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Combined effects of chemical priming and mechanical stimulation on mesenchymal stem cell differentiation on nanofiber scaffolds

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ABSTRACT

Functional tissue engineering of connective tissues such as the anterior cruciate ligament (ACL) remains a significant clinical challenge, largely due to the need for mechanically competent scaffold systems for grafting, as well as a reliable cell source for tissue formation. We have designed an aligned, polylactide-co-glycolide (PLGA) nanofiber-based scaffold with physiologically relevant mechanical properties for ligament regeneration. The objective of this study is to identify optimal tissue engineering strategies for fibroblastic induction of human mesenchymal stem cells (hMSC), testing the hypothesis that basic fibroblast growth factor (bFGF) priming coupled with tensile loading will enhance hMSC-mediated ligament regeneration. It was observed that compared to the unloaded, as well as growth factor-primed but unloaded controls, bFGF stimulation followed by physiologically relevant tensile loading enhanced hMSC proliferation, collagen production and subsequent differentiation into ligament fibroblast-like cells, upregulating the expression of types I and III collagen, as well as tenasin-C and tenomodulin. The results of this study suggest that bFGF priming increases cell proliferation, while mechanical stimulation of the hMSCs on the aligned nanofiber scaffold promotes fibroblastic induction of these cells. In addition to demonstrating the potential of nanofiber scaffolds for hMSC-mediated functional ligament tissue engineering, this study yields new insights into the interactive effects of chemical and mechanical stimuli on stem cell differentiation.

1. Introduction

The anterior cruciate ligament (ACL) is the primary knee joint stabilizer and is the most frequently injured ligament of the knee, with upwards of 100,000 reconstruction procedures performed annually in the United States (United States Department of Health and Human Services, 1996; Marrale et al., 2007). Due to the limited healing capacity of the ACL, surgical intervention is required to restore normal knee function in the event of injury (Bray et al., 2002). The bone-patellar tendon-bone (BPTB) autograft is considered to be the gold standard for this procedure but its use often results in donor site morbidity and anterior knee pain (Beynnon et al., 2002; Barrett et al., 2002). Hamstring tendon autografts have been used as alternatives for ACL replacement but frequently result in hamstring muscle weakness and bone tunnel enlargement, among other complications (Clatworthy et al., 1999; Feller and Webster, 2003). Furthermore, these grafts lack a functional tendon–bone interface, normally present in a bone-patellar tendon-bone graft, and must be mechanically fixed to the tibial and femoral bone tunnels resulting in insufficient integration with the subchondral bone, the primary cause of graft failure (Kurosaka et al., 1987; Robertson et al., 1986). Synthetic grafts have also been evaluated for ACL reconstruction but have shown limited success, largely due to their insufficient mechanical strength and the accumulation of wear particles in the joint capsule which can result in associated morbidity, such as synovitis (Richmond and Weitzel, 2010; Legnani et al., 2010; Ventura et al., 2010).

Recent studies have shown that tissue engineering is a promising method by which musculoskeletal tissues can be regenerated (Langer and Vacanti, 1993; Laurencin et al., 1999). For the purpose of ligament tissue engineering, various polymers, both natural (Fan et al., 2009; Horan et al., 2009; Tischer et al., 2007; Noth et al., 2005; Altman et al., 2002a) and synthetic (Freeman et al., 2009; Van et al., 2009; Shao et al., 2009; Fan et al., 2008; Cooper et al., 2007, 2005; Lu et al., 2005; Bourke et al., 2004; Lin et al., 1999; Amis et al., 1988, 1992), have been investigated to replace the ACL with promising results. Our long term goal is to develop a functional and integrative scaffold for ACL tissue engineering. To

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this end, the ideal scaffold for ACL reconstruction should be biocompatible so as not to elicit an adverse biological response, biodegradable to be replaced by new tissue formation, exhibit physiologically relevant mechanical properties and should promote the formation of a ligamentous tissue matrix (Cooper et al., 2005). Guided by these design criteria, this study sought to evaluate the potential of a poly(lactide-*co*-glycolide) nanofiber-based scaffold for ligament tissue engineering. Nanofibers are considered to be advantageous for ligament tissue engineering because of their superior biomimetic potential and physiological relevance. Such scaffolds have been previously investigated for applications in bone (Yoshimoto et al., 2003; Garreta et al., 2007), meniscus (Baker and Mauck, 2007), intervertebral disk (Nerurkar et al., 2007), cartilage (Li et al., 2003), ligament (Lee et al., 2005) and tendon (Moffat et al., 2009) tissue engineering with promising results. The primary advantage of nanofiber scaffolds is that they can be engineered to resemble the native ligament extracellular matrix, and designed to exhibit high aspect ratio, surface area, permeability and porosity. Additionally, fiber organization and alignment can be readily controlled during fabrication (Pham et al., 2006; Murugan and Ramakrishna, 2007), thereby allowing for tailored structural and material properties suitable for the demands of the anterior cruciate ligament.

