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Additive and synergistic effects of bFGF and hypoxia on leporine meniscus cell-seeded PLLA scaffolds

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Abstract

Injuries to avascular regions of menisci do not heal and result in significant discomfort to patients. Current treatments, such as partial meniscectomy, alleviate these symptoms in the short-term, but lead to premature osteoarthritis as a result of compromised stability and changes in knee biomechanics. Thus, tissue engineering of the meniscus may provide an alternative treatment modality to overcome this problem. In this experiment, a scaffold-based tissue engineering approach was utilized to regenerate the meniscus. Meniscus cells were cultured on poly-L-lactic acid scaffolds in normoxic (~21% oxygen) or hypoxic (~2% oxygen) conditions in the presence or absence of the growth factor, basic fibroblast growth factor (bFGF). At $t = 4$ wks, histological sections of constructs showed presence of collagen and glycosaminoglycan (GAG) in all groups. Immunohistochemical staining showed presence of collagen I in all groups, and collagen II in groups cultured in hypoxic conditions. bFGF in the culture medium significantly increased cell number/construct by 25%, regardless of culturing condition. For GAG/construct, synergistic increases were observed in constructs cultured in hypoxic conditions and bFGF (2-fold) when compared to constructs cultured in normoxic conditions. Compressive tests showed synergistic increases in the relaxation modulus and coefficient of viscosity, and additive increases in the instantaneous modulus for constructs cultured in hypoxic conditions and bFGF when compared to constructs cultured in normoxic conditions. Overall, these results demonstrate that bFGF and hypoxia can significantly enhance the ability of meniscus cells to produce GAGs and improve the compressive properties of tissue engineered meniscus constructs *in vitro*.

Keywords

Knee meniscus; tissue engineering; hypoxia; bFGF; PLLA; synergy

1. Introduction

Injuries to avascular portions of the knee meniscus do not heal and can result in significant pain, swelling and loss of range of motion to patients. Current arthroscopic treatments to remove all or part of the meniscus alleviate these symptoms but lead to premature osteoarthritis as a result of compromised stability and changes in knee biomechanics (Maletius and Messner, 1996). Functional tissue engineering of the meniscus may provide an alternative treatment modality to overcome this problem. To obtain high cell numbers necessary in cartilage tissue engineering experiments, cells are usually passaged multiple times in standard Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS). The expansion of these cells, however, results in

phenotypic loss with rapid drop in cellular collagen II and COMP expression (Allen and Athanasiou, 2007; Darling and Athanasiou, 2005; Gunja and Athanasiou, 2007b). Researchers have employed several vehicles to recover losses in gene expression by culturing cells in three dimensional alginate gels, plating cells on two dimensional surface coatings and adding growth factors to the expansion medium (Allen *et al.*, 2008; Benya and Shaffer, 1982; Darling and Athanasiou, 2005; Gunja and Athanasiou, 2007b; Martin *et al.*, 1999). These techniques have been generally successful in reversing the effects of markers such as COMP and collagen I; however, collagen II expression reversal has been particularly challenging.

Recent work has suggested that low oxygen tension (hypoxia) may aid in meniscus cell (MC) and articular chondrocyte (AC) phenotype maintenance and enhancement (Adesida *et al.*, 2006; Hansen *et al.*, 2001; Murphy and Sambanis, 2001; Scherer *et al.*, 2004), with increases in expression of hypoxia inducible factor-1 α (HIF-1 α) and SOX-9. These transcription factors play important roles in collagen II synthesis (Adesida *et al.*, 2007; Pfander *et al.*, 2003). Furthermore, *in vivo*, cartilaginous tissues reside in hypoxic conditions where the lack of the blood supply creates a low oxygen environment (1 to 8%) for cartilage cells (Haselgrove *et al.*, 1993; Heppenstall *et al.*, 1976). The tissue engineering strategy used in this experiment aims to mimic *in vivo* conditions by culturing meniscus cells on poly-l-lactic acid (PLLA) scaffolds under hypoxic conditions. Other physiologically-relevant loading environments such as direct compression and hydrostatic pressure have also been studied in the literature with beneficial results obtained at 3-D level (Elder and Athanasiou, 2008; Mauck *et al.*, 2003; Natsu-Ume *et al.*, 2005).

