ARTICLE

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Creating a Spectrum of Fibrocartilages Through Different Cell Sources and Biochemical Stimuli

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ABSTRACT: In this study a scaffoldless approach was employed with two different cell sources and biochemical stimuli to engineer a spectrum of fibrocartilages representative of the different regions of the knee meniscus. Constructs composed of 100% fibrochondrocytes (FC) or a 50:50 co-culture of fibrochondrocytes and chondrocytes (CC) were cultured in 10% fetal bovine serum medium or serum-free "chondrogenic" medium, each ±10 ng/mL TGF-β1 (+T). Constructs from these two cell groups and four culture conditions were cultured for 6 weeks. By varying the cell type and presence of the growth factor, GAG per dry weight of the constructs spanned that of native tissue, ranging 16–45% and 1–7% in the CC and FC groups, respectively. Collagen density was most dependent on cell type and was significantly lower than tissue values. The collagen I/II ratio could be manipulated by cell type and serum presence to span the native range, from 3.5 in the serum-free CC group to over 1,000 in the FC groups treated with serum-containing medium. Using the CC cell group in the presence of serum-free medium dramatically increased the compressive stiffness to 128 ± 34 kPa, similar to native tissue. Similarly, serum-free medium or TGF-β1 treatment enhanced the tensile modulus by an order of magnitude, up to 3,000 kPa. Using two cell sources and manipulating biochemical stimuli, a range of fibrocartilaginous neotissues was engineered. Fibrocartilages such as the knee meniscus are characterized by heterogeneity in matrix and functional properties, and this work demonstrates a strategy for recreating these heterogeneous tissues.

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KEYWORDS: fibrocartilage; fibrochondrocytes; chondrocytes; tissue engineering; transforming growth factor; serum-free

Introduction

As long as skiing and rugby remain popular pastimes, a tissue engineered meniscus will be an important goal in orthopedic research. Recent years have seen clinical trials examining the collagen meniscus implant (Steadman and Rodkey, 2005) and a wealth of in vitro and animal studies using natural and artificial scaffolds (Buma et al., 2004; Hoben and Athanasiou, 2006). These implants have shown success in decreasing pain and disability to some extent; however, a fully functional meniscal tissue replacement has yet to be realized (Rodkey et al., 1999; Steadman and Rodkey, 2005; Stone et al., 1997). A large component to the challenge of meniscal tissue engineering is the inherent heterogeneity of the tissue. A collagen type I-rich outer zone with high tensile properties is contrasted with a collagen I and II containing inner zone with greater compressive properties, and both are necessary to fully recreate a functional tissue replacement (Cheung, 1987; Sweigart and Athanasiou, 2001). A step-wise approach would consist of first determining the individual culture conditions (cells, medium, and growth factors) capable of yielding this diverse spectrum of properties. These components could then be used for specific injuries or integrated to form a whole replacement tissue.

While much work has been done on scaffold-based therapies, advances in cell culture technology and the growing field of stem cell biology point toward cell-based therapies. Fibrochondrocytes have been used many times in the past (Aufderheide and Athanasiou, 2005; Hidaka et al., 2002; Ibarra et al., 1997; Isoda and Saito, 1998; Martinek et al., 2005; Mueller et al., 1999; Pangborn and Athanasiou, 2005b; Walsh et al., 1999) and can be used to create constructs with a predominantly collagen type I matrix, similar to the outer meniscus (Hoben et al., 2006). For the inner meniscus, chondrocytes have recently received increased attention (Peretti et al., 2001, 2004; Puelacher et al., 1994; Weinand et al., 2006). With the goal of increasing the collagen type II and proteoglycan components of the neotissue, the two cell types have been co-cultured to create an extracellular matrix composed of collagen I, collagen II, and proteoglycans, thus

Tissue engineering a spectrum of fibrocartilages.

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expanding the breadth of tissue characteristics that could be generated (Hoben et al., 2006). As the technology of stem cell differentiation continues to advance, any tissue engineering strategy developed with primary cells may conceivably be translated to stem cells.

Fetal bovine serum (FBS) content is another cell culture component that can be manipulated to change the neotissue's characteristics. FBS, although largely undefined, enhances fibrochondrocyte and chondrocyte growth, but in recent years a "chondrogenic" chemically defined medium (Johnstone et al., 1998; Mackay et al., 1998; Yoo et al., 1998) has been used to increase proteoglycan synthesis (Adkisson et al., 2001; Chua et al., 2005; Fortier et al., 1997; Giannoni et al., 2005; Schwartz et al., 1974; Yates et al., 2005) and may lend itself to producing the more cartilaginous portion of the fibrocartilage spectrum. This serum-free medium uses insulin-transferrin-selenium (ITS+) and dexamethasone instead of FBS, and the effects of the insulin in ITS+, as well as the absence of the many undefined growth factors and other proteins normally found in FBS, may result in profoundly different effects on the growth of cell-based constructs. This serum-free formulation also poses potential advantages to later clinical translation in that there are no animal products that may cause immune reactions (Johnson et al., 1991; Meyer et al., 1988), nor are there the potentially large batch-to-batch variations (Yaeger et al., 1997) commonly seen with FBS.

Additional biochemical stimuli can be used in concert with the cells and serum level to enhance and modulate tissue regeneration. Several studies in meniscal tissue engineering have demonstrated the superiority of transforming growth factor β1 (TGF-β1) over a variety of growth factors including IGF-1, PDGF, bFGF, and BMP-7 (Imler et al., 2004; Lietman et al., 2003; Pangborn and Athanasiou, 2005a,b). In both 2D and 3D studies, 5–10 ng/mL of TGF-β1 appears to provide a saturation effect for enhancing collagen and GAG synthesis (Imler et al., 2004; Lietman et al., 2003; Pangborn and Athanasiou, 2005a). In particular, by increasing collagen production, this growth factor could serve to enhance the tensile properties of the neotissue to better replace the outer zone of the meniscus. While serum and cell source change the character of the neotissue, use of this growth factor in a tissue engineering approach could result in enhanced extracellular matrix synthesis and corresponding functional properties.

In this study, we take a scaffoldless approach, termed self assembly (Hu and Athanasiou, 2006), to examine multiple cell types and biochemical treatments for fibrocartilage tissue engineering. Self assembly has been used successfully in the past to create neofibrocartilage constructs and moreover, avoids the challenges of scaffold-based studies (Hoben et al., 2006). In this cell-based approach, we utilized fibrochondrocytes (FC) and co-cultures of fibrochondrocytes and chondrocytes (CC) to create neofibrocartilage constructs. We then evaluate the application of 10 ng/mL TGF-β1 in either a 10% FBS medium (FM) or a serum-free chondrogenic medium (CM) (Table I). We hypothesize that these different cell sources will respond with distinct changes in matrix production to the different biochemical stimuli, and a range of neofibrocartilages with different histological, biochemical, and functional properties will be created. In creating such a spectrum of constructs, the heterogeneous regions of the meniscus can be specifically regenerated as an exciting step forward in creating a functional tissue replacement.