It has been well established that constructs containing relevant cell types can be utilized to augment neo-tissue formation on tissue engineered scaffolds. Cooper et al. (2005) demonstrated that the unique response of primary ligament fibroblasts is the most desirable for ACL tissue engineering, however clinically, autologous ligament fibroblasts are difficult to obtain. Additionally, there are no unique markers which can be used to readily distinguish ligament fibroblasts from other conventional tissue fibroblasts for the purpose of in vitro selection and purification. As a result, mesenchymal stem cells (MSC) have emerged as a promising cell source for tissue engineering (Pittenger et al., 1999). These cells have been shown to respond to a variety of stimuli, both chemical (Pittenger et al., 1999) and mechanical (Altman et al., 2002b), and are capable of differentiating into chondrocytes (Barry et al., 2001), osteoblasts (Toquet et al., 1999) and fibroblasts (Moreau et al., 2005). In addition, they can be routinely harvested via bone marrow biopsy techniques and are able to maintain their differentiation capabilities even after long term culture (Pittenger et al., 1999).

Despite the versatility of MSCs for tissue engineering purposes, standardized methods to differentiate these cells towards a fibroblastic lineage are not well established. To this end, several groups have demonstrated that mechanical stimulation can be used to drive fibroblastic differentiation of MSCs. Among the earliest to evaluate *fibrogenic* differentiation of MSC were Altman et al. (2002b) who demonstrated that MSCs could be induced into a fibroblastic phenotype when seeded in type I collagen gels and subjected to a combination of tensile and rotational strain. Specifically, It was reported that the application of mechanical stress upregulated the expression of ligament fibroblast markers, resulted in the production of type III collagen and guided cell alignment in the direction of applied load. Studies performed by Butler et al. evaluating a range of mechanical stimulation parameters using a type I collagen sponge system have similarly demonstrated that tensile strain can upregulate the expression of fibroblastic markers and enhance MSC matrix deposition (Nirmalanandhan et al., 2008; Juncosa-Melvin et al., 2006; Butler et al., 2007; Butler et al., 2004). Recently, we reported that the application of dynamic mechanical stimulation to human MSCs cultured on aligned nanofiber scaffolds results in fibroblastic differentiation and the production of a ligament-like matrix (Subramony et al., 2013). Mechanical loading resulted in the upregulation of several fibroblastic genes including type III collagen, fibronectin, tenascin-C and scleraxis while also resulting in the production of a matrix rich in types I and III collagen.

To augment ligament engineering strategies, methods to further enhance MSC response and biosynthesis have been explored in order to facilitate the formation of functional ligament tissue. Mitogens such as the transforming growth factor-beta (TGF-β) family, basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) have been shown to induce proliferation both *in vitro* and *in vivo* for cell types including fibroblasts and MSCs and have also been shown to affect MSC differentiation and biosynthesis. In particular, bFGF has been shown to maintain MSC differentiation potential, stimulate proliferation, and induce fibroblastic differentiation. Studies performed by Hankemeier et al. (2005) demonstrated that low-dose (3 ng/ml) bFGF increased MSC proliferation as measured after 7 days and, on days 14 and 28, upregulated the expression of type I and III collagen, fibronectin and smooth muscle actin. Furthermore, chemical stimulation has been shown to synergistically enhance cell response when combined with mechanical stimulation. For example, Moreau et al. (2008) evaluated the sequential application of biochemical and mechanical stimulation to MSCs cultured on silk fiber-based scaffolds. It was reported that stimulating cells with bFGF or EGF for five days prior to the application of mechanical load modulated matrix protein expression and cell activity.

Building upon these observations, bFGF was selected for further investigation to enhance MSC-based ligament engineering on a nanofiber scaffold system. Specifically, the objective of this study is to evaluate the synergistic effect of chemical and mechanical stimulation on the fibroblastic differentiation of human MSCs when seeded on nanofiber scaffolds. It is hypothesized that chemical priming of hMSCs with bFGF prior to the application of mechanical stimulation will enhance hMSC matrix production and upregulate the expression of relevant fibroblastic genes.

2. Materials and methods

2.1. Nanofiber scaffold fabrication

Aligned nanofiber scaffolds composed of PLGA (85:15, M_w=123.6 kDa; Lakeshore Biomaterials, Birmingham, AL) were produced using electrospinning (Reneker and Chun, 1996; Moffat et al., 2009). Briefly, PLGA scaffolds were fabricated by producing a solution composed of 35% PLGA (v/v) in 55% N,N-dimethylformamide (Sigma-Aldrich, St. Louis, MO) and 10% ethyl alcohol. The solution was loaded into a syringe with an 18.5-gauge stainless steel blunt tip needle and electrospun at 8–10 kV and 1 mL/hour using a custom electrospinning device. Aligned fibers were produced by electrospinning onto a custom rotating mandrel (20 m/s) with polymer dispensation via a syringe pump (Harvard Apparatus, Holliston, MA; 1 ml/hr).

