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RESEARCH ARTICLE

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 $t = 4$ weeks, histological sections of constructs showed presence of collagen and glycosaminoglycan (GAG) in all groups. Immunohistochemical staining showed the presence of collagen I in all groups and collagen II in groups cultured under hypoxic conditions. bFGF in the culture medium significantly increased cell number/construct by 25%, regardless of culture conditions. For GAG/construct, synergistic increases were observed in constructs cultured in hypoxic conditions and bFGF (two-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 under hypoxic conditions and bFGF, when compared to constructs cultured under 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*.

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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 the high cell numbers necessary in cartilage tissue-engineering experiments, cells are usually passaged multiple times in standard Dulbecco's modified Eagle's medium (DMEM) 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 (3D) alginate gels, plating cells on two-dimensional (2D) surface coatings

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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 the expression of hypoxia-inducible factor-1α (HIF-1α) 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 (1–8%) 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 the 3D 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 the 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 the lateral and medial menisci of eight skeletally mature New Zealand white rabbits <12 h 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 <1 mm^3 sections and digested with collagenase II overnight. The cells were then pooled from the rabbits the following day, to reduce animal variability, and counted using a haemocytometer. Cell viability (>95%) was assessed using a Trypan blue exclusion test. The cells were then frozen at −80 °C in DMEM supplemented with 20% FBS and 10% dimethyl sulphoxide for 1 month. At the start of the experiment, the 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, 100 nM dexamethasone, 1% penicillin/streptomycin/fungizone, 50 μg/m 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. The cells used in the experiment were passaged at 90% confluence, using trypsin–EDTA, and counted using a haemocytometer.

2.2. Cell seeding

Cylindrical (2 mm thick × 3 mm wide) non-woven PLLA scaffolds were obtained from a commercially available PLLA sheet (Biomedical Structures Warwick, RI, USA), using a 3 mm dermal punch. The density of the scaffold, as determined by the manufacturer, was 60 mg/ml, with an average molecular weight of 100 kDa, porosity of approximately 95%, crystallinity of 45–55% and fibre 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 had previously been coated with 0.5 ml 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 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% CO_2 and approximately 21% O_2 (normoxic; no bFGF group). Constructs in the second group were housed in the same incubator; however, the culture medium was supplemented with

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5 ng/ml 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 (Del Mar, CA, USA) modular incubator chamber that contained a custom gas mixture of 5% CO_2, 93% N_2 and 2% O_2 (hypoxic; no bFGF group). An oxygen concentration of 2% O_2 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 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 weeks post-seeding.

2.4. Histology and immunohistochemistry (IHC)

At $t = 4$ weeks, 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 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 the samples of peroxidase activity. The samples were then blocked with horse serum (Vectastain ABC kit). The slides were then incubated with mouse anti-COL 1 antibodies (1 : 1000 dilution; Accurate Chemicals, Westbury, NY, USA) or mouse anti-COL 2 antibodies (1 : 1000 dilution; Chondrex, Redmond, WA, USA) for 1 h. A secondary mouse IgG antibody (Vectastain ABC kit) was then added for 30 min and colour was developed using the Vectastain ABC reagent and DAB (Vector Labs, Burlingame, CA, USA) for 8 min.

2.5. Quantitative biochemistry

At $t = 0$ (5 days post-seeding) and $t = 4$ weeks, 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, USA) was used to determine the 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 hydrolysed with NaOH at 121 °C and neutralized with HCl. They were then combined with *p*-dimethylaminobenzaldehyde in perchloric acid. For the 4 week samples, the 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 $t = 4$ weeks, 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 min, 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_i), 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: (a) presence of hypoxia; and (b) 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 two-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 two-way ANOVA were also used to confirm whether 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 ± standard deviation (SD).

