Clin Orthop Relat Res (2008) 466:1930–1937
DOI 10.1007/s11999-008-0300-x

SYMPOSIUM: NEW APPROACHES TO ALLOGRAFT TRANSPLANTATION

The Effects of GDF-5 and Uniaxial Strain on Mesenchymal Stem Cells in 3-D Culture

Eugene Farng MD, Alfonso R. Urdaneta BS, David Barba BS, Sean Esmende BS, David R. McAllister MD

Published online: 6 June 2008

Abstract Recent endeavors in tissue engineering have attempted to identify the optimal parameters to create an artificial ligament. Both mechanical and biochemical stimulation have been used by others to independently modulate growth and differentiation, although few studies have explored their interactions. We applied previously described fabrication techniques to create a highly porous (90%–95% porosity, 212–300 μm), 3-D, bioabsorbable polymer scaffold (polycaprolactone). Scaffolds were coated with bovine collagen, and growth and differentiation factor 5 (GDF-5) was added to half of the scaffolds. Scaffolds were seeded with mesenchymal stem cells and cultured in a custom bioreactor under static or cyclic strain (10% strain, 0.33 Hz) conditions. After 48 hours, both mechanical stimulation and GDF-5 increased mRNA production of collagen I, II, and scleraxis compared to control; tenascin C production was not increased. Combining stimuli did not change gene expression; however, cellular metabolism was 1.7 times higher in scaffolds treated with both stimuli. We successfully grew a line of mesenchymal stem cells in 3-D culture, and our initial data indicate mechanical stimulation and GDF-5 influenced cellular activity and mRNA production; we did not, however, observe additive synergism with the mechanical and biological stimuli.

Each author certifies that he or she has no commercial associations (eg, consultancies, stock ownership equity interest, patent/licensing arrangements, etc) that might pose a conflict of interest in connection with the submitted article. The institution of the authors has received funding from the Orthopaedic Hospital Foundation.

E. Farng (✉), D. R. McAllister
Department of Orthopaedic Surgery, David Geffen School of Medicine, University of California Medical Center, 10833 Le Conte Avenue, Room 16-155 CHS, Los Angeles, CA 90095, USA
e-mail: efarng@mednet.ucla.edu

A. R. Urdaneta, D. Barba, S. Esmende
David Geffen School of Medicine, University of California Medical Center, Los Angeles, CA, USA

Introduction

The anterior cruciate ligament (ACL) is one of the most frequently injured ligaments in the knee, with over 100,000 reconstructions performed annually [40]. Poor results with nonoperative management [26], particularly in those who wish to remain active [4], has led to increased numbers of surgical reconstruction. Today, surgical reconstruction can restore function and prevent instability and early cartilage deterioration in the knee with 90% success reported in one study [49]. There are, however, limitations to autografts (donor site morbidity such as muscle weakness, patellar fracture, and anterior knee pain) [49] and allografts (donor availability, fear of disease transmission) [16, 49]. As a result, artificially engineered ligaments suitable for ACL reconstruction have emerged as one promising alternative.

Current approaches to ligament and tissue engineering are aimed at optimizing the growth of an autologous population of cells grown on a biocompatible scaffold. First, an appropriate cell source with high proliferative potential that can be easily harvested and cultured must be identified. These cells are then grown on a platform that enables cell adhesion, proliferation, and the bulk production of organized collagen matrix. However, the ideal growth conditions for ligamentogenesis must be still identified, and the interplay between different stimuli is largely unknown. The growth conditions are selected based on their ability to enhance proliferation, direct differentiation, and promote

the production of an organized extracellular matrix. Ligaments and tendons have a specific alignment of their collagen fibrils and serve to transmit forces, and therefore, it is anticipated mechanical strain in the external environment can upregulate or direct ligamentogenesis. Indeed, cyclic strain is promising since it upregulates fibroblast markers [2, 41]. Another category of promising stimuli is growth factors, including members of the TGF-B superfamily such as bone morphogenic protein (BMP) and growth and differentiation factor (GDF). GDF-5, in particular, shows great promise in ligament- and tendon-specific differentiation [44, 50].