2.2. Cells and cell culture

Human mesenchymal stem cells (MSC) were obtained commercially (Lonza, Walkersville, MD) and maintained in culture with DMEM containing 10% fetal bovine serum (FBS, embryonic stem cell certified, Atlanta Biologicals, Atlanta, GA), 1% penicillin–streptomycin, 1% non-essential amino acids, 0.1% amphotericin B and 0.1% gentamicin. Cells were cultured to 80% confluence and then passaged using 0.25% trypsin/1 mM ethylenediaminetetraacetate (EDTA) and re-plated at a density of 5×10^3 cells/cm^2. Passage 2 cells were used for scaffold seeding.

All studies were performed using low-serum medium as adapted from previously published studies. This medium was identical to MSC maintenance media but contained 5% FBS and was utilized for the duration of the culture period. To evaluate chemical stimulation, the low-serum medium was supplemented with 10 ng/mL bFGF (Invitrogen).

Scaffolds were secured in a custom bioreactor to apply uniaxial tensile strain. Briefly, electrospun scaffolds (5×6 cm^2) were excised after fabrication and subsequently sterilized via ultraviolet irradiation (15 min/side). Scaffolds were secured in loading cartridges via Teflon clamps, sectioned into 5 cm × 1 strips and pre-incubated in culture medium at 37 °C and 5% carbon dioxide for 16 h. Cells were seeded on the scaffolds at a density of 3×10^4 cells/cm^2 and allowed to attach for 15 min before the addition of culture medium.

2.3. Cell attachment, alignment and proliferation

Cell viability and morphology ($n=3$/group) was evaluated using live/dead staining (Invitrogen, Carlsbad, CA) following the manufacturer's suggested protocol. The samples were imaged under confocal microscopy (Leica TCS SP5, Bannockburn, IL) at excitation wavelengths of 488 nm and 594 nm. Cell penetration was evaluated by taking a z-series of confocal images over a depth of 20 μm, equivalent to 15 to 20 layers of nanofibers. Total DNA content was measured using the PicoGreen dsDNA assay (Invitogen). At each time point, the samples ($n=5$/group) were homogenized in 0.1% Triton-X (Sigma-Aldrich) and subjected to 20 s of ultrasonication at 5 W. Fluorescence was measured using a microplate reader (Tecan) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. A standard curve was derived and used to correlate DNA concentration to fluorescence intensity, and cell number was determined based on a conversion factor of 8 pg DNA/cell (Lu et al., 2005).

2.4. Matrix production

Total collagen content per sample ($n=5$/group) was calculated using the Sircol Collagen Assay. The assay was performed following a 16-h sample digestion in Papain (Sigma-Aldrich) to solubilize extracellular matrix (ECM) proteins. Absorbance was measured using a microplate reader (Tecan) at 555 nm. A standard curve was generated and used to correlate total collagen content to absorbance.

Collagen penetration was also visualized using picrosirius red staining of frozen sections ($n=2$/group) after 28 days of culture. Briefly, after fixation, samples were embedded in 5% polyvinyl alcohol (PVA, Sigma-Aldrich) and 7-micrometer thick sections (spanning the depth and width of the scaffold) were obtained using a cryostat (Hacker-Bright OTF model, Hacker Instruments and Industries, Winnsboro, SC). Collagen distribution was visualized with picrosirius red staining under light microscopy (Axiovert 25, Zeiss).

2.5. Gene expression

Gene expression ($n=5$/group) was measured using quantitative real-time reverse transcriptase polymerase chain reaction at 1, 7, 14 and 28 days. Total RNA was isolated using the Trizol extraction method (Invitrogen). Isolated RNA was then reverse-transcribed into complementary DNA using the SuperScript First-Strand Synthesis System (Invitrogen), and the cDNA product was amplified using recombinant Taq DNA polymerase (Invitrogen). Expression of fibroblastic markers type I collagen, type III collagen, fibronectin tenascin-C, tenomodulin and scleraxis was determined. GAPDH served as the house-keeping gene. All primer sequences are based on published studies (Subramony et al., 2013). All genes were amplified for 50 cycles in a thermocycler (Bio-Rad iCycler, Hercules, CA) with a fluorescent probe (SYBR Green, Invitrogen). Quantitative analysis of gene expression was performed using the delta–delta CT method.

2.6. Mechanical properties

Mechanical properties of the loaded and unloaded scaffolds ($n=5$ samples/group) were determined at 1, 7, 14 and 28 days after priming. At each time point, samples were tested to failure under uniaxial tension. Scaffolds were secured with custom clamps tested to failure at a strain rate of 5 mm/min (Instron, Model 8841, Norwood, MA) with an average gauge length of 3 cm. Samples were evaluated to failure at a strain rate of 5 mm/min with load applied in the direction of fiber alignment. Scaffold yield strength and ultimate tensile stress were determined, and elastic modulus was calculated from the linear region of the stress–strain curve.

