analysis of all possible combinations of three ratios showed Agg/Col-1 to be the dominantly appearing ratio over all others in the top performing 5 % in the discrimination of passage number, while the LDA model identified the ratios TS4/Agg followed by Agg/Col-1 and TS4/Col-2. This set of three gene ratios correctly identified the passage number for 78 % of the samples, and all misidentifications were within one passage of the actual passage number (Table 3b). The set of ratios that performed best for identifying zonal origin of tissue samples, Col-6/Col-2, TS5/Col-2 and Col-1/Col-2, did not perform as well, with 67 % correct identification and all misidentifications also within one passage (Fig. 7).

Discussion

Achieving heterogeneity of the native meniscus will be important to the functionality of engineered tissue, but objective assessment of the success of meniscal tissue engineering efforts is difficult without quantitative metrics of meniscal phenotypes. Although differences among meniscal cells from different regions have been generally described, no single marker definitively distinguishes between meniscal cells and chondrocytes or among cells from different meniscal regions. Statistical analyses of gene expression profiles integrating expressions of multiple genes may provide a quantitative basis for distinguishing

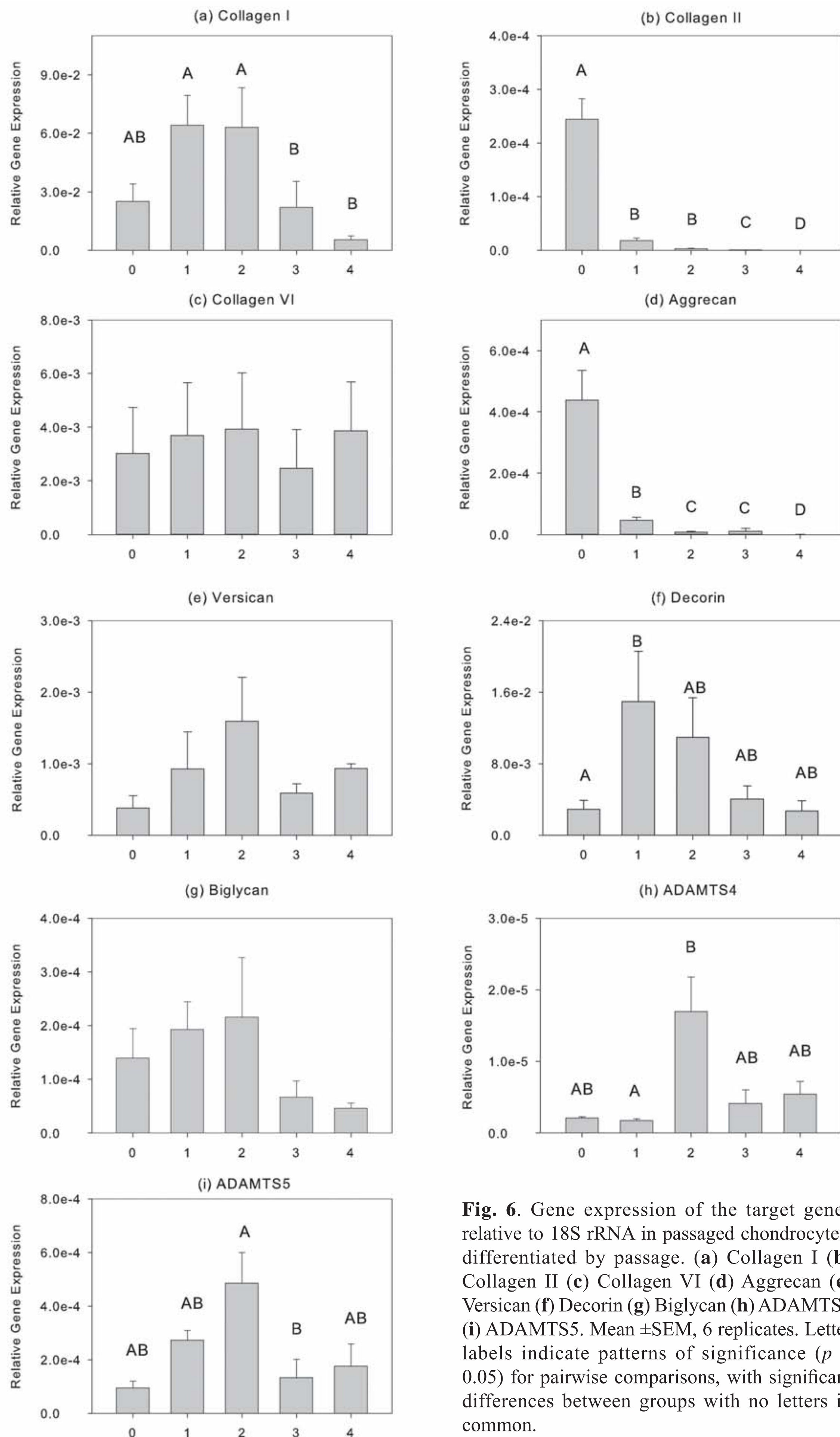

Fig. 6. Gene expression of the target genes relative to 18S rRNA in passaged chondrocytes, differentiated by passage. (**a**) Collagen I (**b**) Collagen II (**c**) Collagen VI (**d**) Aggrecan (**e**) Versican (**f**) Decorin (**g**) Biglycan (**h**) ADAMTS4 (**i**) ADAMTS5. Mean ±SEM, 6 replicates. Letter labels indicate patterns of significance ($p \leq 0.05$) for pairwise comparisons, with significant differences between groups with no letters in common.