Table I. Experimental groups.

FC-FM	100% fibrochondrocytes in 10% FBS medium
FC-FM + T	100% fibrochondrocytes in 10% FBS medium + TGF-β1
FC-CM	100% fibrochondrocytes in serum-free chondrogenic medium
FC-CM + T	100% fibrochondrocytes in serum-free chondrogenic medium + TGF-β1
CC-FM	50:50 co-cultures in 10% FBS medium
CC-FM + T	50:50 co-cultures in 10% FBS medium + TGF-β1
CC-CM	50:50 co-cultures in serum-free chondrogenic medium
CC-CM + T	50:50 co-cultures in serum-free chondrogenic medium + TGF-β1

Methods

Cell Isolation and Seeding

Fibrochondrocytes and chondrocytes were harvested from the inner two-thirds of the medial and lateral menisci and the distal femora, respectively, of approximately 1-week-old male calves (Research 87, Boston, MA) less than 36 h after slaughter. Tissue was minced and digested overnight with 0.2% collagenase II (Worthington, Lakewood, NJ) in the FBS culture medium. The FBS culture medium (FM) is DMEM with 4.5 g/L-glucose and L-glutamine (Gibco, Grand Island, NY), 10% FBS (Biowhittaker, Walkersville, MD), 1% fungizone, 1% Penicillin/Streptomycin, 1% non-essential amino acids, 0.4 mM proline, 10 mM HEPES, and 50 μg/mL L-ascorbic acid. To obtain sufficient cells for the experiment, cells from multiple animals were frozen at −80°C and later pooled to create the constructs. In addition to FM, the CM was composed of DMEM with 4.5 g/L-glucose and L-glutamine, 1% fungizone, 1% Penicillin/Streptomycin, 1% non-essential amino acids, 0.4 mM proline, 10 mM HEPES, and 50 μg/mL L-ascorbic acid, 1% ITS+ (BD Biosciences, San Jose, CA), and 100 nM dexamethasone. TGF-β1 (Peprotech, Rocky Hill, NJ) was diluted in sterile water to a concentration of 2,000 ng/mL and added to media to obtain a 10 ng/mL dilution. Media containing TGF-β1 will be indicated with "+T."

Cylindrical agarose molds with a 5 mm diameter were made as previously described in a 48 well plate (Hoben et al., 2006). To each agarose well, 5.5×10^6 cells were added in 150 μL of appropriate medium with or without TGF-β1. The 100% fibrochondrocyte groups will be indicated "FC" and the 50:50 fibrochondrocyte/chondrocyte co-cultures will be indicated "CC." After 5 h, 450 μL of additional

medium was added. Constructs were given daily complete media changes of 500 μL. After 2 weeks of culture, the constructs were gently lifted out of the wells and placed in agarose coated wells of a 6 well plate and given 500 μL media change every day. Constructs were taken out after 3 and 6 weeks total culture time for evaluation. Gross measurements including diameter, thickness, and wet weight were taken, and then constructs were split into 2–3 sectors to perform the different analyses.

Histology and Immunohistochemistry

Samples were frozen and sectioned at 12 μm. Safranin-O and fast green were used to examine GAG distribution and picrosirius red staining was used to analyze collagen distribution. Immunohistochemical analysis was performed by fixing sections in chilled acetone, rehydrating, treating with 3% H_2O_2 in methanol, and blocking with horse serum. The following primary antibodies were diluted in PBS and applied for 1 h: 1:300 rabbit anti-human collagen VI pAb (US Biological, Swampscott, MA) and 1:200 mouse anti-human anti-αSMA mAb (Sigma, St.Louis, MO). Visualization using a secondary biotinylated antibody, the ABC reagent, and DAB was performed using the Vectastain kit (Vector Laboratories, Burlingame, CA), and counterstaining was done with Harris's Hematoxylin. Sections of articular cartilage, meniscal fibrocartilage, aorta tissue, and skin tissue were run as positive controls, while samples were stained without application of the primary antibody as negative controls.

Quantitative Biochemistry

Samples were lyophilized for 48 h and digested in 125 μg/mL papain (Sigma) for 18 h at 60°C. Cell number was determined using Picogreen® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). A hydroxyproline assay was performed to gauge total collagen using bovine collagen standards (Biocolor, Newtonabbey, Northern Ireland). Sulfated GAG was measured with the Blyscan GAG Assay Kit (Biocolor).

Samples for enzyme linked immunosorbent assay (ELISA) were digested in papain at 4°C for 4 days and then a 1/10 volume of elastase (Sigma) solution in 10× TBS buffer was added to achieve a concentration of 0.1 mg/mL elastase. Samples were allowed to digest an additional 48 h. Between each incubation step in the ELISA, plates were washed using PBS with 0.05% Tween-20. For the collagen I ELISA, plates were incubated overnight at 4°C with 1:400 mouse anti-human capture mAb (US Biological), blocked with 2% bovine serum albumin (BSA), samples and standards were added, then exposed to rabbit anti-human pAb (US Biological). Finally, goat anti-rabbit pAb was added, and the color developed in TMB as a liquid peroxidase substrate. The collagen II ELISA was performed using the Chondrex (Redmond, Washington) capture mAb, the Chondrex biotinylated mAb, and streptavidin peroxidase was used with TMB to develop the color. Absorbance was read at 450 nm in a Genios plate reader (Tecan, San Jose, CA).

Biomechanics

The disc-shaped samples were cut to a dog-bone shape using biopsy punches (Miltex, York, PA) and then glued into paper frames that were placed into the grips of a uniaxial materials testing machine (Instron 5565, Norwood, MA). Strain was applied at 1% of the gauge length (ranged 0.5–1 mm) per second, until failure. Samples were kept hydrated during testing with PBS. Compression testing was done on an automated indentation apparatus (Athanasiou et al., 1994). A 3 mm biopsy punch was taken from the center of the sample, attached to the sample holder with cyanoacrylate glue, and submerged in PBS solution. The sample was positioned to be perpendicular to the porous indenter tip and the tip diameter was chosen to be <30% of the construct diameter, if possible, from 0.7 to 1.0 mm. The specimen was automatically loaded with a tare mass of 0.2 g and allowed to reach tare creep equilibrium, defined as deformation $<10^{-6}$ mm/s or a maximum tare time of 25 min. When tare equilibrium was reached, a step mass of 0.7 g was applied. Displacement of the sample surface was measured until equilibrium was reached or a maximum creep time of 60 min elapsed. The step load was then removed, and the displacement recorded until equilibrium was again reached. The intrinsic mechanical properties of the samples were then determined using the linear biphasic theory (Mow et al., 1980). All testing was performed at room temperature.

