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## ABSTRACT

Tissue-engineering of the temporomandibular joint (TMJ) disc aims to provide patients with TMJ disorders an option to replace diseased tissue with autologous, functional tissue. This study examined clinically relevant cell sources by comparing costal chondrocytes, dermal fibroblasts, a mixture of the two, and TMJ disc cells in a scaffoldless tissue-engineering approach. It was hypothesized that all constructs would produce matrix relevant to the TMJ disc, but the mixture constructs were expected to appear most like the TMJ disc constructs. Costal chondrocyte and mixture constructs were morphologically and biochemically superior to the TMJ disc and dermal fibroblast constructs, and their compressive properties were not significantly different. Costal chondrocyte constructs produced almost 40 times more collagen and 800 times more glycosaminoglycans than did TMJ constructs. This study demonstrates the ability of costal chondrocytes to produce extracellular matrix that may function in a TMJ disc replacement.

**KEY WORDS:** tissue engineering, TMJ disc, costal chondrocyte, dermal fibroblast.

# Clinically Relevant Cell Sources for TMJ Disc Engineering

## INTRODUCTION

There are approximately 10 million individuals in the United States who suffer from TMJ disorders (NIDCR, 2006). In severely diseased joints, surgical options are met with various degrees of success, as reviewed previously (Dimitroulis, 2005; Wong *et al.*, 2006). Efforts to engineer the TMJ disc may create a viable alternative to current treatment options.

While previous work with TMJ disc cells characterized their *in vitro* behavior for the purpose of tissue engineering, a clinical solution will likely not involve these cells. This conclusion was reached following the experience of our laboratory and others, demonstrating the difficulty in culturing these cells, failure of constructs to achieve sufficient morphology, and poor mechanical strength of the engineered tissue (Puelacher *et al.*, 1994; Springer *et al.*, 2001; Almarza and Athanasiou, 2005, 2006; Detamore and Athanasiou, 2005; Allen and Athanasiou, 2006; Johns and Athanasiou, 2007).

A clinically feasible cell source should be abundant, healthy, and leave little donor site morbidity. Selection of an alternative source must also consider the functionality of the cells. Previous characterization data guide this selection; the TMJ disc has properties of both fibrous tissue and cartilage, indicating the need for a fibrochondrocytic cell source (Almarza and Athanasiou, 2004). Specifically, the cells should produce tissue containing collagen type I, type II, and glycosaminoglycans, and should support both tensile and compressive loads. Dermal fibroblasts exhibit chondrogenic potential, despite being inherently fibrogenic (French *et al.*, 2004; Deng *et al.*, 2007). Costal cartilage contains both collagen types II and I (in a ratio of 5:1) and glycosaminoglycans, suggesting its potential to function as a fibrocartilage replacement (Safronova *et al.*, 1991). Indeed, costochondral grafts are already used in mandibular reconstruction (Lindqvist *et al.*, 1988). In addition to their functional potential, costochondral cartilage and dermal fibroblasts are easily harvested and more abundant than TMJ disc cells, making them a more clinically feasible source of cells for tissue engineering.

Constructs of costal chondrocytes, dermal fibroblasts, TMJ disc cells, and a 50/50 costal chondrocyte/dermal fibroblast mixture were examined in this study. Based on known tissue characteristics, it was hypothesized that all cell types would produce extracellular matrix similar to the TMJ disc cell construct. However, the costal chondrocyte/dermal fibroblast mixture was expected to produce the most TMJ disc cell-like construct. Specifically, mechanical and biochemical properties that are most similar to the TMJ would be observed *via* glycosaminoglycan and collagen II production by the costal chondrocytes and collagen I production by the dermal fibroblasts.