In addition to hypoxia, we also examined whether basic fibroblast growth factor (bFGF), an ubiquitous growth factor with several different functions (Fernig and Gallagher, 1994; Nugent and Iozzo, 2000), would affect extracellular matrix (ECM) production on a PLLA scaffold and influence the final biomechanical properties of the cell-seeded construct. bFGF has been shown to promote glycosaminoglycan (GAG) synthesis in costal chondrocytes and MCs and aid in maintaining their phenotype (Adesida *et al.*, 2006; Kato and Gospodarowicz, 1985). bFGF is also a regular component of serum-free medium used for cartilaginous tissue engineering as well as for chondro-differentiation of mesenchymal stem cells (Hoben and Athanasiou, 2007; Hofmann *et al.*, 2006).

Thus, the goal of this experiment was to investigate whether bFGF and hypoxia would influence the matrix and functional properties of MC seeded-PLLA scaffolds. It was hypothesized that the individual application of bFGF and hypoxia would enhance the biochemical and compressive biomechanical properties of the constructs and that their combination would result in an additive or synergistic effect.

2. Materials and Methods

2.1 Cell harvesting, culture and passage

Cells were obtained from avascular portions of lateral and medial menisci of eight skeletally mature New Zealand white rabbits less than 12 hr after slaughter using a protocol described previously (Gunja *et al.*, 2009). Briefly, meniscus tissue was aseptically harvested from the knee joint and transferred to a sterile cell culture hood. The tissue was minced to less than 1 mm³ sections and digested with collagenase II overnight. Cells were then pooled the following day from the rabbits to reduce animal variability and counted using a hemocytometer. Cell viability (over 95%) was assessed using a trypan blue exclusion test. Cells were then frozen at -80°C in DMEM supplemented with 20% FBS and 10% dimethyl sulfoxide for one month. At the start of the experiment, cells were thawed and cell viability was determined to be 90%. The cells were plated on T-225 flasks at approximately 25%

confluence in chemically defined culture medium. The medium consisted of DMEM, 4.5 g/L-glucose and L-glutamine, 40 µg/mL L-proline, 100nM dexamethasone, 1% penicillin/streptomycin/fungizone, 50 µg/mL, ascorbate-2-phosphate, 100 µg/mL sodium pyruvate, 1% ITS+ and 1% FBS. FBS was used to promote cell attachment and proliferation during monolayer expansion. Cells used in the experiment were passaged at 90% confluence using trypsin/EDTA and counted with a hemocytometer.

2.2 Cell seeding

Cylindrical 2 mm thick and 3 mm wide non-woven PLLA scaffolds were obtained from a commercially available PLLA sheet (Biomedical Structures Warwick, RI) using a 3 mm dermal punch. The density of the scaffold as determined by the manufacturer was 60 mg/cc with an average molecular weight of 100 kDa, porosity of approximately 95%, crystallinity of 45-55%, and fiber diameter of 25 µm (Allen and Athanasiou, 2008). The scaffolds were sterilized using ethylene oxide followed by 70% ethanol. They were then washed twice with phosphate buffered saline (PBS) and housed in individual wells of 12 well plates that were previously coated with 0.5 ml of 2% sterile molten agarose and incubated overnight in culture medium. Prior to seeding, the culture medium was changed and passaged cells were injected into the scaffolds at a density of 1 million cells/scaffold. The 12-well plates were placed on an orbital shaker (80 rpm) in the incubator for 3 days. The plates were then removed from the orbital shaker and placed in static culture for an additional 2 days to allow for the cells to adhere to and infiltrate the PLLA scaffold.