Statistics

For all quantitative biochemistry and biomechanical measurements, 4–6 samples were analyzed. All data were compiled as mean ± standard deviation and a two-factor ANOVA was used when comparing means for time point and biochemical treatment (base media ± TGF-β1). Cell type was used as a block. If analysis showed a significant difference, a Tukey's post hoc analysis was performed to compare sample sets. A significance level of $P < 0.05$ was used in all statistical tests performed.The R^2 values were calculated using linear regression of the data sets under evaluation (Rosner, 2000).

Results

Gross Characteristics

Within 12 h of seeding in the agarose wells, all groups formed 5 mm discs. After 3 and 6 weeks of culture, all of the constructs had a cartilaginous appearance; those grown in FM had a smooth gleaming surface, while the other groups tended to have a rougher surface (Fig. 1). The CC-FM constructs were the largest at 6.7 ± 0.3 mm in diameter and

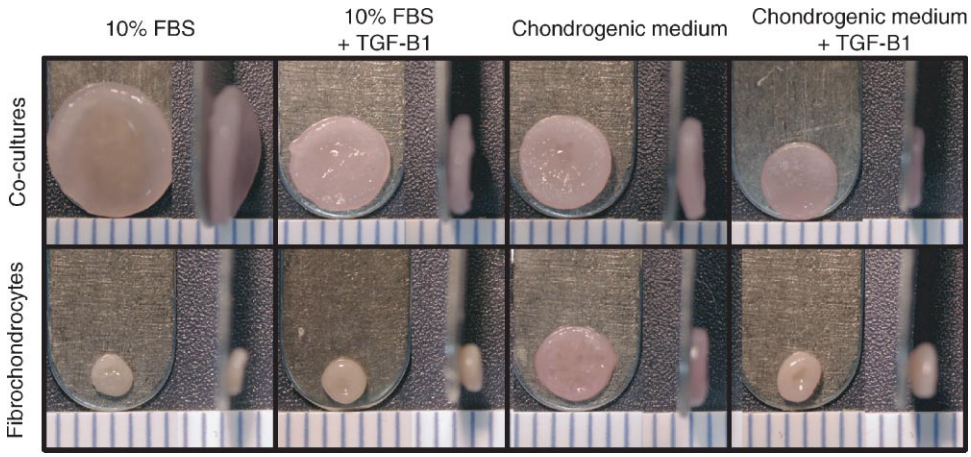

Figure 1. Gross photographs of constructs following 6 weeks of culture, top and side views. The CC constructs grew beyond the original dimensions, while the FC constructs show contraction from the original 5 mm diameter. The constructs have a cartilaginous appearance and are of clinically relevant dimensions. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

0.68 ± 0.13 mm in thickness at 6 weeks (Table II). Addition of the growth factor resulted in significant reductions ($P < 0.0001$) in the diameters of CC constructs cultured in either base medium. The FC-CM group contained the largest constructs of the FC groups, which at 3.4 ± 0.4 mm was smaller than any of the CC constructs at either time point. While the CC groups grew in diameter over time, the FC constructs remained the same diameter over the time points following the initial contraction from 5 mm. The CC-FM constructs had the greatest wet weight, nearly double that of the next highest, CC-FM + T; all co-culture groups except CC-CM + T increased significantly in wet weight over time. In contrast, only the FC groups grown in CM increased in wet weight over time to 8.0 ± 0.5 mg, twofold greater than the other FC groups.

Histology/Immunohistochemistry

CC constructs grown in FM show the development of a superficial layer with decreased cell density, while such a region was absent in the CC constructs cultured in CM

Table II. Gross and biochemical data for the constructs.

	Time (weeks)	Diameter (mm)	Thickness (mm)	Wet wt (mg)	10^6 Cells per construct	GAG/DW (%)	Collagen/DW (%)
Co-cultues							
FM	3	5.5 ± 0.1^{b}	0.59 ± 0.04^{bc}	15.9 ± 1.9^{b}	3.15 ± 0.14	17.97 ± 4.54^{c}	7.13 ± 2.23
	6	6.7 ± 0.3^{a}	0.68 ± 0.13^{ab}	32.5 ± 4.2^{a}	2.50 ± 0.34	18.25 ± 3.69^{c}	5.86 ± 1.29
FMT	3	4.7 ± 0.2^{de}	0.45 ± 0.05^{c}	8.8 ± 0.9^{cd}	3.65 ± 0.28	9.66 ± 1.46^{c}	6.99 ± 1.72
	6	5.1 ± 0.2^{c}	0.68 ± 0.10^{ab}	16.6 ± 1.7^{b}	3.96 ± 0.25	15.75 ± 2.90^{c}	$11.70 \pm 1.92^{*}$
CM	3	4.6 ± 0.1^{e}	0.66 ± 0.10^{ab}	10.1 ± 0.6^{cd}	3.52 ± 0.11	34.37 ± 1.93^{b}	7.67 ± 1.00
	6	5.0 ± 0.2^{cd}	0.81 ± 0.05^{a}	15.2 ± 1.3^{b}	3.96 ± 0.41	44.91 ± 3.72^{a}	9.39 ± 1.47
CMT	3	3.9 ± 0.2^{f}	0.46 ± 0.08^{c}	5.5 ± 0.2^{d}	4.13 ± 0.50	13.58 ± 6.35^{c}	5.98 ± 2.67
	6	4.0 ± 0.1f	0.53 ± 0.11^{bc}	6.2 ± 1.3^{d}	4.71 ± 0.78	16.75 ± 2.12^{c}	9.12 ± 1.90
Fibrochondrocytes							
FM	3	2.4 ± 0.1^{c}	0.47 ± 0.07^{B}	1.7 ± 0.3^{D}	2.93 ± 0.89	2.23 ± 0.82^{B}	0.88 ± 0.49
	6	1.8 ± 0.1^{D}	0.93 ± 0.34^{AB}	1.9 ± 0.3^{D}	1.88 ± 0.42	1.02 ± 0.27^{B}	1.44 ± 1.01
FMT	3	2.4 ± 0.2^{C}	0.47 ± 0.06^{B}	2.6 ± 0.4^{CD}	2.67 ± 0.58	2.50 ± 0.48^{B}	1.41 ± 1.06
	6	2.2 ± 0.1^{C}	0.97 ± 0.14^{A}	3.6 ± 0.4^{CB}	$5.01 \pm 1.50^{*}$	2.88 ± 1.00^{B}	3.15 ± 1.83
CM	3	3.4 ± 0.4^{B}	0.45 ± 0.20^{B}	3.9 ± 0.9^{B}	2.32 ± 0.31	3.04 ± 1.29^{B}	1.60 ± 0.50
	6	4.2 ± 0.3^{A}	0.55 ± 0.06^{B}	8.0 ± 0.5^{A}	2.02 ± 0.24	1.10 ± 0.26^{B}	0.87 ± 0.49
CMT	3	2.3 ± 0.3^{C}	0.69 ± 0.06^{AB}	2.2 ± 0.2^{D}	3.03 ± 0.43	3.05 ± 1.35^{B}	2.15 ± 1.21
	6	2.2 ± 0.1^{C}	0.97 ± 0.12^{A}	3.9 ± 0.3^{B}	3.04 ± 0.64	6.51 ± 1.81^{A}	6.67 ± 0.98