## MATERIALS & METHODS

### Cell Isolations

Cells were isolated from 3 skeletally mature, Spanish, female goats. (The use of animal tissues followed an animal use protocol approved by the institutional review board.) TMJ disc cells were isolated as previously described and cultured

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until 70-90% confluent (Johns and Athanasiou, 2007). They were dissociated with trypsin-EDTA (Gibco, Carlsbad, CA, USA) and grown to passage 2. Culture medium was Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine and 4.5 g/L glucose (BioWhittaker, Walkersville, MD, USA), 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA, USA), 1% penicillin-streptomycin-fungizone (Cambrex, Walkersville, MD, USA), 1% non-essential amino acids (Gibco), 25  $\mu$ L/mL L-ascorbic acid (Sigma, St. Louis, MO, USA), and 1  $\mu$ L/mL insulin (Sigma).

Skin was cut into 1-cm<sup>2</sup> squares and digested in 0.5% dispase (Gibco) at 4°C overnight, and then epidermis and adipose layers were removed. The remaining dermis was placed in 0.05% type II collagenase (Worthington, Lakewood, NJ, USA). After 24 hrs, samples were passed through a 70- $\mu$ m cell strainer, yielding a single-cell suspension, which was plated and cultured in DMEM containing Glutamax, 10% fetal bovine serum, 1% penicillin-streptomycin-fungizone, and 1% non-essential amino acids. When confluent, cultures were exposed to 0.5% dispase for 30 min to remove keratinocytes. The purified dermis cells were allowed to expand, and passage 2 cells were used.

Cartilage was scraped from non-floating ribs, minced into cubes of approximately 1 mm<sup>3</sup>, and digested overnight with 0.2% collagenase in DMEM. After isolation, cells were frozen in DMEM with 10% dimethyl sulfoxide, 20% fetal bovine serum, 1% penicillin-streptomycin-fungizone, and 1% non-essential amino acids to allow for concurrent seeding of costal cartilage constructs with the other groups.

### Construct Culture

Constructs were formed by a scaffoldless method described previously (Hu and Athanasiou, 2006). Two million cells (TMJ, costal chondrocytes, dermal fibroblasts, or a 50:50 costal chondrocyte:dermal fibroblast mix) were seeded in 3-mm wells formed with 2% agarose (Fisher Scientific, Pittsburgh, PA, USA). Media changes occurred every day with DMEM with 1% penicillin-streptomycin-fungizone, 1% NEAA, 1% insulin-transferrin-selenium + premix (BD Biosciences, San Jose, CA, USA), 0.1  $\mu$ M dexamethasone, 40  $\mu$ g/mL L-proline (EMD Chemicals, Gibbstown, NJ, USA), 50  $\mu$ g/mL ascorbate 2-phosphate (Sigma), and 100  $\mu$ g/mL sodium pyruvate (Fisher). After 2 wks, constructs were transferred to agarose-coated plates. Samples were removed for biochemistry and histology at 3 wks and 6 wks. Additionally, mechanical testing was performed at 6 wks.

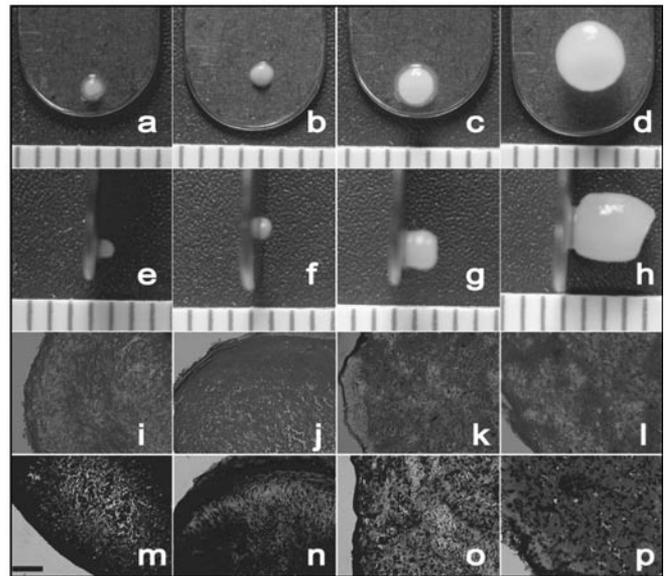
### Histology

Samples were frozen in HistoPrep™ (Fisher), and 14- $\mu$ m sections were prepared. Sections were stained with picrosirius red for collagen or with safranin-O/fast green for glycosaminoglycans. Sections were immunohistochemically stained for collagen types I and II, as described previously (Detamore *et al.*, 2005).