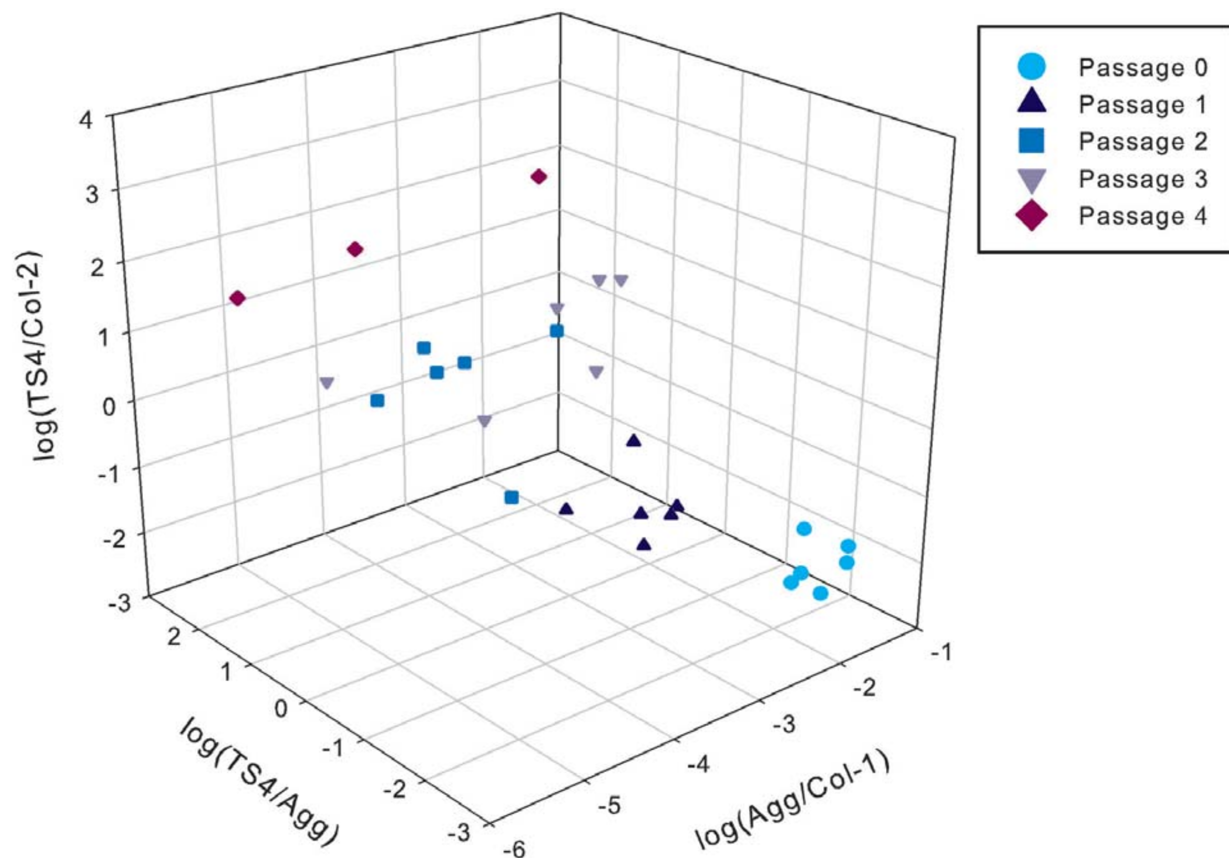

Fig. 7. Passaged chondrocytes in the best passage predictor space of log(Agg/Col-1) *vs*. log(TS4/Agg) *vs*. log(TS4/Col-2). Although not as distinct as with the tissue zonal origin, there is an apparent progression with increasing passage to higher TS4/Col-2 and TS4/Agg and lower Agg/Col-1.

regional phenotypes, establishing a foundation for describing changes during disease and defining targets for tissue engineering strategies.

In this study, the heterogeneity of meniscal cell phenotypes was examined using combined gene expression profiles of different types of collagens, proteoglycans and aggrecanases, revealing regional patterns within the meniscus that have not previously been described. In addition to examining the medial and lateral meniscus separately, each tissue was separated into three zones, providing more zonal detail, including the transitional zone between inner and outer regions. In particular, the results of this study present new information on the regional distribution of Col-6, VS, TS4 and TS5 gene expression level in the meniscus which complement previous studies at the protein level (Marsano *et al.*, 2006; Chevrier *et al.*, 2009). The major large proteoglycan, aggrecan, was concentrated toward the inner part of the meniscus, which is reflective of the compressive stresses experienced by that region. In contrast, the small proteoglycan biglycan did not significantly vary with zone and decorin showed the opposite pattern from aggrecan, with greater expression in the outer region. This finding may be related to the role of decorin in organizing collagen fibers and maintaining the integrity of fibrous connective tissue (Reed and Iozzo, 2003), as the