Biochemical characterization was normalized to dry weight (DW) to facilitate comparison to native tissue. Values reported as mean ± standard deviation, $n = 4$–6 samples. Statistics were blocked by cell group (capital letters for FC vs. lower case letters for CC) and data separated by different letters indicate significant differences, $P < 0.05$.

*Significant change within the treatment group over time.

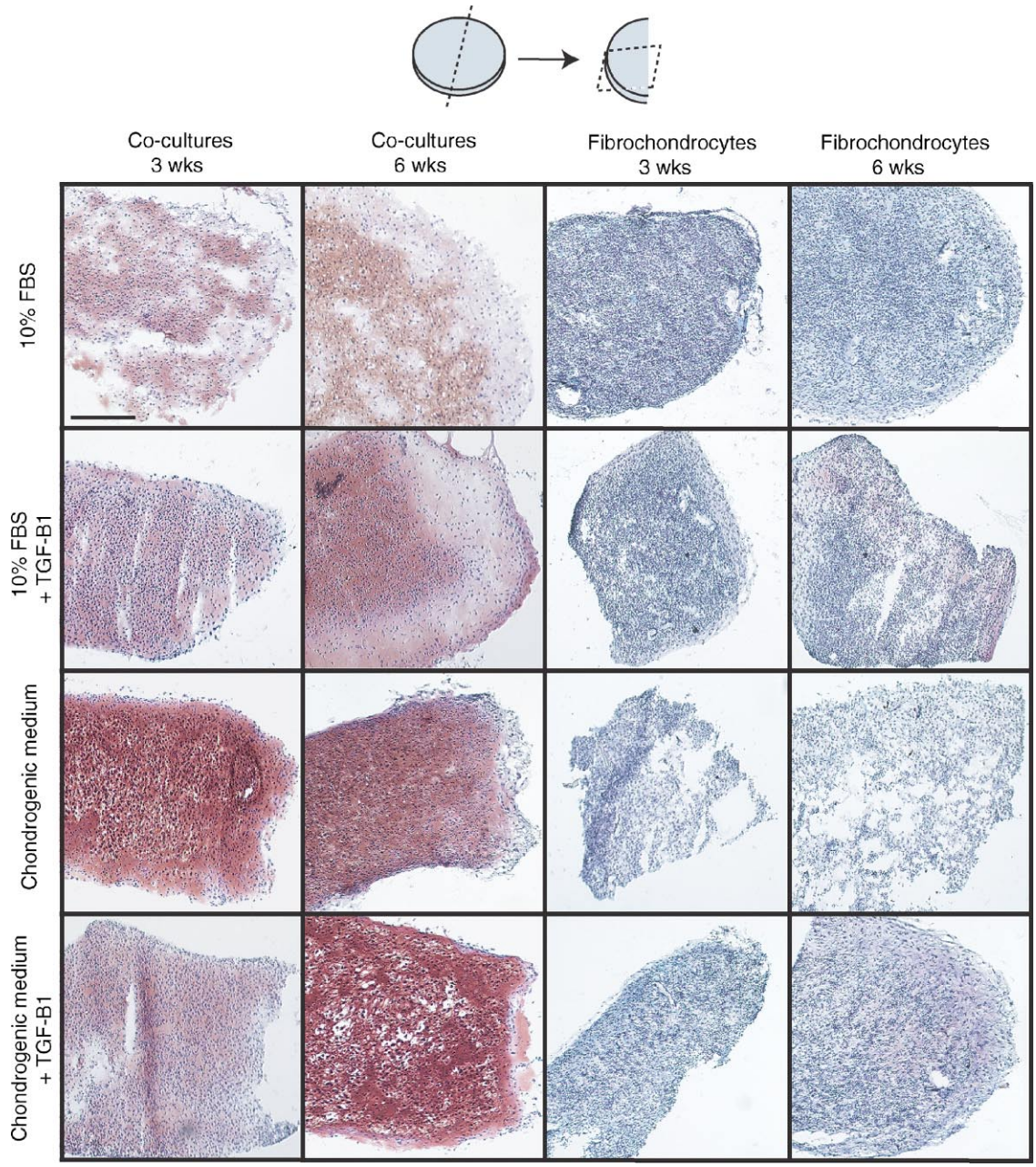

Figure 2. Safranin-O staining of the constructs illustrates the changes in morphology as they mature through 3–6 weeks of culture. The fibrochondrocyte constructs are cell-dense with sparse matrix while the co-culture constructs show striking differences based on the treatment group. Co-cultures groups cultured in FM ± T show the development of a superficial layer while those prepared in CM are more compact with proteoglycan-rich matrix. Scale bar = 250 μm. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

(Fig. 2). Overall, the FC constructs appear very cell dense, with the exception of a thin (~100 μm) hypocellular superficial layer in the FC-FM + T group. Safranin-O staining indicates the deep portion of the CC-FM/FM + T groups is more proteoglycan-rich than the superficial layer. The CM treated CC groups stained intensely for proteoglycans through the entire thickness of the constructs. Only the growth factor treated FC groups appear to have faint proteoglycan staining at the periphery, and only at the later time point was this seen. Picrosirius red staining revealed collagen development in all constructs. Similar to the staining for proteoglycans, the deep portions of the CC-FM + T group showed more intense staining. Staining for collagen VI was particularly intense in the CC-CM group, compared to lighter staining in other groups (Fig. 3). Staining for α-SMA showed this protein was present in all of the constructs; however, the staining appeared with greater intensity in the FC groups (data not shown).