2.3 Tissue culture

Post-seeding, constructs were randomly divided into four experimental groups. Constructs in the first group were housed in an incubator set at 37°C, 5% CO₂ and approximately 21% O₂ (normoxic, no bFGF group). Constructs in the second group were housed in the same incubator; however, the culture medium was supplemented with 5 ng/ml of bFGF (normoxic, bFGF group). The dose was chosen based on prior literature that showed its benefit for meniscus cell culture (Adesida *et al.*, 2006). Constructs in the third group were housed in a Billups-Rothenberg modular incubator chamber (Billups-Rothenberg, Del Mar, CA) that contained a custom gas mixture of 5% CO₂, 93% N₂ and 2% O₂ (hypoxic, no bFGF group). An oxygen concentration of 2% O₂ has been used previously to mimic a hypoxic environment and enhance cartilage matrix synthesis (Coyle *et al.*, 2009; Koay and Athanasiou, 2008; Zaka *et al.*, 2009). Sterile deionized water was used to humidify the chamber and the entire chamber was placed in the regular incubator housing the normoxic constructs. Constructs in the fourth group were housed in the same incubator; however, the culture medium was supplemented with 5 ng/ml of bFGF (hypoxic, bFGF group). The culture medium in all groups was devoid of FBS and was changed once every 3 days. In addition, a fresh dose of bFGF was added during every medium change to groups that were cultured with the growth factor. The custom gas mixture used in the Billups-Rothenberg incubator was flushed once every 3 days during each media change. Constructs were kept in culture for a period of 4 wks post-seeding.

2.4 Histology and immunohistochemistry (IHC)

At t = 4 wks, two samples from each group were frozen using HistoPrep and then sectioned at 14 µm. Safranin-O and fast green stains were used to determine GAG distribution (Rosenberg, 1971). Picro-sirius red staining was used to qualitatively determine the presence of collagen (Battlehner *et al.*, 1996). Collagen I and collagen II distributions were determined using a Biogenex i6000 autostainer. Briefly, sectioned samples were fixed in chilled acetone (4°C) for 20 min and rinsed with IHC buffer. Hydrogen peroxide/methanol was added for 30 min to quench samples of peroxidase activity. The samples were then blocked with horse serum (Vectastain ABC kit). Slides were then incubated with mouse

anti-COL 1 (1:1000 dilution) (Accurate Chemicals, Westbury, NY) or mouse anti-COL 2 (1:1000 dilution) (Chondrex, Redmond, WA) antibodies for one hour. A secondary mouse IgG antibody (Vectastain ABC kit) was then added for 30 min and color was developed using the Vectastain ABC reagent and DAB (Vector Labs, Burlingame, CA) for 8 min.

2.5 Quantitative biochemistry

At $t = 0$ (5 days post-seeding) and $t = 4$ wks, samples were digested at 65 °C overnight with 125 µg/ml papain in 50 mM phosphate buffer (pH = 6.5) containing 2 mM N-acetyl cysteine and 2 mM EDTA. A picogreen cell proliferation assay kit (Invitrogen, Carlsbad, CA) was used to determine total DNA content in each sample. Total GAG was quantified using the manufacturer's protocol provided in Blyscan Glycosaminoglycan Assay kit (Pietila *et al.*, 1999). A modified chloramine-T hydroxyproline assay was used to determine total collagen in the construct (Woessner, 1961). Briefly, samples were hydrolyzed with NaOH at 121 °C and neutralized with HCl. They were then combined with *p*-dimethylaminobenzaldehyde in perchloric acid. For the 4 wk samples, compressive properties of the constructs were first determined prior to biochemical testing.

2.6 Mechanical properties

The viscoelastic compressive properties of samples from each group were tested at $t = 4$ wks using a set up described previously (Allen and Athanasiou, 2006). Briefly, an incremental stress relaxation test at 10%, 20% and 30% strain was designed and implemented after determining the height of the construct. Each sample was held at the chosen strain level for 20 minutes with a 10% strain step. The strain rate was kept constant throughout at 0.5 mm/s. Data obtained from each test were fitted using MATLAB to an incremental stepwise viscoelastic stress relaxation solution for a standard linear solid. (Allen and Athanasiou, 2005) The parameters obtained were converted to instantaneous modulus (E_t), relaxation modulus (E_r) and coefficient of viscosity (μ) for each strain level.