2.7. Experimental design and bioreactor culture

The effect of exogenous bFGF stimulation on MSC response was first evaluated in a 2-week study in which cell viability, proliferation, matrix deposition and differentiation were assessed. Subsequently, a 4-week study was conducted in which MSCs were primed statically for 5 days with bFGF and then subjected to dynamic tensile stimulation in a custom bioreactor. Loaded samples were subjected to 1% strain at 1 Hz for 90 min twice daily and evaluated over a period of 28 days. Control scaffolds, cultured in identical bioreactor cartridges, were subjected to the same growth factor priming regimen without subsequent mechanical stimulation.

2.8. Statistical analysis

Results are presented in the form of mean ± standard deviation, with n equal to the number of samples per group. Two-way ANOVA was used to determine the effects of chemical stimulation and mechanical loading on cell proliferation, matrix deposition, gene expression and mechanical properties. The Tukey–Kramer post-hoc test was used for all pair-wise comparisons and significance was attained at $p<0.05$. Statistical analyses were performed with JMP IN (4.0.4, SAS Institute, Inc., Cary, NC).

3. Results

3.1. MSC response to chemical stimulation

Cell viability and attachment morphology were visualized using confocal microscopy (Fig. 1). The MSCs displayed an elongated fibroblastic morphology that conformed to the alignment of the underlying nanofiber scaffold. Similarly viable cells were observed both with and without the treatment of bFGF. In terms of cell growth, by day 7, a significantly greater number of cells were measured for the group subjected to bFGF stimulation (Fig.1). The total number of cells was also significantly greater than that measured after 1 day of culture for both groups. After 14 days of culture, there remained significant differences in number of cells present in the control and bFGF stimulated group. Total collagen deposition was evaluated for both groups over time, and found to be similar at day 1 with a significant increase in the bFGF stimulated group after 7 days of culture (Fig. 1). A significantly greater amount of collagen was measured on scaffolds subjected to

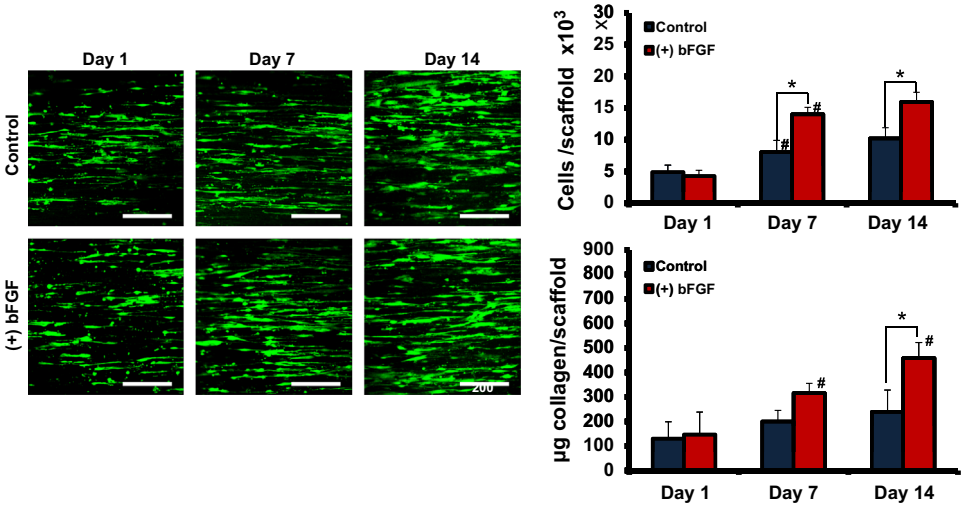

Fig. 1. Effect of bFGF on hMSC proliferation and matrix production. Treatment with exogenous bFGF resulted in significantly greater cells on scaffolds after 7 days of culture. Growth factor treatment also resulted in a significant increase in matrix deposition after 14 days of culture.