Using such an ex vivo ACL substitute we addressed three questions regarding this ligament engineering approach: (1) if a biopolymer scaffold would support the adherence and proliferation of a stem cell line; (2) whether mechanical stimulation and/or GDF-5 would influence cellular proliferation; and (3) whether mechanical stimulation and/or GDF-5 would influence cellular differentiation.

Materials and Methods

We fabricated bioabsorbable polymer scaffolds that we seeded with a multipotent bone marrow stromal cell line, cultured under experimental conditions for 48 hours, then removed for analysis. Four experimental groups were created. In group one (control), no mechanical strain or exogenous growth factor was used. In group two, cyclic uniaxial 10% strain at 0.33 Hz was applied to the scaffolds. In group three, GDF-5 (1600 ng/scaffold) was administered by adding it to the collagen coating of the scaffold. In group 4, both mechanical stimulation and exogenous growth factor were used. Two scaffolds from each group were randomly selected for qualitative histologic analysis in order to confirm the presence of cells and to observe the pattern of growth. Two scaffolds from each group were randomly selected for an MTS-based cellular proliferation assay (3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium—see below) to estimate the size of the live cell population. Two scaffolds from each group were randomly selected for RNA extraction and RT-PCR analysis for specific genes of interest (collagen I, collagen III, tenascin C, and scleraxis) to identify potential cellular differentiation and ECM production. The experiment was repeated for a total $n = 3$.

A multipotent mouse bone marrow stromal cell (BMSC) line was obtained from ATCC (Manassas, VA; Designation D1 ORL UVA). Cells were cultured in medium consisting of Dulbecco's modified Eagle's medium (DMEM) with high glucose, L-glutamine, and sodium pyruvate (Invitrogen, San Diego, CA). The medium was supplemented with 10% fetal bovine serum (Omega Scientific, Tarzana, CA) and Gibco 1% antibiotic-antimycotic solution (Invitrogen). Cells were cultured on standard cell-culture-treated plastic, and culture plates were maintained in a humidified incubator at 37°C and 5% CO_2. The medium was changed every 3 to 4 days, and cells were split 5:1 when plates reached 70% to 80% confluence. At passages 4 and 6, cells were cryopreserved in 10% (v/v) dimethyl sulfoxide. All experiments were performed with cells from passages 8 and 9.

We prepared racetrack-shaped scaffolds according to previously reported techniques [41, 42]. Briefly, a 20% (w/w) solution was created by dissolving polycaprolactone (Birmingham Polymers, Birmingham, AL) in chloroform and stirring on an orbital shaker. Methanol was then added to create a 30% (w/w) methanol concentration, again mixed on an orbital shaker. Sucrose with grain sizes 212–300 μm was mixed in to provide a 90% to 95% porosity scaffold. This mixture was packed into machined Teflon® molds to create racetrack-shaped scaffolds. Scaffolds were then placed in a vacuum freeze-drier overnight and stored in a dessicator until use. Before seeding, sucrose particles were leached in deionized water for 24 hours. Scaffolds were then sterilized in 70% ethanol for 30 minutes, followed by five washes in sterile phosphate-buffered saline (PBS).

Cell adhesion and proliferation were enhanced by the noncovalent coating of bovine collagen and GDF-5 to the scaffolds as previously described [41]. Briefly, purified bovine dermal collagen (Cohesion Technologies, Palo Alto, CA) was neutralized to pH 7.4 with 10X PBS and NaOH in an 8:1:1 ratio. This was then diluted with sterile water to a final concentration of 0.25 mg/mL. For half the scaffolds, GDF-5 (R&D Systems, Minneapolis, MN) was then added. Under sterile conditions, 100 μL of the appropriate solution was then serially added to each arm of the scaffold, with 30 minutes of drying time between coats to allow for collagen and growth factor adhesion. A total of 0.125 mg collagen and 0 versus 800 ng GDF-5 was added to each scaffold arm.