### Biochemistry

Four samples *per* group were lyophilized for 2 days and digested at 4°C with constant agitation for 7 days with 125  $\mu$ g/mL papain (Sigma) digest, followed by 2 days of 1 mg/mL elastase (Sigma) digestion. Samples were stored at -20°C.

Cell numbers were determined with PicoGreen® (Molecular Probes, Eugene, OR, USA), with a conversion factor of 7.7 pg DNA/cell (Kim *et al.*, 1988). Total collagen was measured by a modified hydroxyproline assay (Woessner, 1961). Sulfated glycosaminoglycans were quantified with a dimethylmethylene blue Blyscan kit (Bicolor, Newtownabbey, Ireland). Type I collagen was quantified with an indirect ELISA, described



**Figure 1.** Gross morphology and histology at wk 6 for tissue-engineered constructs with various cell sources: TMJ (a column), dermal fibroblast (b column), costal chondrocyte/dermal fibroblast (c column), and costal chondrocyte (d column). a-h illustrate top and side views of the constructs. Divisions on the ruler below the images are 1 mm. These clearly show the extensive contraction that occurred in TMJ and dermal fibroblast constructs, while costal chondrocyte/dermal fibroblasts had substantial but less contraction. Staining with picrosirius red (i-l) and safranin-O/fast green (m-p) are also shown here. Of particular interest is the glycosaminoglycan staining, which is most apparent in the costal chondrocyte construct and around the edge of the costal chondrocyte/dermal fibroblast construct. Dermal fibroblast and TMJ disc cell constructs did not stain with safranin-O. Scale bar = 0.1 mm.

previously (Darling and Athanasiou, 2005).

### Mechanical Testing

At least 5 samples *per* group were tested in tension and compression. Tensile testing was performed on an Instron 5565 (Norwood, MA, USA) to determine ultimate tensile strength and elastic modulus. Samples were cut into a 'dog bone' shape by means of a scalpel blade and biopsy punch, and tested at 10% strain rate/min until failure.

Specimens were tested in unconfined compression with an indentation apparatus (Athanasiou *et al.*, 1994). Each sample was tare-loaded with 0.00196 N until equilibrium was reached (deformation less than 10<sup>-6</sup> mm/sec) or loading time reached 10 min. A step load of 0.00686 N was then applied until equilibrium or until 1 hr elapsed. Creep data were analyzed with Matlab's (The Math Works, Inc., Natick, MA, USA) curve-fitting tool with the viscoelastic model (Leipzig and Athanasiou, 2005).

### Statistics

Data were analyzed with a two-way analysis of variance, where time and cell type were factors with two and four levels, respectively. When an F-test indicated significance ( $p < 0.05$ ), a Tukey's *post hoc* test was performed.