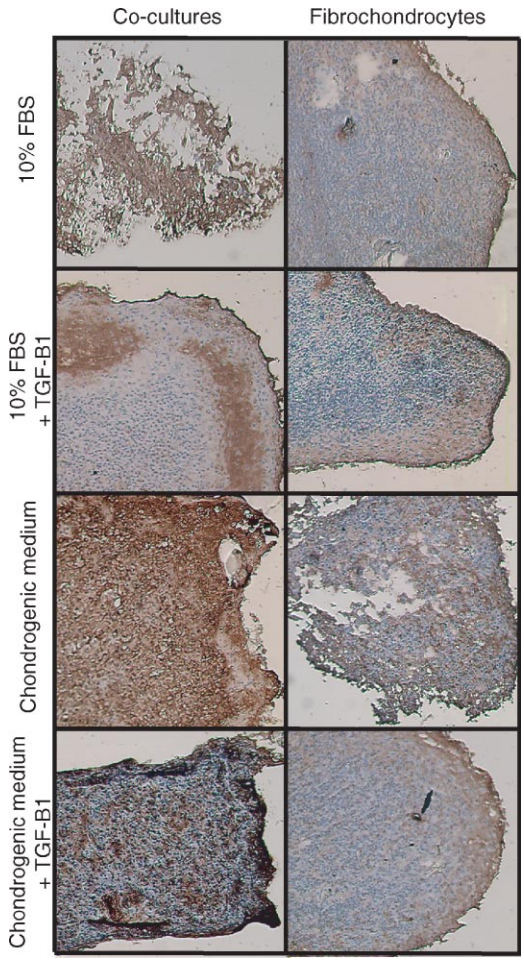

Figure 3. Immunohistochemistry for collagen type VI: brown color indicates positive staining. While all of the constructs show some positive staining, the co-culture CM constructs show the most intense staining, perhaps accounting for the significant proportion of the total collagen not accounted for by collagens I and II. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

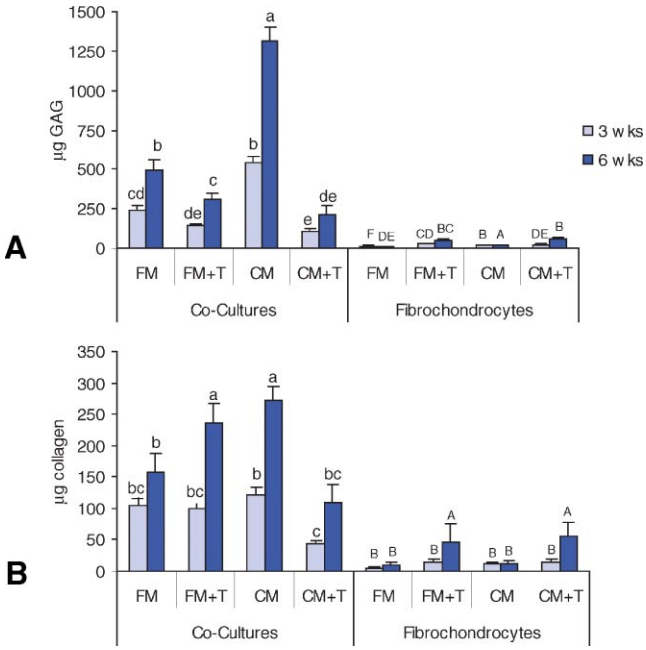

Figure 4. Quantitative (**A**) GAG and (**B**) collagen per construct for the different treatment groups. Co-culture constructs produced significantly more matrix than the fibrochondrocyte groups and there was significant variation according to treatment. Values reported as mean ± standard deviation, $n = 4$–6 samples. Statistics were blocked by cell group (capital vs. lower case letters) and data separated by different letters indicate significant differences, $P < 0.05$. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Quantitative Biochemistry

Construct cell content averaged 3.78×10^6 cells/construct in the CC group, while the FC groups averaged 2.63×10^6 cells/construct (Table II). The FC-FM + T group showed significant proliferation, increasing from $2.67 \pm 0.58 \times 10^6$ to $5.01 \pm 1.50 \times 10^6$ cells/construct from 3 to 6 weeks. Without the growth factor, FC-FM cells/construct decreased significantly over time. The quantity of GAG per construct was greatest in the CM group for the CC constructs, 1314.2 ± 37.6 µg/construct, nearly threefold greater than the next highest group (Fig. 4A). In the FC constructs, the addition of TGF-β1 resulted in three- to sixfold increases in GAG production compared to medium without growth factor, at 45.2 ± 16.0 and 55.26 ± 16.3 µg/construct for FC-FM + T and FC-CM + T, respectively. The GAG density per dry weight (DW) did not change significantly over time for any of the groups, and the density was greatest in the CC-CM and FC-CM + T groups within their respective cell groups (Table II). Collagen per construct was greatest in the CC-FM + T and CC-CM groups at 235.0 ± 32.0 and 272.0 ± 21.2 µg/construct, respectively (Fig. 4B). In contrast, the FC group collagen was significantly lower than the CC groups; however, growth factor addition resulted in four- to fivefold greater collagen production compared to the no-growth factor groups. Collagen density per DW ranged from 6.4% to 11.7% for the CC groups and 0.8–6.6% in the FC groups (Table II). Density in the CC-FM + T and CC-CM groups increased significantly from 3 to 6 weeks.

Collagen type I and collagen II were specifically quantified with ELISA (Fig. 5). Within the FC groups, FM + T resulted in the greatest overall collagen II production, while neither medium nor growth factor influenced collagen I content. In contrast, in the CC groups the CM treatment produced 75% less collagen I than the other groups, and serum-containing medium significantly increased collagen II production. Excepting the CC-CM + T group, the collagen I–II ratio ranged from 3 to 15 for the CC groups and tended to decrease over time (Fig. 5C). In the FC groups, the ratio was an order of magnitude greater, ranging from 72 to 2,206, and, excepting the CM + T group, this ratio increased significantly over time. Total collagens I and II, as assayed individually via ELISA, was lower than or not significantly different from total collagen measured by the hydroxyproline assay in any of the groups.