After the final coat was dry, 1×10^6 BMSCs in 100 μL medium were applied to each scaffold arm, and scaffolds were incubated for 2 hours at 37°C. The scaffolds were then rotated 180° and the process was repeated for the other side of each scaffold arm. A total of 4×10^6 cells were seeded onto each scaffold.

Under sterile conditions, we then mounted scaffolds in a custom bioreactor. Each chamber of the bioreactor houses up to six scaffolds, and linear strain was applied to each chamber by a programmable linear stepper motor (Arrick Robotics, Tyler, TX). Ten percent strain was applied in a triangular waveform at 0.33 Hz. The bioreactor was maintained in a humidified incubator at 37°C and 5% CO_2.

After incubation in the custom bioreactor for 48 hours, two scaffolds from each chamber were randomly selected for histologic sectioning, and they were fixed with 10% formalin for 24 hours. Individual scaffold arms were then excised en bloc using a sterile No. 15 scalpel, and samples were embedded using a gelatin embedding technique developed previously [8]. Briefly, scaffolds were placed in a 5% (w/w) porcine gelatin (G1890; Sigma-Aldrich, St. Louis, MO) with 5% (w/w) sucrose solution in disposable plastic molds and heated in a rocking convection oven at 45°C overnight. Samples were then immersed in an acetone and dry ice bath for rapid freezing and stored at −80°C until sectioned. Samples were taken from four areas throughout the depth of the scaffold. Each area was spaced 2.5 mm apart, and 10 sections were taken from each area. Samples were sectioned at 10 μm on a cryotome, mounted on Superfrost® Plus slides (VWR Scientific, West Chester, PA), and stained with hematoxylin (Fisherbrand Gill #3; Fisher Scientific, Pittsburgh, PA) and eosin (Sigma-Aldrich) by the primary author. We qualitatively examined slides with standard light microscopy.

To quantify relative cell number and metabolism, a modification of Promega's MTS-based assay was used (Promega, Madison, WI). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, also known as MTS, is a tetrazolium compound that undergoes colorimetric change when reduced. In the presence of reducing agents such as NADH and NADPH, MTS turns deep purple with a peak absorbance at 490 nm. Thus, this assay measures cellular reductive capacity as a surrogate for overall cellular metabolism and cell number. Individual scaffold arms were placed into each well of a 12-well plate. Scaffolds were then incubated in 1000 μL culture medium and 100 μL MTS at 37°C and 5% CO_2 for 10 minutes. Media was then transferred into a 96-well plate, and absorbance at 490 nm was measured using a Tecan F-200 multiwell plate reader (Tecan Inc, San Jose, CA).

Scaffolds were randomly selected for RT-PCR analysis and immediately frozen in a liquid nitrogen vapor-phase storage tank. Frozen scaffolds were then excised and crushed, and RNA was extracted using an RNeasy® Mini Kit (Qiagen, Valencia, CA). Briefly, cells from crushed scaffolds were lysed and homogenized using the Qiashredder® (Qiagen) protocol, and the resulting lysate was serially processed and washed using the RNeasy® protocol. To prevent DNA contamination, we included the optional use of RNase-free DNase (Qiagen). Real-time RT-PCR was performed with a QuantiTect® RT-PCR kit (Qiagen) in a 96-well plate configuration. mRNA probes for Mus musculus genes of interest, including procollagen Type I, procollagen Type III, tenascin C, and scleraxis were obtained from Applied Biosystems (Foster City, CA). Internal control was performed with TaqMan® GAPDH rodent control reagents (Applied Biosystems). Quantitative real-time PCR was performed with an ABI Prism® 7900 Sequence Detection System (Applied Biosystems) at UCLA's Sequencing and Genotyping Core facility.

Initial data analysis was performed with Microsoft Excel (Microsoft, Redmond, WA), and statistical analysis was performed with STATA (Stata Corp, College Station, TX). MTS results are reported as mean ± standard deviation relative to control, and one-way ANOVA was performed to assess for differences between treatment groups. Similarly, gene expression is reported as mean ± standard deviation relative to control, and one-way ANOVA was used to assess for differences. When a difference between groups was identified by ANOVA, a comparison of group means was performed using a Student's t test.