## RESULTS

### Morphology and Histology

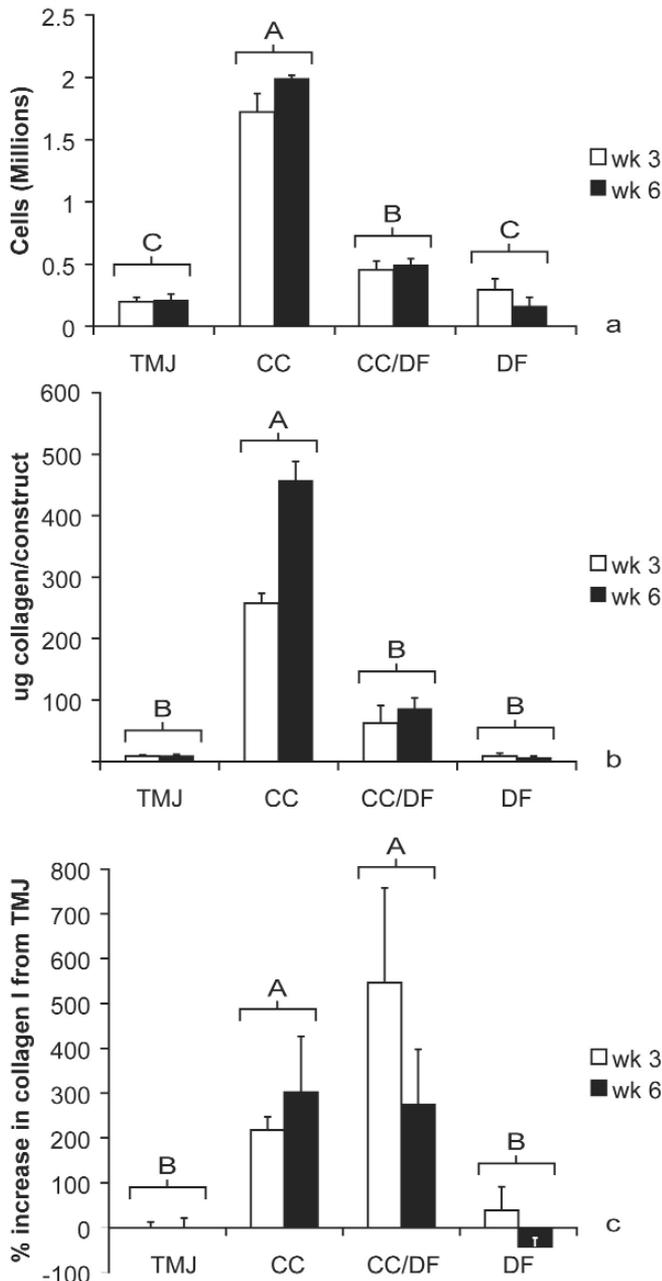
Morphologically, dermal fibroblast and TMJ disc constructs con-

**Table 1.** Quantitative Size Data (mean  $\pm$  standard deviation) of All Groups\*

	Diameter wk 3 (mm)	Diameter wk 6 (mm)	Volume wk 3 (mm <sup>3</sup> )	Volume wk 6 (mm <sup>3</sup> )**
TMJ	0.970 $\pm$ 0.037 <sup>c</sup>	0.863 $\pm$ 0.109 <sup>c</sup>	0.493 $\pm$ 0.135 <sup>c</sup>	0.357 $\pm$ 0.158 <sup>c</sup>
Costal chondrocyte	3.05 $\pm$ 0.088 <sup>a</sup>	3.12 $\pm$ 0.088 <sup>a</sup>	19.5 $\pm$ 1.95 <sup>a</sup>	26.4 $\pm$ 1.77 <sup>a</sup>
Costal chondrocyte/ dermal fibroblast	1.61 $\pm$ 0.166 <sup>b</sup>	1.73 $\pm$ 0.132 <sup>b</sup>	2.38 $\pm$ 0.861 <sup>b</sup>	3.54 $\pm$ 0.564 <sup>b</sup>
Dermal fibroblast	1.03 $\pm$ 0.110 <sup>c</sup>	0.955 $\pm$ 0.090 <sup>c</sup>	0.578 $\pm$ 0.174 <sup>bc</sup>	0.525 $\pm$ 0.132 <sup>bc</sup>

\* Groups separated by different letters are considered significantly different ( $p < 0.05$ ). Volumes were significantly greater at wk 6 than at wk 3, as indicated by the \*\*. Time was not a significant factor for diameter. Costal chondrocyte groups were consistently the largest, followed by the costal chondrocyte/dermal fibroblast mixture.

tracted, yielding mostly spherical constructs measuring about 1 mm in diameter. Mixture constructs contracted to a diameter just less than 2 mm and maintained a more cylindrical shape. Costal



chondrocyte constructs expanded to a diameter and height just over 3 mm (Fig. 1 and Table 1).