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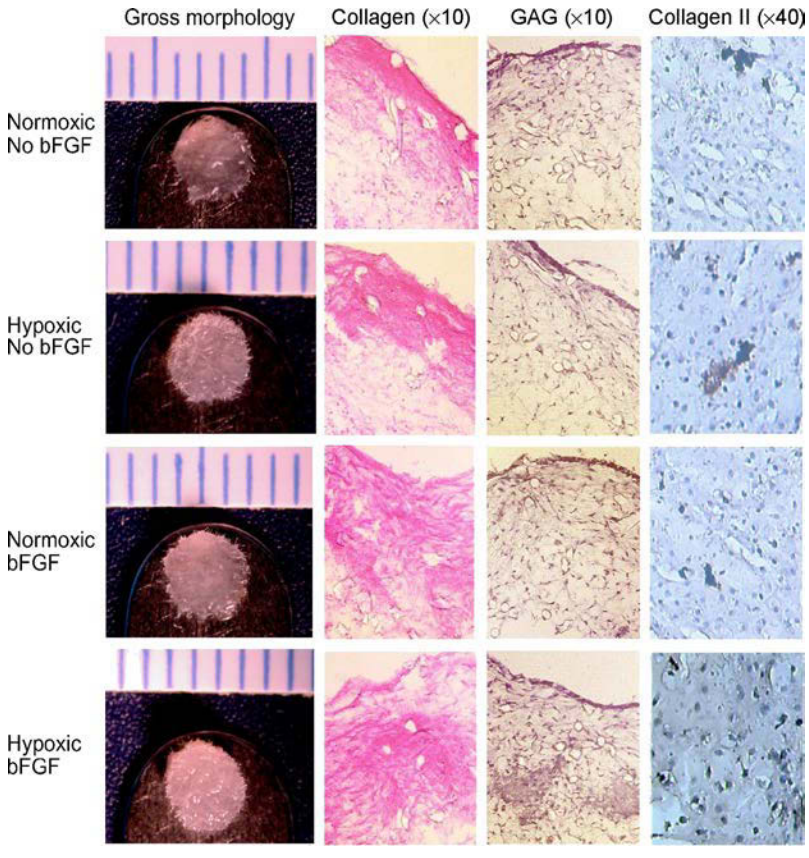

Figure 1. Gross morphological, histological (collagen and GAG) and immunohistochemical (collagen II) sections of constructs at $t = 4$ weeks

Table 1. Wet weight, dry weight, thickness and diameter of constructs at $t = 4$ weeks. $p < 0.05$ was considered significant

	Wet weight		Dry weight		Thickness		Diameter	
Groups	No bFGF (mg)	bFGF (mg)	No bFGF (mg)	bFGF (mg)	No bFGF (mm)	bFGF (mm)	No bFGF (mm)	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
p (Culture condition)	0.63		0.74		0.43		0.52	
p (Growth factor)	0.03		0.55		0.41		0.20	

3. Results

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

Morphological analysis at $t = 4$ weeks showed the presence of a translucent cartilage-like matrix in all groups (Figure 1). The 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 culture condition ($p = 0.52$ and $p = 0.43$) and for the growth factor treatment ($p = 0.20$ and $p = 0.41$) (Table 1). Histological analysis showed presence of collagen and GAG in all constructs (Figure 1). Collagen staining was uniform throughout the constructs 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 under normoxic conditions (Figure 1).

3.2. Biochemistry ($n = 5$)

At $t = 4$ weeks, the wet weight of constructs were significantly higher in groups cultured with bFGF ($p = 0.03$), regardless of the culture 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 culture condition ($p = 0.74$) (Table 1). At $t = 0$, the cell number was found via picogreen analysis to be 0.60 ± 0.2 million cells/construct. At $t = 4$ weeks,

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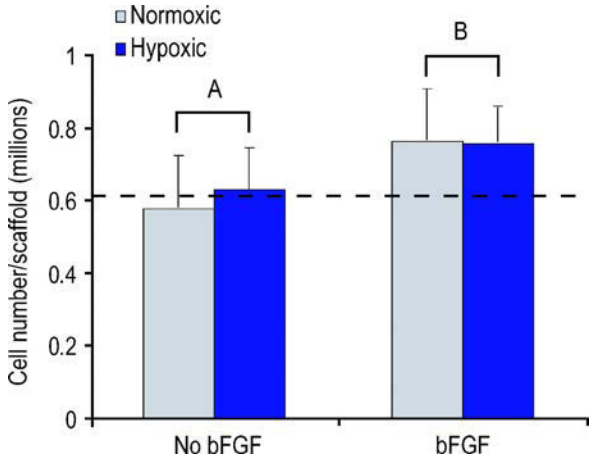

Figure 2. Cell number per construct. At $t = 0$, cell number per construct was found to be 0.60 ± 0.2 million cells, as indicated by the dashed line. A two-way ANOVA was performed followed by a Tukey's *post hoc* analysis to determine significant differences within factors. Groups with different letters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

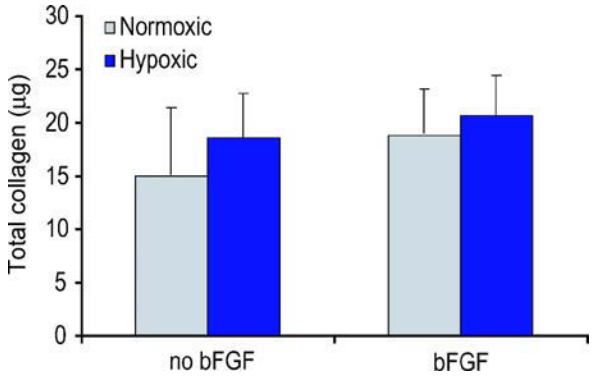

Figure 3. Total collagen per construct. Statistics were conducted using a two-way ANOVA. All values are reported in mean ± SD