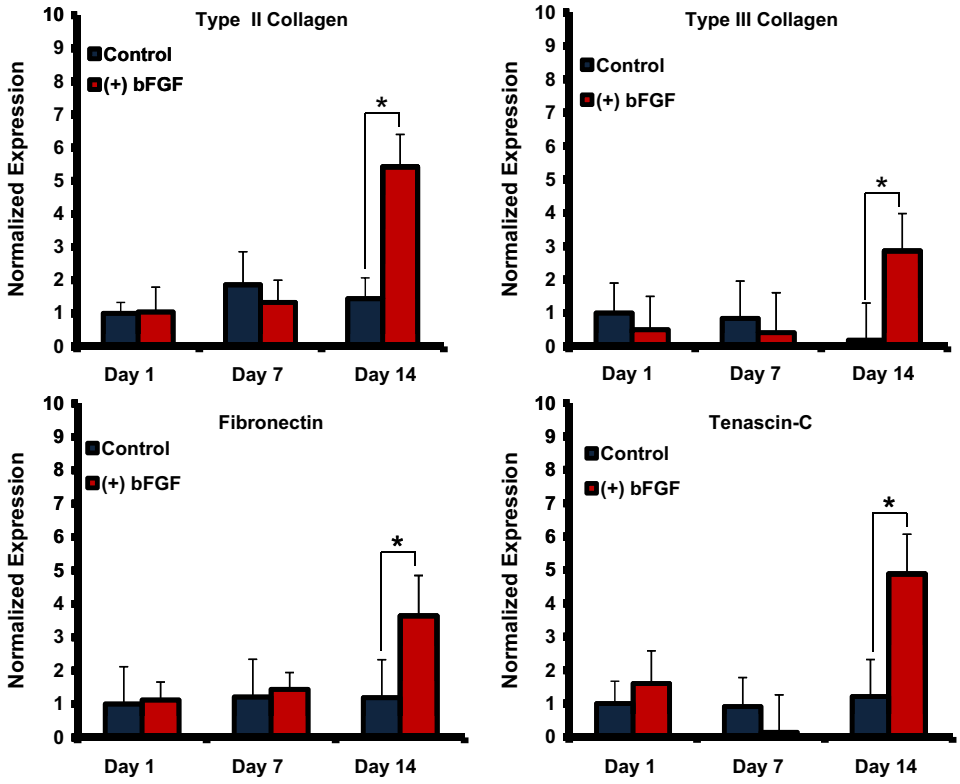

Fig. 2. Effect of bFGF on hMSC differentiation. Growth factor treatment resulted in the upregulation of types I and III collagen, fibronectin and tenascin-C. In contrast, the mean expression of scleraxis and tenomodulin decreased over time.

bFGF stimulation after 14 days. No significant change in total collagen was measured over time in the control group.

Differentiation of MSCs in both groups was assessed over the 14 day culture period by measuring the expression of types I and III collagen, fibronectin, tenascin-C, scleraxis and tenomodulin (Fig. 2). Significant changes in gene expression occurred only after 14 days of culture for the bFGF stimulation group. Specifically, types I and III collagen, fibronectin and tenascin-C were all upregulated after 14 days of chemical stimulation as compared to the control group. The expression of these genes did not change over time in the control group. In both the control and bFGF stimulated groups, the mean expression of scleraxis and teomoudlin decreased over time albeit no significant change was measured.

3.2. MSC response to sequential chemical and mechanical stimulation

After determining the effect of chemical stimulation on MSC response, a 4-week study was conducted to assess MSC response to sequential chemical and mechanical stimulation. Confocal microscopy of cells seeded revealed no differences in cell viability or morphology between the loaded and unloaded control group as cells aligned in the direction of the underlying nanofibers and remained viable over the duration of the study (Fig. 3). A significant increase in total cell number was observed after 7 days of culture for both the loaded and unloaded groups. Total cell number on scaffolds was significantly greater in the loaded group after 7 days of mechanical stimulation, as compared to unloaded controls. No significant difference between groups was measured after 14 days, though a significantly greater number of cells were once again measured in the loaded group after 28 days.

Matrix deposition was evaluated both quantitatively, to determine total collagen content, and qualitatively, to evaluate matrix morphology and penetration (Fig. 3). Total collagen deposition increased over the duration of the culture period with significantly greater collagen deposited in the loaded group as compared to unloaded controls by day 28. In addition, histological analysis of matrix deposition via Picrosirius Red staining revealed deeper matrix penetration into loaded scaffolds after four weeks of culture.

To evaluate MSC differentiation, the expression of several key fibroblastic genes was measured using quantitative PCR. In the unloaded group, type I collagen expression was unregulated significantly at 7 days after the priming culture period but decreased significantly after 28 days. In contrast, type I collagen was upregulated to a greater extent in the loaded group after 7 days of mechanical stimulation and remained elevated after 28 days. The expression of type III collagen was significantly upregulated after 14 days in both groups though the expression levels on mechanically stimulated scaffolds were significantly higher at both 14 and 28 days. Fibronectin expression increased over time in both the unloaded and loaded groups with expression levels remaining similar regardless of the application of mechanical stimulation post chemical priming. Tenascin-C expression increased over time in both groups over the first 14 days following priming. Interestingly, a significant decrease in tenascin-C expression was measured in the unloaded group after 28 days whereas the expression level was maintained with mechanical stimulation. Expression of scleraxis remained similar in both groups over the duration of the study; however the expression of tenomodulin increased significantly after 28 days of loading (Fig. 4).

Scaffold mechanical properties were determined over the duration of the study via uniaxial tensile testing. No differences in mechanical properties were measured between the unloaded and loaded groups in terms of elastic modulus, yield strength, ultimate tensile strength or ductility. A significant decrease in elastic modulus and ductility were observed after 28 days of culture in both groups (Fig. 5).