TMJ and dermal fibroblast groups did not stain with safranin-O (Figs. 1m-1p). All groups stained positive for collagen (Figs. 1i-1l). The costal chondrocyte group stained intensely for glycosaminoglycans throughout the construct, whereas the costal chondrocyte/dermal fibroblast group stained only around the periphery of the construct. Immunohistochemistry demonstrated positive collagen I staining for all groups. Only groups that contained costal chondrocytes, however, stained positive for collagen II (Appendix Fig.).

### Biochemistry

Costal chondrocyte constructs had significantly more cells than any other group, and costal chondrocyte/dermal fibroblast constructs had significantly more than dermal fibroblast or TMJ constructs (Fig. 2a). Cell numbers were not significantly affected by time. The initial seeding was 2 million cells/construct, which only the costal chondrocyte constructs maintained.

Costal chondrocyte constructs had significantly more total collagen than any other group (Fig. 2b). Collagen type I was normalized to the amount in the TMJ constructs, and there was significantly more collagen I for costal chondrocyte and costal chondrocyte/dermal fibroblast compared with the other constructs (Fig. 2c). Both total collagen and collagen I increased significantly at wk 6 from wk 3.

Glycosaminoglycan *per* construct also increased significantly at the later timepoint. For costal chondrocyte constructs, it was orders of magnitude higher than the other groups, with values at 3 and 6 wks of  $640 \pm 17 \mu\text{g}$  and  $1700 \pm 94 \mu\text{g}$ , respectively. TMJ constructs made  $1.7 \pm 0.6 \mu\text{g}$  at 3 wks and  $1.1 \pm 0.4 \mu\text{g}$  at 6 wks. At 3 wks, the dermal fibroblast constructs contained  $2.6 \pm 1.0 \mu\text{g}$  and  $1.1 \pm 0.3 \mu\text{g}$  at 6 wks. The co-culture constructs produced  $1.8 \pm 0.7 \mu\text{g}$  and  $2.0 \pm 0.8 \mu\text{g}$  at 3 and 6 wks, respectively. Despite cell quantity changes, matrix normalization to cell number resulted in trends similar to the *per*-construct normalization.

### Mechanical Properties

There were no statistical differences among any of the groups for compressive properties. For all 3 tensile properties, the TMJ constructs were significantly higher (Table 2).

**Figure 2.** Biochemical quantities for cells (a), collagen per construct (b), and percent increase in collagen type I (c). All data are shown as mean  $\pm$  standard deviation, with a sample size equal to 4 for all groups. Groups separated by different letters are considered significantly different ( $p < 0.05$ ). Costal chondrocyte (CC) constructs had more cells (determined by picogreen quantification) and total collagen (measured with hydroxyproline assay) than any other group. Time was a significant factor for total collagen, with larger values at wk 6. Costal chondrocyte/dermal fibroblast (CC/DF) constructs had significantly more cells than dermal fibroblast (DF) or TMJ constructs, and had a nearly significant increase in collagen over dermal fibroblast and TMJ constructs. Graph c shows the percent increase in collagen I from the TMJ control at the respective timepoint. Costal chondrocyte and costal chondrocyte/dermal fibroblast constructs had significantly more collagen type I.