outer region mostly consists of circumferentially oriented collagen fiber bundles. These patterns are consistent with variations in matrix composition described in semi-quantitative studies of small proteoglycans in the different regions of the meniscus (Nakano *et al.*, 1997; Scott *et al.*, 1997), particularly a greater ratio of biglycan to decorin in the outer meniscus. Interestingly, versican did not show any particular dependence on the region. Versican has been suggested to play a role in cell adhesion, migration and proliferation (Wight, 2002) and thus may be more dependent on the development stage of the tissue, as shown in a study comparing skeletally immature and mature rabbits (Hellio Le Graverand *et al.*, 1999). Collagen VI, a main constituent of the pericellular matrix (Poole *et al.*, 1988; McDevitt and Webber, 1990), exhibits higher expression in the inner region, which can be attributed to the fact that the more fibroblast-like cells of the outer region lack an organized pericellular matrix (McDevitt *et al.*, 2002).

In addition to examining patterns of individual genes, we also examined the ability to distinguish the zonal origin based on combined profiles of multiple genes or gene expression ratios. The resultant best tissue predictor ratios from LDA and MLR, Col-6/Col-2, TS5/Col-2, and Col-1/Col-2, suggest that a balance between certain genes may be necessary to produce a certain type of fibrocartilage. Col-1/Col-2 has already been extensively used as a differentiation marker to characterize the degree of chondrogenesis (Martin *et al.*, 2001; Gebhard *et al.*, 2003; Marlovits *et al.*, 2004). On the other hand, the other two ratios have not been examined previously, and the extent to which the