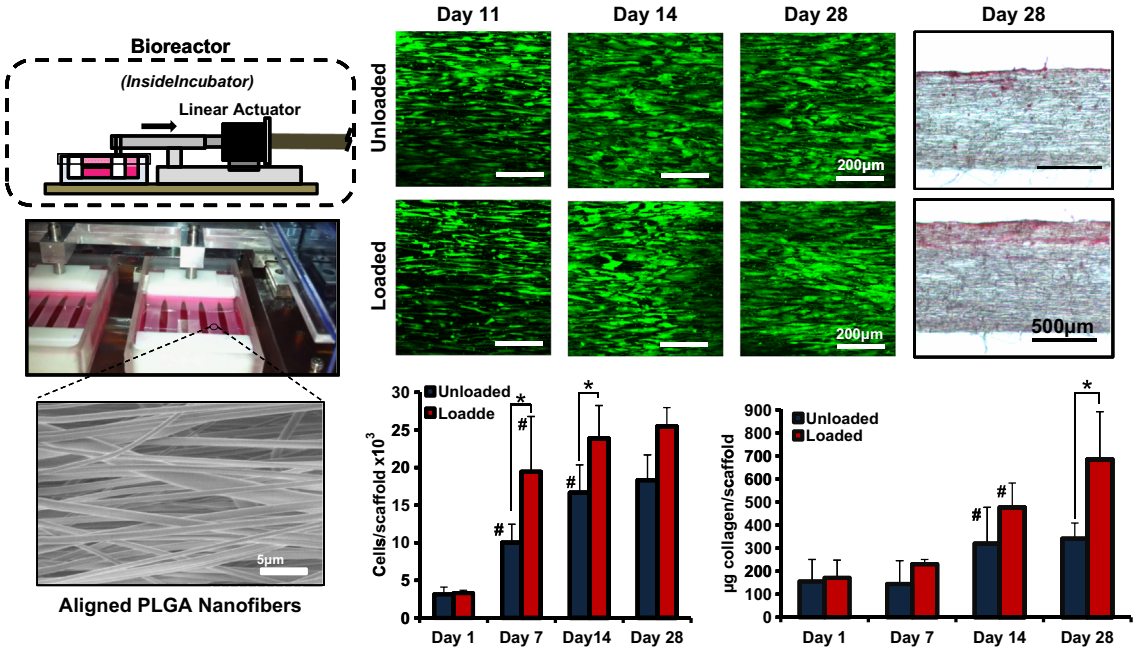

Fig. 3. Effect of chemical and mechanical stimulation on hMSC proliferation and biosynthesis. Cells remained similarly viable on both unloaded and loaded scaffolds. A significantly greater number of cells was measured on loaded scaffolds as compared to unloaded scaffolds after 28 days. Mechanical stimulation in conjunction with growth factor priming resulted in enhanced collagen deposition after 28 days of loading, as compared to unloaded controls. Histological analysis, via picrosirius red staining of collagen, indicated deeper matrix penetration into loaded scaffolds after 28 days. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

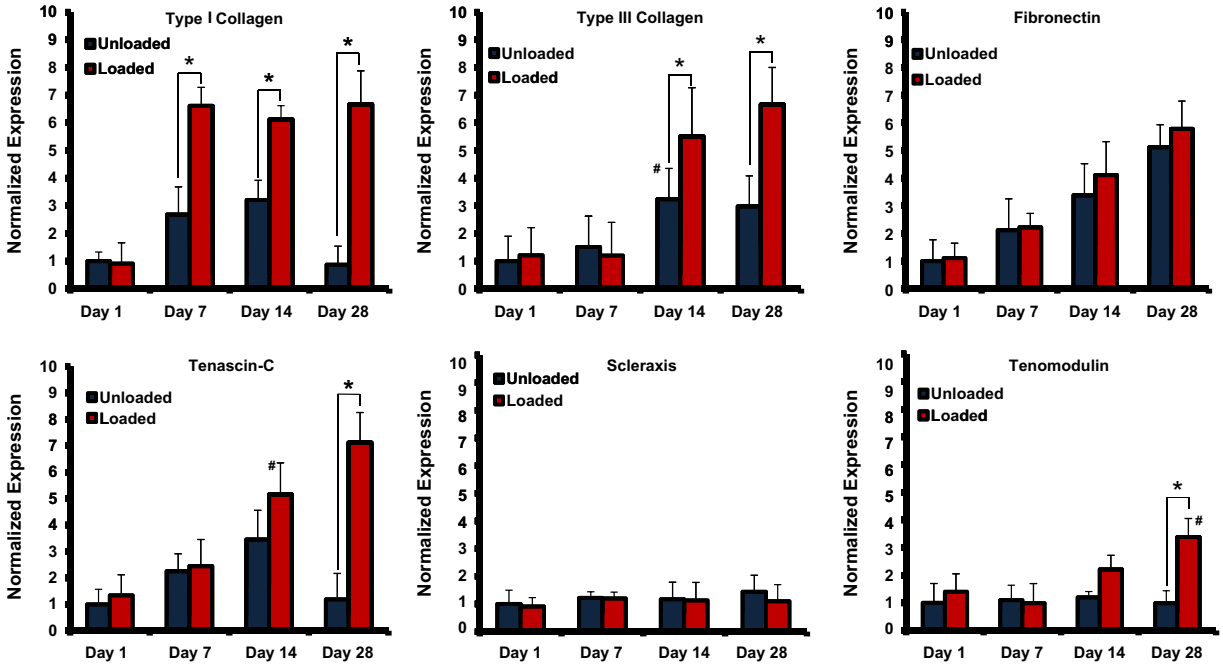

Fig. 4. Effect of chemical and mechanical stimulation on hMSC differentiation. Mechanical stimulation in conjunction with chemical priming resulted in a significant upregulation of types I and III collagen, tenascin-C and tenomodulin expression.