2.7 Additive and synergistic interactions

Biochemical and biomechanical data were examined for synergistic or additive interactions. The two conditions considered in this experiment were 1) presence of hypoxia and 2) presence of bFGF. A synergistic increase in properties was observed if the total effect during the interaction of the hypoxic condition and bFGF was greater than each individual condition. An additive increase was observed if the total effect during an interaction was equivalent to the sum of each individual effect. All calculations performed were normalized to control group values (normoxic, no bFGF group).

2.8 Statistical analyses

The quantitative biochemical and biomechanical data were compared using a 2-way analysis of variance (ANOVA). If significant differences were observed, a Tukey's *post hoc* test was performed to determine specific differences among groups. The interaction terms of a the 2-way ANOVA were also used to confirm if synergistic effects were observed among groups (Slinker, 1998). A significance level of 95% with a *p* value of 0.05 was used in all statistical tests performed. All values are reported as mean $\pm$ standard deviation.

3. Results

3.1 Gross morphology, histology, and IHC (n = 2)

Morphological analysis at $t = 4$ wks showed presence of translucent cartilage-like matrix in all groups (Fig. 1). Constructs retained their structural integrity over the culture period with no observable signs of scaffold degradation. No significant differences were observed for

the construct diameter and thickness for the culturing condition ($p = 0.52$, $p = 0.43$) and for the growth factor treatment ($p = 0.20$, $p = 0.41$) (Table 1). Histological analysis showed presence of collagen and GAG in all constructs (Fig. 1). Collagen staining was uniform throughout the construct in groups exposed to bFGF while staining was limited to the periphery of the constructs in other groups. GAG staining was concentrated to the periphery of the construct with interspersed pockets of GAG observed in constructs cultured simultaneously under hypoxic conditions and bFGF. All constructs stained positively for collagen I (data not shown). Collagen II staining was diffuse in constructs exposed to hypoxia and absent for constructs cultured in normoxic conditions (Fig. 1).

3.2 Biochemistry (n = 5)

At $t = 4$ wks, wet weight of constructs were significantly higher in groups cultured with bFGF ($p = 0.03$), regardless of culturing condition ($p = 0.63$) (Table 1). No significant differences were observed in the dry weight at the same time point for the growth factor treatment ($p = 0.55$) and culturing condition ($p = 0.74$) (Table 1). At $t = 0$, the cell number was found to be 0.60 ± 0.2 million cells/construct via picogreen analysis. At $t = 4$ wks, cell numbers/constructs significantly increased in constructs exposed to bFGF ($p = 0.002$), to approximately 0.74 ± 0.1 million cells/construct (Fig. 2); however, the culturing condition was not found to be significant ($p = 0.82$). Thus, hypoxic conditions did not affect meniscus cell proliferation rates on PLLA constructs.

Collagen and GAG contents were undetectable at $t = 0$ in all groups. At $t = 4$ wks, the neither the culturing condition ($p = 0.81$) nor growth factor treatment ($p = 0.31$) were found to be significantly different for total collagen/construct. Values ranged between $15 \pm 7 \mu\text{g}$ for the normoxic, no bFGF group to $21 \pm 3 \mu\text{g}$ for the hypoxic, no bFGF group (Fig. 3). For GAG/construct, however, both the culturing condition ($p = 0.01$) and the growth factor ($p = 0.004$) were found to be significant factors. Specifically, GAG/construct was at least 2 times higher in the hypoxia + bFGF group ($17 \pm 5 \mu\text{g}$) when compared to both non-bFGF treated groups ($\sim 8 \pm 2 \mu\text{g}$) (Fig. 4). Further, it was determined that a synergistic increase was observed in the total GAG content for constructs stimulated in hypoxic conditions with bFGF when compared to constructs cultured in the normoxic condition with no bFGF.