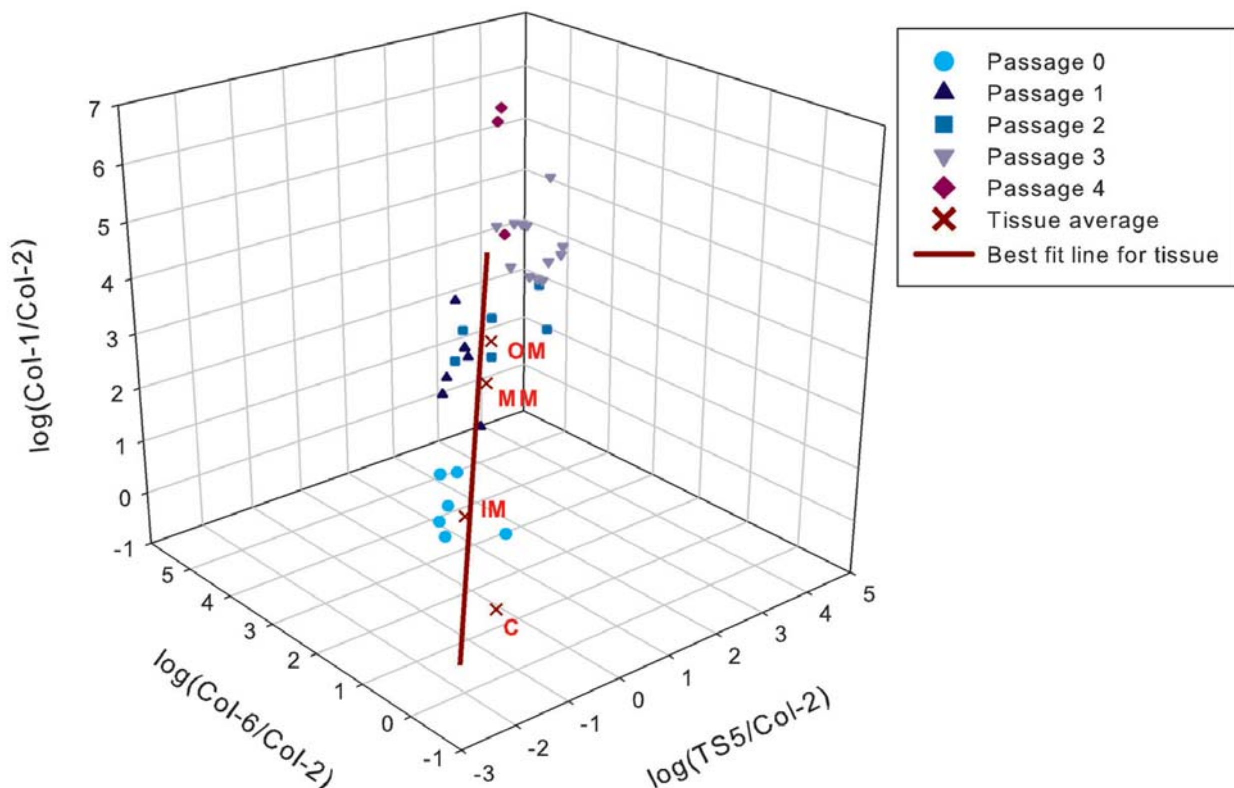

Fig. 8. Passaged chondrocytes in the best tissue predictor space of log(Col-6/Col-2) *vs*. log(TS5/Col-2) *vs*. log(Col-1/Col-2) compared to the tissue samples. Neither freshly isolated (P0) nor passaged chondrocyte groups closely approximate the cells from cartilage or meniscus tissue. C: Cartilage, IM: Inner Meniscus, MM: Middle Meniscus, OM: Outer Meniscus.

ratios of Col-6 or TS5 to Col-2 have biological significance needs to be further studied. The combination of these gene expression profiles in a quantifiable manner may provide some insight into co-regulatory activities of genes that are critical to matrix composition. As no gene can be solely responsible for the general matrix composition and characteristics of a given tissue, the differences between cartilage and meniscus can be more clearly defined when multiple genes are analyzed simultaneously. Such analysis using ratios rather than individual gene expression levels has additional advantage in eliminating dependence on the stability of the housekeeping gene, as the ratios represent the fold difference between different genes within a given sample and the normalization factor is cancelled out. For heterogeneous tissues like the meniscus, identifying this kind of markers can further distinguish cartilage-like regions from those that exhibit more fibrous characteristics, and lead to more tailored fibrocartilage tissue engineering approaches In addition, it could provide insight to the functional difference between the two types of tissues as changes in these gene expression markers should be related to functional changes such as mechanical properties or the composition of the produced extracellular matrix.