4. Discussion

The long-term goal of this study is to engineer a biomimetic, functional scaffold system for anterior cruciate ligament reconstruction. This study focuses on the differentiation of mesenchymal stem cells into fibroblast-like cells on nanofiber scaffolds using a combination of chemical and mechanical stimuli. In this study, the effects of treating MSCs seeded on nanofibers with bFGF alone and also sequentially with bFGF followed by dynamic tensile strain were systematically investigated. It is observed that bFGF stimulation alone can enhance MSC proliferation and matrix deposition, while mechanical stimulation can synergistically enhance these effects to drive MSCs towards a fibroblastic phenotype. Based on these findings, it is apparent that combinatorial stimulation techniques represent promising strategies to engineer ligament tissue *in vitro*.

In this study, biochemical stimulation of MSCs with bFGF resulted in an increase in the total number of cells and collagen

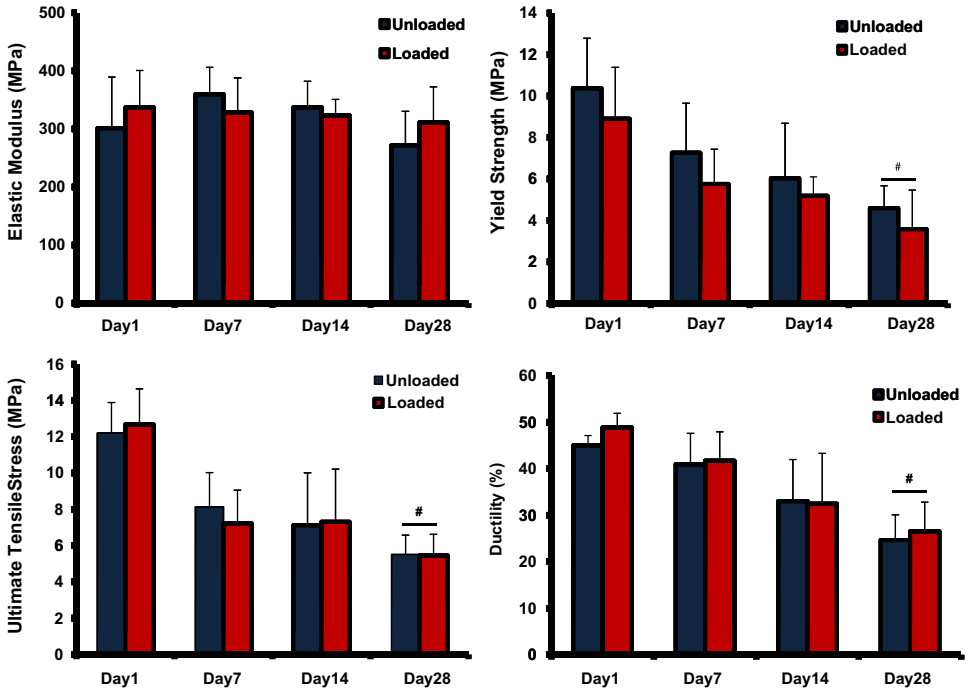

Fig. 5. Effect of chemical and mechanical stimulation on scaffold mechanical properties. No difference in scaffold mechanical properties was measured between groups over time. A significant decrease in yield strength, ultimate tensile strength (UTS) and ductility were measured 4 weeks after priming in both groups as compared to day 1 (#, $p < 0.05$).

on nanofiber meshes after 14 days as well as an increase in the expression of types I and III collagen, fibronectin and tenascin-C. It has previously been established that bFGF is a mitogen which can enhance the proliferation of MSCs. Pitaru et al. (1993) reported that bFGF in conjunction with osteogenic medium containing dexamethasone stimulated proliferation. Hankemeier et al. (2005) also reported that bFGF stimulation results in dose-dependent effects on MSCs and low doses (3 ng/ml) stimulated cell proliferation while upregulating the expression types I and III collagen, fibronectin and alpha-smooth muscle actin (α-SMA). In contrast, high doses (30 ng/ml) decreased proliferation and ECM protein expression. The dose of bFGF used in this study (10 ng/ml) is closer in magnitude to the low-dose condition. In addition, an increase in total collagen was observed on nanofibers with chemical stimulation as compared to unstimulated controls after 14 days. This difference is likely due to the increased number of cells, as normalizing collagen content per-cell indicated no difference in per-cell collagen production. Previous groups have reported similar findings when utilizing bFGF to stimulate MSCs on tissue engineered scaffold systems. For example, Sahoo et al. (2010) extended upon this and evaluated the incorporation of bFGF into a silk/PLGA hybrid fiber scaffold for ligament tissue engineering. Local release from the biohybrid scaffold resulted in an increase in cell number and total collagen and also increased scaffold mechanical properties after three weeks of culture *in vitro*.