Results

Histologic examination (Fig. 1) of the scaffolds demonstrated successful attachment and proliferation of cells in all groups. Qualitative observation suggested cell colonies tended to form along the surface of the scaffold, growing in large clusters. These clusters were noted in all treatment groups (Fig. 1A–B). Individual cells and small groups of cells were occasionally found throughout the scaffolds, including deep within the center of the scaffolds (Fig. 1C–D). Scaffolds treated with both GDF-5 and cyclic strain seemed to form the largest cell colonies, although quantitative analysis was not performed. Cell growth on the internal surfaces of scaffolds was most increased in the combined stimulus group, with groups of cells spanning between scaffold struts (Fig. 1C–D), although most of the cell growth remained confined to the superficial layers of the scaffold. At this early 48-hour time point, no obvious phenotypic changes were noted suggesting differentiation to a known cell or tissue type, and visible extracellular matrix production was essentially nonexistent.

Incubation of MTS in the presence of multiple cell populations of known size confirmed the linear response of MTS to cell number. Reduction of MTS assay in the presence of cultured scaffolds confirmed the presence of live cells in the all scaffold groups but with differences ($p = 0.02$) between groups. Scaffolds treated by either cyclic strain or GDF-5 independently showed no increase ($p > 0.05$) in cellular activity compared to control (Fig. 2). However, scaffolds treated with both GDF-5 and cyclic strain demonstrated 1.7 times ($p = 0.017$) the reductive capacity as untreated controls.

Production of mRNA was increased by both mechanical stimulation and GDF-5 when compared to unstimulated scaffolds (Fig. 3). Between the four groups we observed

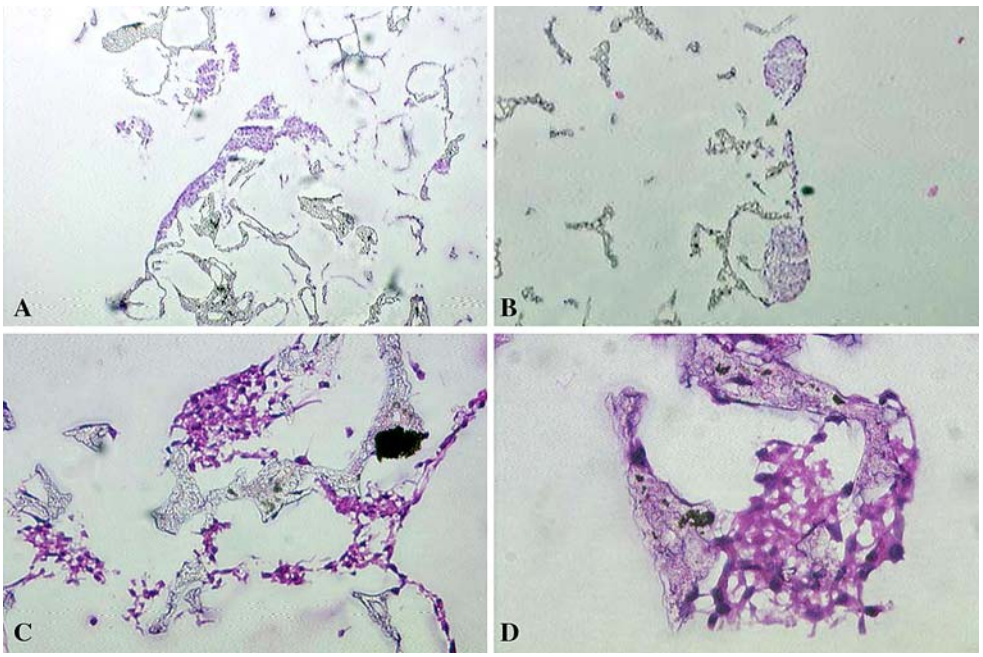

Fig. 1A–D The cultured scaffolds demonstrated successful cell adhesion and growth (Stain, hematoxylin and eosin). (**A**) A low-power (×10) view of a scaffold treated with only mechanical stimulus shows a large cell colony on the surface of the scaffold. (**B**) A medium-power (×100) view of a scaffold treated with only GDF-5 also demonstrates a large cell colony on the surface of the scaffold. (**C**) High-power view (×500) of a scaffold treated with both cyclic strain and GDF-5 shows cell colonies within the substance of the scaffold, spanning scaffold struts. (**D**) Higher-power view (×1000) of a scaffold treated with both cyclic strain and GDF-5 shows a cell colony within the substance of the scaffold.