As a preliminary proof of concept, we additionally evaluated the expression profiles of passaged chondrocytes, a possible source for meniscal tissue engineering. Although this preliminary application involved cells derived from a single animal, our results were in agreement with the widely reported dedifferentiation of chondrocytes with passage; Col-2 rapidly decreased while Col-1 either increased continuously or stabilized after a certain number of passages (von der Mark *et al.*, 1977; Darling and Athanasiou, 2005; Lin *et al*., 2008). However, the set of best predictors for passage number did not match the best set of tissue origin predictors. This suggests that the changes in cell phenotype induced by passaging may be distinct from the differences among the native cells from different regions. Within the 3D space defined by the gene expression ratios providing the best identification of tissue origin, expanded cells became less like either cartilage or meniscus samples with each sequential passage (Fig. 8). In fact, the “distance” between passage 4 chondrocytes and outer zone meniscus was comparable to the distance between outer zone meniscus and native articular cartilage. Thus, while both outer meniscal cells and passaged chondrocytes have lower aggrecan and collagen II and higher collagen I than primary chondrocytes, these cells should not necessarily be viewed as similar. Efforts have been made to reverse the effects of monolayer expansion, through a variety of techniques such as surface protein coatings and return to 3D culture (Benya and Shaffer, 1982; Lee *et al.*, 2003; Darling and Athanasiou, 2005; Gunja and Athanasiou, 2007). Quantitative analysis of gene expression profiles would allow quantitative assessment of the success of such strategies in approaching a desired native cell phenotype.

The gene expression profiles of the passaged chondrocytes were obtained as a proof of concept in

using the tissue predictors as evaluators of the degree of dedifferentiation and should not be viewed as definitive descriptors of chondrocytes in general. While cells exhibiting the defining characteristics of the native cell phenotype would be expected to yield ECM comparable to that of the native tissue, the relationship between the identified gene expression patterns and the resulting ECM composition, reflecting tissue function, needs to be further explored. In addition, as the passaged chondrocytes in this study were derived from only one animal donor, the variations in this study represent procedural variation related to replicate cultures and do not represent variability across a population. Further studies including multiple animals are clearly necessary.

It should also be noted that the passaged chondrocytes could be evaluated using the tissue predictors because their gene expression profiles were obtained in exactly the same manner as those derived from the native tissue. The analysis conditions should be always kept constant between the samples used to construct the model and those tested for proper comparison based on gene expression profiles. The predictors obtained may depend on such conditions, including the amount of RNA used or the concentration of primers in performing RT-PCR. The specific types of samples used, the types and number of genes analyzed will also influence the results.

In this particular study, we used samples extracted from immature bovine sources and nine genes, which were selected for their importance in extracellular matrix production and regulation. We chose collagens and proteoglycans, the two main components of meniscus and cartilage. Aggrecanases were additionally included, as a previous study from this lab has shown aggrecanases-mediated aggrecanolysis may contribute to the development of the meniscus while in articular cartilage, it is associated with degeneration (Wilson *et al.*, 2009). Possible microarray studies with additional genes may provide some insight in the defining characteristics of the various cartilage types. However, such an approach should be undertaken carefully and take into account pre-existing biological information as screening a massive number of genes may yield results that are difficult to interpret and may overlook known relevant genes (Ochi *et al.*, 2003). Differences will likely exist for human samples or for adult and osteoarthritic tissues as well compared to the bovine samples presented here. The ultimate goal should be to establish reliable markers of healthy human tissue, to which other species or disease conditions could be compared.