Based upon the ability of bFGF to enhance cell response and elicit the upregulation of fibroblast-related matrix proteins, chemical priming of MSCs with bFGF prior to mechanical stimulation was investigated. Notably, in this study, it was shown that applying mechanical stimulation following chemical priming resulted in a greater number of cells and total collagen present on nanofiber meshes after 28 days, as compared to applying a bFGF priming regimen alone. These findings indicate that mechanical stimulation can synergistically enhance cell proliferation and biosynthetic response on nanofibers. In addition, mechanical stimulation maintained the expression of types I and III collagen over the four week period following priming. This observation suggests that chemical stimulation alone may not lead to cells fully differentiating into a fibroblastic phenotype, whereas mechanical stimulation could maintain differentiation or potentially result in cells committing to fibrogenesis. Interestingly, combined mechanical and chemical stimulation resulted in the upregulation of tenomodulin 28 days after chemical priming. Tenomodulin is an anti-angiogenic transmembrane protein that has been shown to be predominantly expressed in tendons and ligaments. While previous work evaluating mechanical stimulation without chemical priming of MSCs on nanofibers did not show any effect on tenomodulin expression, this finding indicates that bFGF priming may result in MSCs further differentiating towards a ligament or tendon fibroblast phenotype.

The effect of combined bFGF and mechanical stimulation has been previously investigated on other tissue engineered constructs with similar results. Petrigliano et al. (2007) reported on the development of a bFGF-coated, porous polycaprolactone-based scaffold. It was shown that bone marrow stromal cells subjected to both chemical and mechanical stimulation resulted in the greatest upregulation of types I and III collagen and tenascin-C. Moreau et al. (2008) demonstrated that bFGF priming followed by mechanical stimulation enhanced matrix development and better supported overall tissue development on silk-based scaffolds, as compared to EGF priming.

It has also been reported that mechanical stimulation may result in increased cell responsiveness to growth factors, potentially due to mechanical forces initiating enhanced matrix production and remodeling. In a study performed by Shin et al. (2004), bFGF signaling was investigated as a mechanotransduction pathway for human umbilical vein endothelial cells (HUVEC) subjected to cyclic pressure. It was shown that the enhanced proliferation of cells was associated with rapid tyrosine phosphorylation of the bFGF receptor, fibroblast growth receptor 2 (FGFR-2), but not with increased synthesis of bFGF. Vincent et al. (2002, 2004) also demonstrated that cyclic loading of porcine cartilage resulted in

the rapid activation of the bFGF-dependent extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase pathway. These findings collectively implicate bFGF in mechanical signaling and suggest that priming of cells with this factor may increase cell response to mechanical stimulation.

Sequential chemical and mechanical stimulation resulted in no significant difference in nanofiber mesh mechanical properties as compared to chemical priming alone. While it is emphasized that post *in vitro* culture, the resultant nanofiber mechanical properties remain within range of those of ligaments and tendons, it is likely that longer-term culture, a higher bFGF dose or the use of multiple factors to enhance matrix deposition may be necessary in order for the cells to produce sufficient ECM to further augment nanofiber mesh mechanical properties. For example, an increase in mechanical properties of cell-seeded nanofiber scaffolds was only observed after 10 weeks of *in vitro* culture (Baker and Mauck, 2007). In addition to bFGF, other growth factors, such as transforming growth factor-β (Moreau et al., 2005; Jenner et al., 2007) or growth/differentiation factor-5 (Jenner et al., 2007; James et al., 2011), may be able to further enhance cell response, either simultaneously or in sequence, as these proteins have been shown to promote MSC biosynthesis as well as ligament and tendon tissue formation.

Moreover, the temporal optimizations of chemical and mechanical stimulation on MSC response, as well as those of dose, priming duration and variations in mechanical stimulation parameters can be used to enhance and maintain MSC differentiation into fibroblast-like cells on nanofiber scaffolds. The findings of this study represent a foundation upon which these future studies can be performed and demonstrate that combined stem cell stimulation strategies (chemical and mechanical) on nanofiber-based scaffolds is a promising approach for stem cell-mediated ligament tissue engineering.

5. Conclusions

The study investigated the effects of chemical and mechanical stimulation on stem cell differentiation, and the results demonstrate that bFGF priming coupled with physiologically relevant tensile loading enhance MSC differentiation into ligament fibroblast-like cells. While bFGF priming increases cell proliferation, mechanical stimulation of the MSCs on the aligned nanofiber scaffold promotes fibroblastic induction of these cells.

Conflict of interest

The authors have no conflicts of interest to report.

Acknowledgments

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