**Table 2.** Mechanical Properties\*\* (mean  $\pm$  standard deviation) of All Groups at wk 6

	Compressive Properties			Ultimate Tensile Strength (MPa)	Tensile Properties	
	Relaxed Modulus (kPa)	Instantaneous Modulus (kPa)	Viscosity (s*kPa)		Elastic Modulus (MPa)	Yield Stress (MPa)
TMJ	41.2 $\pm$ 9.37	179 $\pm$ 40.3	6570 $\pm$ 3320	0.910 $\pm$ 0.585 <sup>†</sup>	0.935 $\pm$ 0.565 <sup>†</sup>	1.85 $\pm$ 1.46 <sup>†</sup>
Costal chondrocyte	36.4 $\pm$ 25.0	851 $\pm$ 1470	4470 $\pm$ 9810	0.111 $\pm$ 0.0581	0.133 $\pm$ 0.0449	0.277 $\pm$ 0.135
Costal chondrocyte/ dermal fibroblast	23.3 $\pm$ 9.05	77.1 $\pm$ 36.3	2320 $\pm$ 517	0.306 $\pm$ 0.161	0.339 $\pm$ 0.167	0.584 $\pm$ 0.819
Dermal fibroblast	41.9 $\pm$ 11.1	135 $\pm$ 58.0	6230 $\pm$ 2790	0.246 $\pm$ 0.163	0.244 $\pm$ 0.160	0.168 $\pm$ 0.102

\*\* TMJ disc cell constructs were significantly stronger in tension than any other group, as indicated by the <sup>†</sup> ( $p < 0.05$ ). No other statistical differences were observed.

## DISCUSSION

Due to the prevalence of TMJ disorders and limited treatment options, it is essential to examine possible alternatives to current surgical techniques, such as the engineering of a replacement disc (Wong *et al.*, 2006). The use of TMJ disc cells in this approach has numerous drawbacks, such as a limited population of healthy cells and donor site morbidity. The current study examines the potential of dermal fibroblasts and costal chondrocytes as alternative cell sources for fibrocartilage tissue-engineering. The results show significant increases in extracellular matrix produced by costal chondrocytes compared with that produced by dermal fibroblasts or TMJ disc cells, while a co-culture of costal chondrocytes and dermal fibroblasts made extracellular matrix in quantities between those made by either individual cell type. These distinctions were also apparent in construct size and weight, where costal chondrocyte constructs were significantly larger. The hypothesis that a costal chondrocyte/dermal fibroblast co-culture would produce constructs most similar to those produced by the TMJ cells was not supported by this work. Instead, dermal fibroblast constructs were most like TMJ constructs. Costal chondrocyte/dermal fibroblasts showed a trend toward improved biochemical content in addition to improved morphology over TMJ cells and dermal fibroblasts. However, costal chondrocyte constructs exceeded expectations by producing significantly more glycosaminoglycans, total collagen, and collagen type I than any other construct.

The extracellular matrix results obtained with the TMJ disc cells are representative of those reported from previous studies, which suggests that these cells do not exhibit a robust synthetic capacity. In contrast, costal chondrocyte constructs demonstrate that cells derived from this source are highly productive relative to the others tested. The most collagen produced previously with TMJ disc cells was approximately 60  $\mu$ g total collagen *per* construct—made with over 6 million cells (Almarza and Athanasiou, 2005). At the same timepoint (6 wks), the costal chondrocyte constructs produced over 450  $\mu$ g of total collagen, with an initial seeding density of 2 million cells. Additionally, the costal chondrocyte constructs yielded almost 100 times more glycosaminoglycans than previous TMJ disc cell constructs (Almarza and Athanasiou, 2005).

The 200-300% increase in collagen I of costal chondrocyte constructs over TMJ constructs further illustrates the productive capacity of the costal chondrocytes. Since rib cartilage contains both collagen types I and II, and skin

contains primarily type I, the dermal fibroblast constructs were expected to produce the most collagen type I, followed by costal chondrocyte/dermal fibroblast constructs and, finally, costal chondrocyte constructs. However, like total collagen, dermal fibroblast constructs contained the least collagen I, and no statistical difference was seen between the costal chondrocyte and costal chondrocyte/dermal fibroblast group. This reinforces the total extracellular matrix data suggesting that costal chondrocytes alone may be a viable cell source for functional tissue-engineering of the TMJ disc.