3.3 Mechanical properties (n = 5)

Compressive viscoelastic tests were performed at three different strain levels (10%, 20%, and 30%) and the instantaneous modulus, relaxation modulus, and coefficient of viscosity were determined at each strain level (Fig. 5). At each strain level, the constructs exposed to hypoxia and bFGF were found to have significantly higher instantaneous modulus, relaxation modulus, and coefficient of viscosity when compared to the no growth factor control groups. At 30% strain, for example, the relaxation modulus and the instantaneous modulus for the hypoxia + bFGF group were $31 \pm 7 \text{ kPa}$, and $80 \pm 10 \text{ kPa}$ respectively, approximately 35% and 50% higher than their corresponding normoxia – bFGF control group values. The coefficient of viscosity at the same strain for the hypoxia + bFGF group was $205 \pm 58 \text{ kPa}$, approximately 38% higher than the normoxia – bFGF group. Further, it was determined that an additive increase was observed for the instantaneous modulus, and synergistic increases were observed for the relaxation modulus, and the coefficient of viscosity at all strain levels.

3.4 Correlation between biochemical and biomechanical data

The compressive properties obtained from incremental stress relaxation curves were correlated with the GAG and collagen content at each strain level. At 30% strain, univariate regression analysis showed a significant correlation between instantaneous modulus and GAG/construct ($p < 0.0001$, $R^2 = 0.56$), and collagen/construct ($p = 0.002$, $R^2 = 0.24$).

Similar results were obtained when correlating the relaxation modulus and GAG/construct ($p < 0.0001$, $R^2 = 0.61$), and collagen/construct ($p < 0.001$, $R^2 = 0.31$). Further, the coefficient of viscosity correlated significantly with GAG/construct ($p = 0.001$, $R^2 = 0.45$), and collagen/construct ($p = 0.01$, $R^2 = 0.32$) as well. Similar significant correlations were obtained at 20 and 30% strain levels.

4. Discussion

This study demonstrates the potential benefit of hypoxia and bFGF for knee meniscus tissue engineering. The biochemical content was enhanced with synergistic increases in total GAG content for constructs exposed to hypoxia and bFGF. The compressive properties of the construct also increased, with additive increases in the instantaneous modulus and synergistic increases in the relaxation modulus and the coefficient of viscosity observed for constructs exposed to both hypoxia and bFGF. Although, significant differences were not observed for total collagen content/construct, scaffolds exposed to hypoxia and a combination of hypoxia and bFGF showed diffuse staining for collagen II, which was absent in the normoxic groups. Together, these results demonstrate that growth factors and oxygen concentrations can significantly enhance the ability of meniscus cells to produce relevant ECM *in vitro*.

Cells in the inner regions of a skeletally mature meniscus reside in an avascular, low oxygen tension environment that may induce their chondrogenic-like phenotype.(Adesida *et al.*, 2007) Studies have shown that under hypoxic conditions, MCs express HIF-1 α that controls SOX-9 expression (Adesida *et al.*, 2006; Adesida *et al.*, 2007) which has been implicated in modulating aggrecan and collagen II gene expression (Kypriotou *et al.*, 2003; Sekiya *et al.*, 2000). In this experiment, a significant increase in GAG was observed in constructs cultured in hypoxic conditions suggesting increases in the production of aggrecan and other proteoglycans with sulfated GAG chains such as decorin and biglycan. Further, IHC data showed that collagen II production was enhanced relative to collagen I production in the hypoxic constructs. In a previous experiment, we showed that inner avascular portions of the meniscus that consist of mainly chondrocyte-like cells rapidly dedifferentiate during monolayer and express very low levels of collagen II (Gunja and Athanasiou, 2007b). Thus, the observed collagen II increase in this study was exciting and suggests that post-translational processing of collagen II (Koivunen *et al.*, 2007) relative to collagen I was increased under hypoxic conditions, even though total collagen processed remained the same.