This study describes a novel, analytical approach to characterize a cartilaginous tissue and create a profile of markers that could serve as a standard in comparing different types of tissues and evaluating various tissue engineering strategies, as shown with the passaged chondrocytes. Future studies could include validating this approach and applying it to the context of cellular changes during the development or treatment of pathologies such as osteoarthritis, or cellular changes during *in vitro* conditioning or *in vivo* implantation of tissue engineered replacements. The results are consistent with the view that cells from articular cartilage and the fibrocartilaginous meniscus can be viewed on a continuous spectrum with progressive, consistent variations in phenotype (Benjamin and Ralphs, 2004; Hoben *et al.*, 2007). From this perspective, fibrocartilage has been described as a transitional tissue that lies between hyaline cartilage and dense, fibrous connective tissue (Benjamin and Ralphs, 2004). The analysis presented in this study could clearly be extended to include fibrous tissues such as ligaments and tendons to expand the range of this spectrum that can be evaluated. Other genes known to play critical roles in cartilage and fibrocartilage formation and regulation, such as metalloproteinases, SOX9 or transforming growth factors (Dean *et al.*, 1989; Bi *et al.*, 1999), as well as genes such as collagen X suggesting undesirable hypertrophic differentiation, could also be included. Refinement of this approach will provide a broad context for quantitatively describing cellular changes in different meniscal tissue engineering strategies, and may assist in identification of optimal cell sources and culture conditions leading to clinically viable treatment strategies.

Acknowledgements

This research was funded by NIH AR052861, AR054938 and a Stanford Bio-X Fellowship. We thank Dr. Eric Vanderploeg for providing the images of cell morphology in Fig. 1, and we thank Dr. Christopher Wilson for providing the images of sulfated glycosaminoglycan distribution in Fig. 2.

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Discussion with Reviewers

Reviewer I: What changes in gene expression ratios would you predict in menisci from osteoarthritic knees?

Authors: As with articular chondrocytes, it is likely that meniscal cells exhibit responses characteristic of both matrix repair and matrix destruction at various stages of osteoarthritis (OA). In order to properly characterize the changes in OA, the pool of candidate genes may need to be expanded to include genes involved in these processes. Animal studies have examined some patterns of gene expression following anterior cruciate ligament (ACL) transection, with elevation of meniscal collagen-I and –II expression in ACL-deficient rabbits (Hellio Le Graverand *et al*., 2001, text reference) and sustained elevation of collagen-I and -VI expression in ACL-deficient dogs (Wildey *et al*., 2001, additional reference). Similarly, elevated expression of procollagens-I, -II and -III were found in OA human menisci, although this was not accompanied by elevated rates of collagen synthesis (Katsuragawa *et al*., 2010, additional reference). Outer regions of degenerate menisci have been described as more cartilage-like than normal tissue, with a higher proportion of chondrocytic cells and regions of elevated sulfated glycosaminoglycan content (Ishihara *et al*., 2009; Declercq *et al*., 2011; Pauli *et al*., 2011, additional references), suggesting that elevation in proteoglycan gene expression may occur during OA development. OA menisci exhibit increased fragmentation of multiple proteoglycans (Melrose *et al*., 2008, additional reference), and both ADAMTS and MMP proteases have been implicated in meniscal proteoglycan degradation (Ishihara *et al*., 2009; Fuller *et al*., 2012, additional references; Wilson *et al*., 2009, text reference). It is difficult to predict *a priori* the regional changes in meniscal gene expression ratios in OA, or which ratios may be most useful in describing early degenerative changes. While studies focused specifically on OA menisci will be required to identify the most appropriate subset of candidate gene expression ratios, we believe that a similar profiling approach involving genes related to both anabolism and catabolism will aid in the quantitative analysis of degenerative changes and responses to potential therapeutic approaches.

Reviewer II: How would you test this system to see if the profile you developed has functional significance?