While analysis of extracellular matrix data indicates that costal chondrocytes are most likely to succeed in fibrocartilage tissue-engineering, mechanical data did not correspond to extracellular matrix changes. Generally, an increase in glycosaminoglycans increases compressive resilience, while more collagen improves tensile strength. In this experiment, the TMJ disc cell constructs had significantly higher tensile properties. This could be due to tighter cell packing or better organization of the extracellular matrix. However, even the largest of any of the constructs' tensile properties were still orders of magnitude below the native values for the TMJ disc (Beatty *et al.*, 2001). Despite the lower tensile strength, the high quantities of extracellular matrix suggest that, with the proper stimuli (biochemical or mechanical), the costal chondrocytes can produce a more mechanically robust construct, perhaps through better organization of the collagen fibers. Mechanical stimuli are particularly well-suited for altering organizational changes, as seen with a variety of tissue-engineering studies, and will be an important area of future research for TMJ disc tissue-engineering (Huang *et al.*, 1993; Eastwood *et al.*, 1998; Seliktar *et al.*, 2000; Aufderheide and Athanasiou, 2006).

While the scaffoldless approach used in this study has clear advantages, such as avoiding immune responses to biomaterials, many of the TMJ disc cells and dermal fibroblasts were not retained at even the first timepoint. Low cell retention has also been seen previously with TMJ disc cells on scaffolds (Almarza and Athanasiou, 2005). At both timepoints, only one-eighth of the original cells were measured for the TMJ and dermal fibroblast constructs, and one-fourth remained in the costal chondrocyte/dermal fibroblast constructs. Dermal fibroblast and TMJ constructs also contracted significantly from the initial well diameter, while the costal chondrocyte constructs retained their initial size or grew slightly. A reduction in size makes it more difficult to engineer a replacement tissue with functional dimensions. Considering the retention of cells, overall

extracellular matrix production, and ability to create a replacement tissue with clinically relevant dimensions, the costal chondrocytes appear to be the most likely cell source candidate, of those studied here, for TMJ disc replacement, particularly with this scaffoldless approach.

Finally, the costal chondrocyte constructs offer several other advantages as a cell source for TMJ disc reconstruction. Large quantities of costal cartilage can be obtained from almost any person with a minimally invasive harvest technique, producing limited morbidity and complications (Yotsuyanagi et al., 2006). While costal cartilage is a relatively acellular tissue (Stockwell, 1967), the protocol could be optimized to limit the amount of needed tissue—for example, by expanding the cells before construct formation. However, individuals requiring a tissue-engineered disc would not have a sufficient quantity of non-pathological tissue to provide an adequate number of TMJ disc cells, even with passaging, particularly considering the size reduction discussed previously (Detamore et al., 2006). While the use of costal cartilage without *in vitro* manipulation is appealing, previous work reveals complications, like tissue overgrowth (Samman et al., 1995; Baek and Song, 2006) and undesirable calcification. These concerns can be addressed by controlling the *in vitro* environment used in a tissue-engineering approach. By influencing growth conditions and applied stimuli, we can engineer constructs to produce the appropriate dimensions, mechanical properties, and biochemical properties. After examination of the integrative capacities of engineered neotissue at different maturities, grafts may be more readily integrated with the native joint.

Although, in this experiment, costal chondrocytes were clearly superior as a highly productive and feasible cell source for tissue engineering, the simplicity of dermal fibroblast harvest warrants its continued examination. Further optimization is also needed to improve the costal chondrocyte constructs' mechanical properties. With the application of external stimuli, like growth factors or mechanical forces, scaffoldless costal chondrocyte constructs may produce sufficient quantities of organized matrix to function as a TMJ disc replacement and serve as a feasible alternative for persons seeking treatment for TMJ disorders.

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