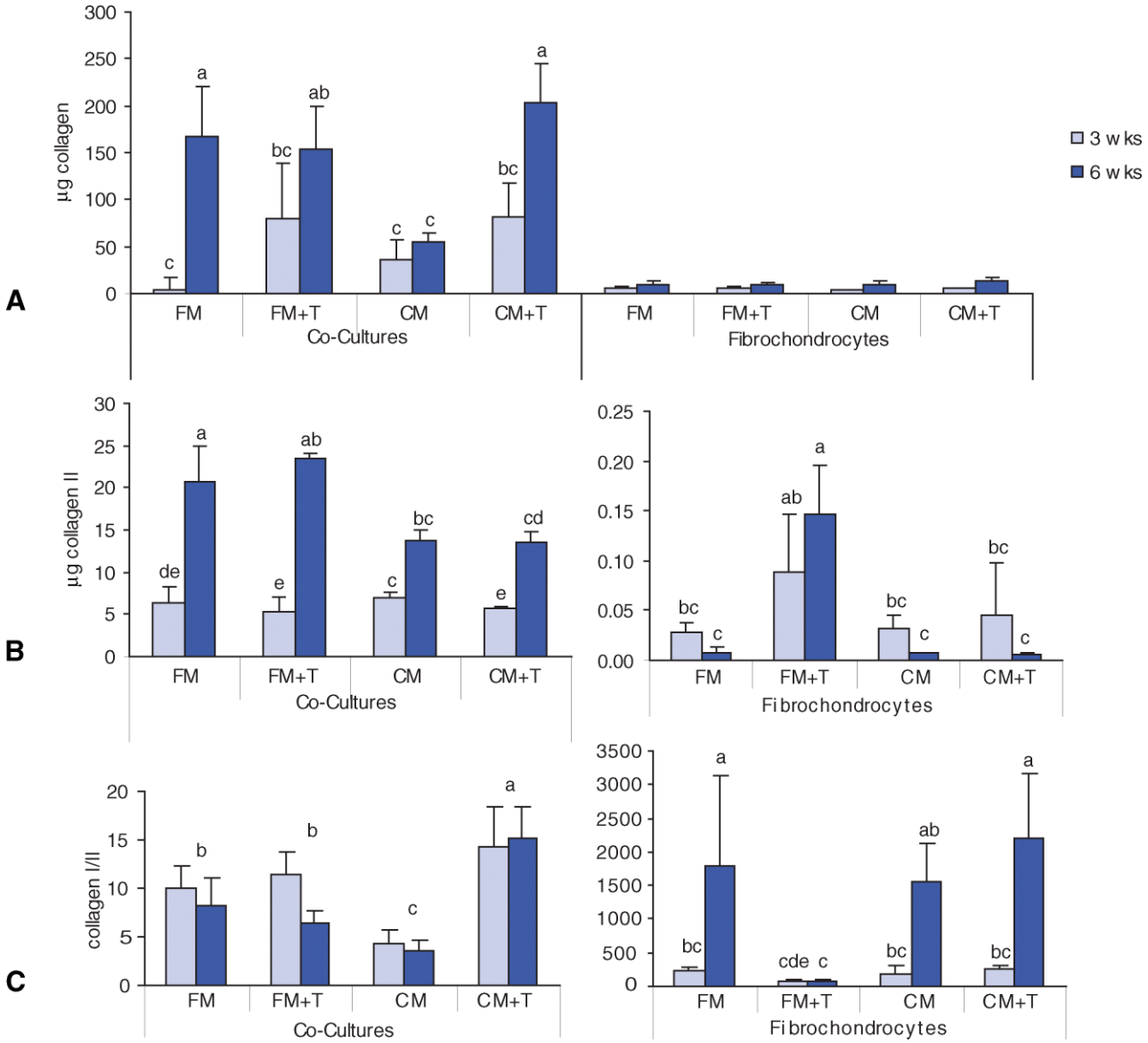

Figure 5. **A**: Collagen type I per construct. The predominant collagen in the constructs is collagen type I; there were no significant differences amongst the fibrochondrocyte groups. **B**: Collagen II per construct. Collagen II, while more abundant in the co-culture constructs, was significantly affected by treatment in the fibrochondrocyte groups. **C**: These variations led to dramatic differences in the collagen I/II ratios mirroring those found in the heterogeneous regions of the native tissue. Values reported as mean ± standard deviation, $n = 4$–6 samples. Statistics were blocked by cell group (capital vs. lower case letters) and data separated by different letters indicate significant differences, $P < 0.05$. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Biomechanics

The tensile modulus was greatest in the CC groups, with the highest being 3,046 ± 1,825 kPa for CC-CM, while the largest value for the FC groups was an order of magnitude lower at 626 ± 242 kPa (Fig. 6A). The ultimate tensile strength was lowest in the FC-FM group at 41 ± 26 kPa and greatest for the CC-CM group at 907 ± 256 kPa (Fig. 6B). The aggregate modulus ranged from 14 to 29 kPa for all but the CC-CM group which was fourfold greater at128 ± 34 kPa (Fig. 6C).

Discussion

This work aims to create an enabling technology of utilizing different cell sources and biochemical stimuli to create a spectrum of fibrocartilages amenable to repairing injuries to any of the heterogeneous regions of the meniscus. Construct variation in terms of morphology existed in terms of zonal development and evidence of construct contraction. Construct composition ranged from predominantly collagen I to constructs containing collagens I, II, and VI. Proteoglycan density varied tremendously amongst the