Authors: In order to relate the proposed gene expression profiles to tissue function, the connection between gene expression levels and actual extracellular matrix (ECM) production needs to be further explored. For collagen I and collagen II, two of the four genes that comprise the identified gene ratio markers in this study, the trends in the gene expression level on regional distribution have shown to translate into trends in the protein level. Many studies have shown that the outer zone of the meniscus has higher content of collagen I and lower content of collagen II than the inner meniscus (Cheung, 1987; Kambic and McDevitt, 2005; Chevrier *et al*., 2009, text references). However, a more quantitative approach where matrix production can be analyzed and linked back to the gene expression levels is needed. For example, matrix produced from cells with the inner meniscal cell phenotypes, as defined by the gene expression profile, can be compared to the matrix produced from cells with the outer cell phenotypes. Quantitative analysis of ECM composition alone, however, is not sufficient to evaluate functional metrics such as mechanical properties, which are dependent on matrix structure. The arrangement of collagen fibers and ECM assembly may

involve maintaining some level of catabolic activity, and exploring this area in the context of gene expression profiles will also be important in evaluating its functional significance. Unlike articular cartilage, high aggrecan turnover has been observed in the middle and outer regions of the immature bovine meniscus (Wilson *et al*., 2009, text reference), indicating that the homeostatic balance that needs to be achieved for the meniscus is different from that of cartilage.

Reviewer II: The authors propose a goal of achieving heterogeneity in functionally engineered meniscus that reflects heterogeneity of native meniscus. How would they use their profiling system to achieve this?
Authors: The profiling system that we propose would be used as a tool that can be used to quantitatively describe the phenotype of the cells. In this study, we limited the number of genes to investigate and chose a subset of three ratios; however, it may be necessary to develop a more comprehensive set of metrics based on other genes of interest in order to fully characterize the cells from different regions of the meniscus. A quantitative measure representing the degree of distinction in cell phenotype can be defined as the "distance" between the samples in any best predictor n-dimensional space. Each gene expression ratio that contributes to this distance could be normalized to the largest difference between the different cell types within the native heterogeneous tissue. For example, in the meniscus, the normalization factor would be based on the difference between inner and outer meniscal cells. One could then define a numerical metric of outcome based on the weighted distance in this n-dimensional space, and explore different culture strategies based on the ability to minimize the distance from the desired outcome. Much additional study would be required to validate such a measure, but the ability to provide an objective, quantitative measure for comparing strategies would be quite valuable.

Reviewer II: Would it be beneficial to increase the panel of gene ratios for more accurate definition of chondrocyte phenotype? If so can the authors speculate on the type of genes that might represent further targets for this approach?
Authors: In this paper, we used combinations of gene expression ratios rather than individual genes to establish an objective metric for distinguishing cells from different regions of the normal meniscus and cartilage. While meniscal phenotypes have been qualitatively described in various studies, we establish here that this approach can not only distinguish between cells from cartilage and meniscus, but can also discriminate among meniscal cells from different tissue regions. An obvious direct application of this approach would be to analyze cells in engineered meniscal tissue, allowing objective analysis of the extent to which various *in vitro* or *in vivo* strategies can drive cells towards replicating the native phenotype. Selection of any subset of markers will always leave out other potentially important or interesting markers, but we believe that the profiling approach described in this manuscript will allow a more integrated perspective on the maturation of engineered meniscus or cartilage tissues, which commonly are analyzed based on a limited set of individual markers (most commonly, collagens-I, -II and aggrecan, or the collagen-I/collagen-II ratio).

That being said, the candidate genes for characterization of phenotypic differences or changes would differ depending on the specific topic under study, and would be guided by the existing knowledge of a particular issue. As discussed above, implementing the profiling approach to study OA changes would likely begin by considering genes involved in matrix synthesis and matrix degradation, and could also include genes in important cytokine signaling pathways. In studies involving stem cell-based cartilage or fibrocartilage tissue engineering, genes associated with the target phenotypes (e.g., collagen-I, collagen-II, aggrecan) would likely be combined with genes associated with undesired phenotypic changes (e.g., collagen X, a marker of hypertrophy) or stem cell markers that would be expected to decrease in a successful strategy. Through pilot studies, the profiling approach discussed in this manuscript will help to identify which of many candidate genes have the greatest capacity to identify differences relevant to a given application.

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