Lateral and medial meniscus cells under hypoxic conditions have also been shown to upregulate TGF- β 1 gene expression after 8 and 24 hrs of hypoxia (Hofstaetter *et al.*, 2005). TGF- β 1 has been shown previously to be a potent regulator of GAG synthesis in meniscus cells (Pangborn and Athanasiou, 2005; Uthamanthil and Athanasiou, 2006). In chondrocytes, TGF- β 1 has been shown to directly influence the synthesis of GAGs by accelerating glucose transport via extracellular signal-regulated kinase-dependent signaling and protein kinase C pathways (Shikhman *et al.*, 2004). Further, hypoxia and TGF- β pathways have also been shown to function synergistically (Sanchez-Elsner *et al.*, 2001), and may supplement SOX-9 pathways, to enhance GAG production on the meniscus-cell seeded scaffolds.

The presence of bFGF in the medium significantly increased the GAG production in the constructs. Furthermore, in the presence of hypoxia and bFGF, a synergistic increase was observed in GAG content over the normoxic control. These results build upon a prior study that showed increases in GAG/DNA in 3-D MC aggregates in the presence of bFGF alone or bFGF and hypoxia (Adesida *et al.*, 2006). The exact mechanism by which bFGF might

modulate GAG synthesis in meniscus cells is unclear. It is known that bFGF has the ability to maintain meniscus cells in a plastic state in monolayer and make them more responsive to a chondrogenic stimulus (Adesida *et al.*, 2006). In addition, bFGF also plays an important role in modulating intracellular Ca^{2+} levels through protein kinase C activity (Peluso, 2003). Changes in intracellular Ca^{2+} levels can affect downstream signaling pathways that influence gene expression and protein synthesis of various extracellular matrix molecules (Wicks *et al.*, 2000). In the vascular literature, the combination of hypoxia and bFGF has been shown to induce tube formation by human microvascular endothelial cells in fibrin clots by activating transcription factor NF- κ B (p65) and increasing phosphorylated mitogen-activated protein kinases ERK1/2 over normoxic levels (Kroon *et al.*, 2001). NF- κ B, usually associated with inflammation pathways, has also been shown to be activated in differentiated chondrocytes (Ulivi *et al.*, 2008). This suggests that the combination of hypoxia and bFGF may be responsible in enhancing cartilaginous markers, such as GAGs and collagen II, in differentiated MCs used in this experiment.

This study also showed that bFGF in the culture medium resulted in increased cell number/construct for both hypoxic and normoxic groups. This increase is likely mediated through transmembrane surface receptors with tyrosine kinase activity (Klagsbrun and Baird, 1991). Other groups have also reported increases in cell number in the presence of bFGF for MCs (Pangborn and Athanasiou, 2005), fibroblasts (Basilico and Moscatelli, 1992), chondrocytes (Chua *et al.*, 2007) and dedifferentiated chondrocytes (Hill *et al.*, 1991). It is yet unclear whether bFGF treatment results in selective proliferation of chondrocyte-like cells from the inner meniscus over fibroblast-like cells from the outer meniscus or whether it maintains both cell types in a plastic state as they proliferate and makes them more responsive to a chondrogenic stimulus. Future studies will have to examine inner and outer meniscus cells separately to isolate the effects of cell type. It is known that inner and outer meniscus cells respond differently to hypoxic treatment with stark changes in gene expression of SOX-9 and collagen I (Adesida *et al.*, 2007); however, whether bFGF elicits a similar response has not been shown.