cell numbers/constructs significantly increased in constructs exposed to bFGF ($p = 0.002$) to approximately 0.74 ± 0.1 million cells/construct (Figure 2); however, the culture 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 $t = 4$ weeks, the neither the culture 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 ± 7 µg for the normoxic, no-bFGF group to 21 ± 3 µg for the hypoxic, no-bFGF group (Figure 3). For GAG/construct, however, both the culture condition ($p = 0.01$) and the growth factor ($p = 0.004$) were found to be significant factors. Specifically, the GAG/construct was at least two-fold higher in the hypoxia + bFGF group (17 ± 5 µg) when compared to both non-bFGF-treated groups ($\sim 8 \pm 2$ µg) (Figure 4). Further, it was determined that a synergistic increase was observed in the total GAG content for constructs stimulated under hypoxic conditions with

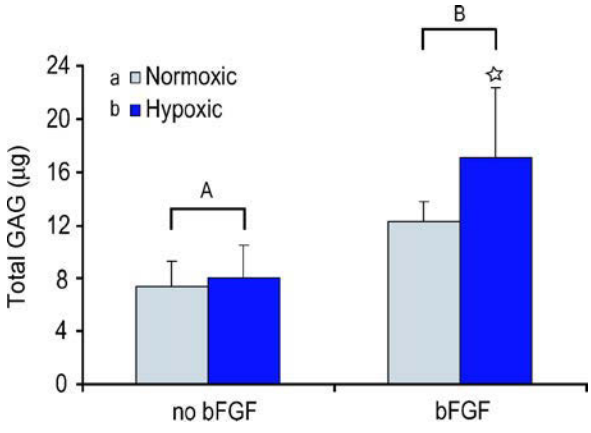

Figure 4. Total GAG content per construct. Statistical analysis was conducted using two-way ANOVA. Groups with different letters (capital or small) are significantly different from each other. Star symbol refers to a synergistic increase in GAG production. All values are reported as mean ± SD

bFGF when compared to constructs cultured under 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 (Figure 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 ± 7 kPa and 80 ± 10 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 ± 58 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 ($p <$

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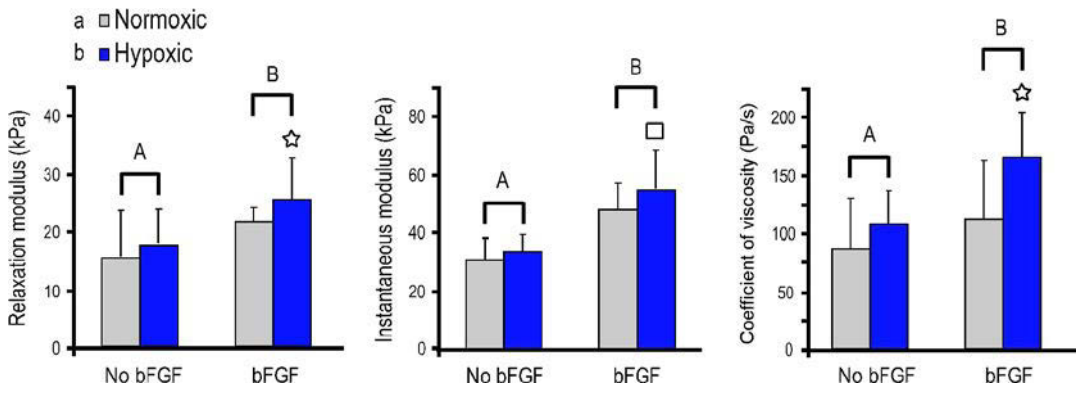

Figure 5. Compressive properties of the constructs (30% strain) at $t = 4$ weeks. Statistics were conducted using a two-way ANOVA. Groups with different letters (capital or small) are significantly different from each other. Star, synergistic increase in relaxation modulus and coefficient of viscosity; box, additive increase in instantaneous modulus. All values are reported as mean ± SD

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 the 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 contents/constructs, 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-1α, which controls SOX-9 expression (Adesida *et al.*, 2006, 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 sulphated 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 avascular inner portions of the meniscus, which 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 *TGFβ1* gene expression after 8 and 24 h of hypoxia (Hofstaetter *et al.*, 2005). TGFβ1 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, TGFβ1 has been shown to directly influence the synthesis of GAGs by accelerating glucose transport via extracellular signal-regulated kinase-dependent signalling and protein kinase C pathways (Shikhman *et al.*, 2004). Further, hypoxia and TGFβ 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 3D 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 signalling pathways that influence gene expression and protein synthesis of various extracellular matrix molecules (Wicks *et al.*, 2000). In the vascular

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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-κB (p65) and increasing phosphorylated mitogen-activated protein kinases ERK1/2 over normoxic levels (Kroon *et al*., 2001). NF-κB, 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 for 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 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 could 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 behaviour.

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).

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