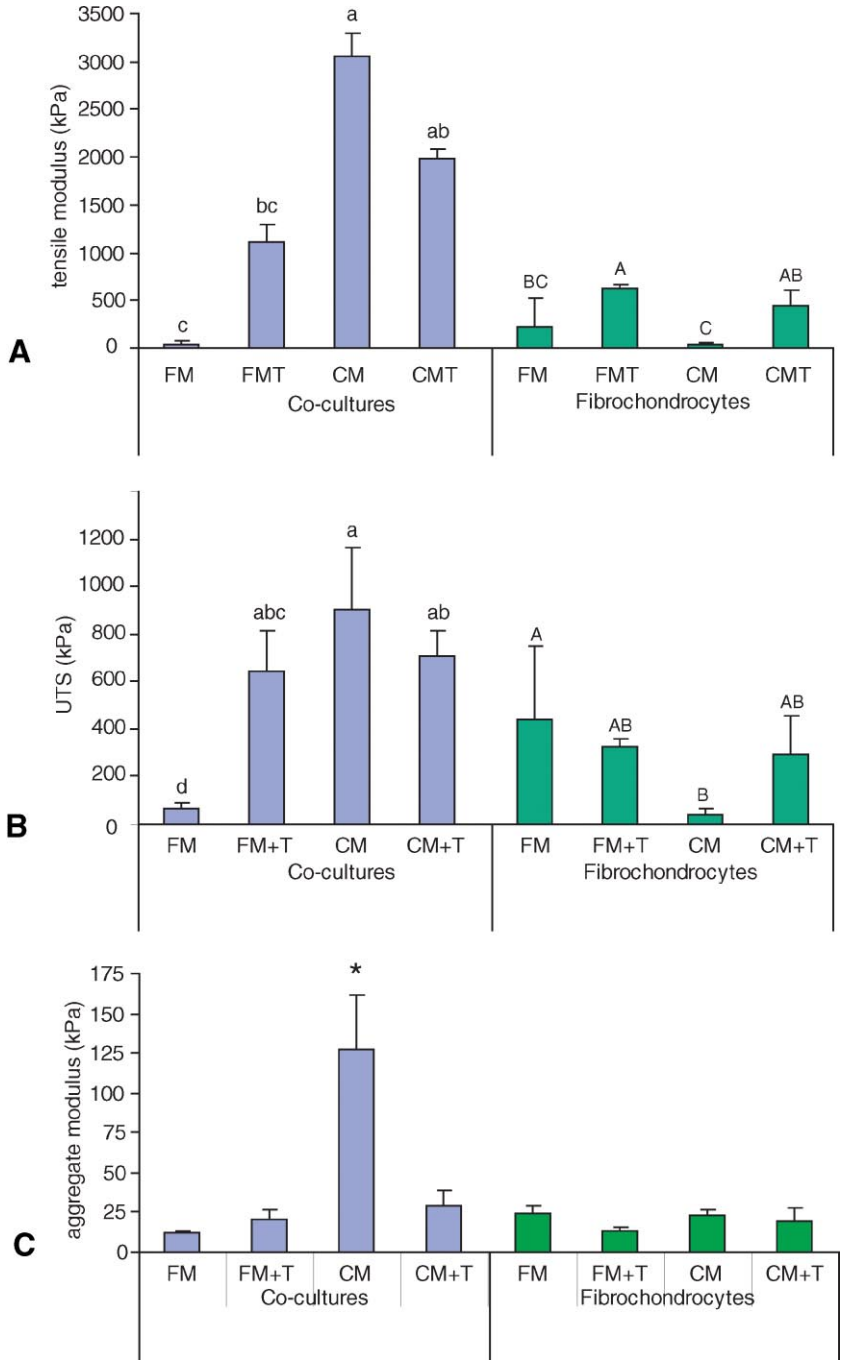

Figure 6. Mechanical properties of the constructs: (**A**) tensile modulus, (**B**) ultimate tensile strength, and (**C**) aggregate modulus. The co-culture constructs treated with CM had the greatest tensile strength, while FBS or growth factor presence was more important in the fibrochondrocyte groups. The co-culture CM group showed dramatically greater compressive stiffness, on par with the stiffness of native tissue. Values reported as mean ± standard deviation, $n = 4$–6 samples. Statistics were blocked by cell group (capital vs. lower case letters) and data separated by different letters or * indicate significant differences, $P < 0.05$. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

groups ($P < 0.0001$) and shows a clear correlation to compressive stiffness ($R^2 = 0.80$). In contrast, all of the groups show significant tensile stiffness that did not directly correlate to collagen I content ($R^2 = 0.08$). Of the treatments, the combination of serum-free medium with the co-culture resulted in constructs with enhanced GAG density, intense collagen VI staining, and the most superior compressive and tensile properties. With properties within and spanning the range of native tissue both in terms of specific biochemical and mechanical properties, these

constructs demonstrate a promising advance in meniscal tissue engineering. The results of this study support the hypothesis that cell source and biochemical stimuli can be used to manipulate cell proliferation and the synthesis of extracellular matrix to produce varying morphological, biochemical, and mechanical properties in a scaffoldless tissue engineering strategy.

The foundation for the heterogeneities developed in these constructs is the cell source. The intuitive cell choice for a tissue-engineered meniscus is the fibrochondrocyte. Previous scaffold-free and scaffold-based studies have shown that chondrocytes tend to synthesize more matrix more rapidly than fibrochondrocytes (Hoben et al., 2006; Vanderploeg et al., 2004), and such was also borne out in this study. The wet weights of the co-cultures were up to 18-fold greater than those obtained by the fibrochondrocyte constructs. It is also notable that the FC constructs tended to contract down to 35–80% of their original diameter, while the CC constructs maintained or grew in diameter. Additionally, the FC groups predominantly produced collagen type I, leading to collagen I/collagen II ratios over 100-fold greater than those of the CC constructs. One might expect that if the natural synthetic capacity of the fibrochondrocytes could be further enhanced, they could produce neotissue with tensile properties surpassing that of the co-cultures. Especially since the co-cultures tend to produce significantly greater proportions of collagen other than collagen type I: for example, in the CC-CM group only 19% of the total collagen was collagen type I. While the CC groups consistently produced more matrix than the FC groups, the response of the fibrochondrocytes versus that of the co-cultures to the different base media and the addition of the growth factor was both marked and interesting.

There were several important findings in these data related to the use of FBS in creating a spectrum of fibrocartilages. The CC constructs grown in FM were significantly larger, developed a zonal structure, and produced more collagen type II. This zonal structure was characterized by an outer layer of lower cellularity, decreased GAG staining, and intense collagen VI staining. These features show similarity to the superficial zone of articular cartilage which is characterized by dense collagen fibers, a lower GAG density, and relative hypocellularity (Buckwalter, 1991). The FC constructs grown in FM were surprisingly smaller but also showed a larger proportion of collagen II. Use of CM resulted in more compact constructs with homogeneous structure and significantly greater GAG content in the CC constructs. It appears that both serum and co-culture conditions need to be present for spatial variations to be observed in the construct morphology. We can further speculate that serum may either be playing a role in potentially segregating the cells or that serum is influencing tissue structure development. Many recent studies have examined a variety of base media including Nutridoma (Glowacki et al., 2005; Yates et al., 2005), HL-1 (Adkisson et al., 2001), Ultraculture (Kita et al., 2006), RPMI (Roswell Park Memorial Institute) Media (Fedewa et al., 1998), as well as numerous combinations of growth factors and other additives to replace serum, as done in this work. Replacing the stimuli of serum with additives such as ITS and varying steroids has been shown by several groups to enhance GAG synthesis in chondrocytes (Adkisson et al., 2001; Chua et al., 2005; Fortier et al., 1997; Giannoni et al., 2005; Kisiday et al., 2005; Schwartz et al., 1974; Yates et al., 2005), just as it was shown in this study with fibrochondrocytes and co-cultures. Indeed, Adkisson et al. (2001) actually found a dose-dependent decrease in GAG with increasing FBS supplementation. Increases in collagen II mRNA expression and enhanced qualitative collagen II protein levels have also been reported associated with serum-free formulations (Adkisson et al., 2001; Chua et al., 2005; Fortier et al., 1997; Giannoni et al., 2005; Gruber et al., 2004; Schwartz et al., 1974; Yates et al., 2005). This increased collagen type II is generally attributed to IGF-I-like action of the insulin supplementation in these media (Foley et al., 1982; Sadick et al., 1999). Although increased collagen II synthesis was not seen in this study, there was a significant decrease in the quantity of collagen I in the CC-CM group compared to CC-FM, which correlates well to evidence that IGF-1 treatment inhibits collagen I expression (Demarquay et al., 1990). With respect to increasing overall matrix production and cell proliferation, however, the use of FBS has clear benefits, as seen in this and other studies (Chua et al., 2005; Glowacki et al., 2005; Gruber et al., 2004; Kamil et al., 2007; Kisiday et al., 2005; Kita et al., 2006; Masuda et al., 2003; Richmon et al., 2005; Sun et al., 2001; Yates et al., 2005). Thus, rather than viewing the use of FBS as an obligatory cell culture medium ingredient, it may be more useful to view it as another biochemical tool. In the case of fibrocartilage tissue engineering it can be used to increase collagen type II content. There are particular challenges to using serum in this fashion, most significantly concerns over immune reaction to animal products (Johnson et al., 1991; Meyer et al., 1988), but recent work examining the use of autologous serum (Tallheden et al., 2005) makes the case that serum should not be left out of the tissue engineering armamentarium.