The compressive properties of meniscus constructs increased when exposed to bFGF in the presence of hypoxia with synergistic increases in the relaxation modulus and coefficient of viscosity and additive increases to the instantaneous modulus. Compressive properties of the meniscus can be correlated with the concentration of GAGs in the tissues. In this study, the instantaneous modulus, the relaxation modulus, and the coefficient of viscosity were all found to be strongly correlated to GAG/construct. GAGs are negatively charged particles that attract water molecules into the tissue and increase the overall construct stiffness (Sweigart and Athanasiou, 2001). A weaker correlation was observed with collagen/construct and the instantaneous modulus, relaxation modulus, and the coefficient of viscosity. Collagen provides tensile resistance to the tissue (Skaggs *et al.*, 1994) and although we did not perform tensile tests in this experiment, we hypothesize that a stronger correlation to collagen would exist with tensile properties such as Young's modulus and ultimate tensile strength when compared to compressive properties. It is of interest to note that a weak but significant correlation between the coefficient of viscosity and GAG was also observed. We are not aware of any such correlation in the literature. This result may be explained via the enhanced electrostatic forces generated by GAGs which may in turn inhibit fluid flow and render the construct more viscous in behavior.

5. Conclusions

Overall, these results demonstrate the effectiveness of combining bFGF and low oxygen tension (2% O_2) to synergistically enhance matrix and functional properties of tissue engineered meniscus constructs *in vitro*. Specifically, we were able to observe significant

enhancements in GAG content, collagen II content, and cell number/construct in groups exposed to hypoxia and bFGF when compared to the controls. In future studies, additional anabolic stimuli, such as hydrostatic pressure (Gunja and Athanasiou, 2007a) or direct compression (Mauck *et al.*, 2003) can be employed in conjunction with hypoxia + bFGF to further leverage potential synergies between the systems and further enhance the overall functional properties of the meniscus constructs towards native values. In addition, a functional assessment technique, such as a functionality index, previously used with articular cartilage constructs, may be used to predict an engineered meniscus tissue's similarity to native meniscus tissue (Sanchez-Adams and Athanasiou, 2009).

Acknowledgments

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Figure 1. Gross morphological, histological (collagen and GAG) and immunohistochemical (collagen II) sections of constructs at t = 4 wks.

Figure 2.

Cell number per construct. At $t = 0$, cell number per construct was found to be 0.60 ± 0.2 million cells, as indicated by the dashed line. A 2-way ANOVA was performed followed by a Tukey's post hoc analysis to determine significant differences within factors. Groups with different letters are significantly different from each other. All values are reported as mean $\pm$ SD.

Figure 3. Total collagen per construct. Statistics were conducted using a 2-way ANOVA. All values are reported in mean $\pm$ SD.

Figure 4. Total GAG content per construct. Statistics were conducted using a 2-way ANOVA. Groups with different letters (capital or small) are significantly different from each other. Star symbol refers to a synergistic increase in GAG production. All values are reported as mean $\pm$ SD.

Figure 5.

Compressive properties of the constructs (30% strain) at t = 4 wks. Statistics were conducted using a 2-way ANOVA. Groups with different letters (capital or small) are significantly different from each other. Star symbol refers to a synergistic increase in relaxation modulus and coefficient of viscosity. Box symbol refers to an additive increase in instantaneous modulus. All values are reported as mean $\pm$ SD.

Table 1

Wet weight, dry weight, thickness, and diameter of constructs at t = 4 weeks. $p < 0.05$ was considered significant.

Groups	Wet weight		Dry weight		Thickness		Diameter	
	No	bFGF (mg)	No	bFGF (mg)	No	bFGF (mm)	No	bFGF (mm)
Normoxic	11.4 ± 1.1	12.8 ± 1.2	0.59 ± 0.2	0.60 ± 0.1	1.9 ± 0.2	2.2 ± 0.1	2.8 ± 0.2	3.0 ± 0.2
Hypoxic	11.5 ± 1.3	13.8 ± 1.4	0.61 ± 0.2	0.62 ± 0.3	2.0 ± 0.1	2.0 ± 0.3	2.9 ± 0.2	3.1 ± 0.3
<i>p</i> (Culture condition)	0.63		0.74		0.43		0.52	
<i>p</i> (Growth factor)	0.03		0.55		0.41		0.20	