The effects of the growth factor, TGF-β1, were particularly surprising in this study. Based on previous, scaffold-based and explant studies (Collier and Ghosh, 1995; Imler et al., 2004; Pangborn and Athanasiou, 2005b), it was hypothesized that growth factor addition would increase both collagen and GAG synthesis, and that it would do so in both types of base media, though this was not exactly the case. In the CC groups, TGF-β1 increased synthesis of specific collagens, particularly collagen type I in serum-free base medium, perhaps overcoming the IGF-I-like suppression brought about by the ITS component of the CM (Foley et al., 1982; Sadick et al., 1999). However, the total GAG and GAG density were lower, whereas most prior studies have shown increased proteoglycan synthesis or precursor uptake due to this growth factor (Imler et al., 2004; Lietman et al., 2003; Pangborn and Athanasiou, 2005a,b; Verbruggen et al., 1995). Work from Lima et al. (2007) with agarose

encapsulated chondrocytes showed that varying the dosing regimen of TGF-β3 was more beneficial than continuous dosing, suggesting a direction for future work with TGF-β1 and the co-cultures. The expected increase in proteoglycan synthesis was, however, borne out in the FC groups: both GAG and collagen production was increased, and this is well corroborated with prior work using fibrochondrocytes in both monolayer, explant, and 3D culture (Collier and Ghosh, 1995; Imler et al., 2004; Lietman et al., 2003; Mauck et al., 2007; Pangborn and Athanasiou, 2005a,b). It is notable that the FC-FM + T group also had fourfold greater collagen II production over FC-FM, and there was also greater cell proliferation. Likewise, an increasing cell proliferation trend was seen in the CC groups treated with TGF-β1 ($P = 0.079$). Several groups have similarly reported increased cell proliferation using TGF-β1 in the presence of FBS but not in serum-free or low serum medium (Fortier et al., 1997; Landesberg et al., 1996; Vivien et al., 1992). Overall, the co-cultures appeared less sensitive to the presence of the growth factor, while it clearly modulated the type and quantity of matrix produced in the fibrochondrocyte groups, thus contributing to the spectrum of tissue properties that can be engineered with fibrochondrocytes.

An additional component to this tissue engineering strategy that may have contributed to the varying response to TGF-β1 when comparing to prior explant, monolayer, and 3D studies is the scaffold-free approach. Chondrocytes respond to their adhesive environment with changes in matrix synthesis and part of that change is that integrins are linked to activation of certain growth factor receptors (Kim et al., 2005; Miyamoto et al., 1996; Thannickal et al., 2003). Proteoglycan production mediated by TGF-β1 has been found to be related to the differentiation status of the chondrocytes, culture-mediated changes in the size of the TGF-β1 receptor, and changes in the expression of proteins related to the TGF-β1 signaling pathway (Bogdanowicz et al., 1996; Glansbeek et al., 1993; Guerne et al., 1994; Lafeber et al., 1997; Scharstuhl et al., 2003). These complex interactions between cells, substrates, and growth factors indicates there are significant challenges in translating results between pellet cultures, monolayer studies, scaffold-based work, and scaffoldless strategies. Moreover, given the unique response of the cells within this culture modality to biochemical stimuli, future work employing mechanical stimulation to further increase the spectrum of engineered tissues is warranted.

While recapitulating the native structure is an important guide to regeneration, functional assessments are certainly the bottom line. This work clearly illustrated the cartilage structure/function paradigm that increased GAG is linked to compressive stiffness: the combination of co-cultures and serum-free medium resulted in a twofold increase in GAG over the next highest group and a compressive stiffness within the range of native neonatal bovine tissue (110 ± 40 to 210 ± 60 kPa) (Sweigart et al., 2004). Moreover, there is increasing evidence that collagen type VI, found particularly in the CC-CM constructs and present in the native meniscus, may be an important player in fibrocartilage function (Carvalho et al., 2006; Vanderploeg et al., 2006; Wildey and McDevitt, 1998). In contrast, the construct with the greatest collagen I content was expected to exhibit the most superior tensile properties, and this was not precisely the case. Three of the co-culture groups (the top three total collagen producers) resulted in the greatest tensile properties, but it is interesting to note that their proportions of different types of collagen were quite different. The collagen I/II ratio in these constructs ranged from 2 to 18. Collagen type VI in particular appears to have been a major component of the CC-CM group. The spectrum of neotissues created here illustrates the interplay of extracellular matrix composition, organization, and function. The goal of future studies will be to better elucidate these interactions in order to exert greater control over the process of engineering various fibrocartilages.

In conclusion, this study showed that different cell sources and biochemical treatments could be used to create fibrocartilaginous constructs of varying characteristics, preparing the way for a tailored tissue engineering approach. For example, the FM + T treatment could be used with co-cultures or fibrochondrocytes to create a largely collagen type I outer zone, while serum-free medium could be used with co-cultures to create a more collagen II rich inner zone of the meniscus with high compressive stiffness. While challenges remain to increase tensile properties to more closely match those of the native tissue which range 60–198 MPa (Proctor et al., 1989), this work presents a model for creating heterogeneous fibrocartilages using primary cells. Future translation to more practical cell sources, such as differentiated stem cells or dermal fibroblasts, will allow these methods to be employed in tissue engineering a range of fibrocartilages from the knee meniscus to the TMJ disc and intervertebral disc.

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