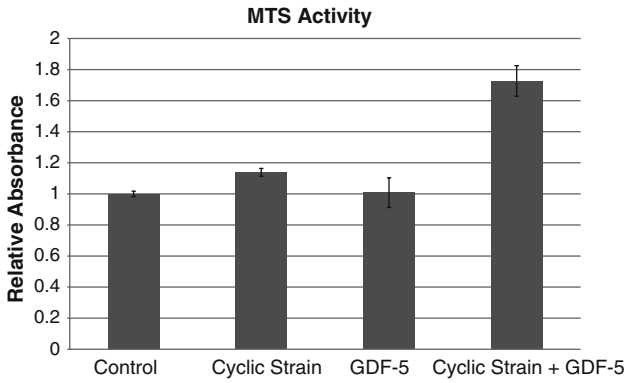

Fig. 2 Cellular proliferation was quantified by MTS reduction. Cultured scaffolds were incubated in the presence of MTS, which undergoes colorimetric change in the presence of cellular reductive agents. Scaffolds treated with both cyclic strain and GDF-5 demonstrated a 70% increase in cellular activity ($p = 0.017$). Values reported represent increases compared to nonstimulated controls. Error bars indicate 95% confidence interval of the mean.

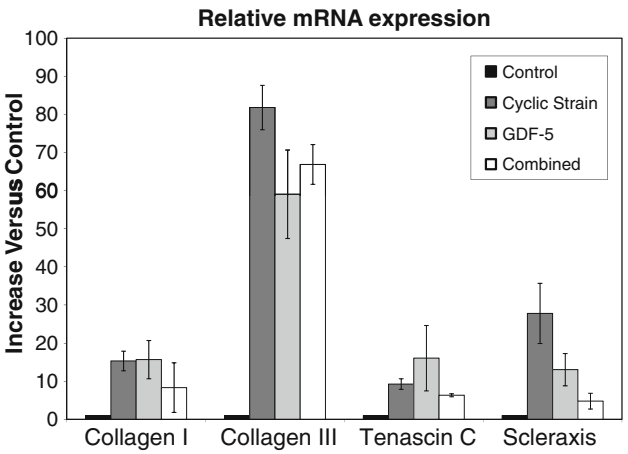

Fig. 3 RT-PCR analysis of mRNA gene expression was performed on scaffolds that were either unstimulated or stimulated with uniaxial 10% cyclic strain at 0.33 Hz, GDF-5, or both. Either mechanical stimulation or GDF-5 increased gene expression of collagen I, collagen III, and scleraxis ($p < 0.05$), without increasing tenascin C ($p > 0.05$). Combined stimuli only increased expression of collagen III ($p < 0.05$). We observed no synergy. Values reported represent increases compared to nonstimulated controls. Error bars indicate standard deviation of the mean.

differences in collagen I ($p = 0.0494$), collagen III ($p = 0.0009$), and scleraxis production ($p = 0.0096$) but not tenascin C production ($p = 0.07$). Mechanical stimulation increased collagen I (15.3 ± 2.3; $p = 0.012$), collagen III (81.7 ± 5.2; $p = 0.002$), and scleraxis (27.8 ± 7; $p = 0.03$) mRNA production compared to control. GDF-5 increased collagen I (15.7 ± 4.4; $p = 0.04$), collagen III (59.1 ± 10.3; $p = 0.01$), and scleraxis (13 ± 3.8; $p = 0.045$) mRNA production. Combining the stimuli produced an increase ($p = 0.004$) only in the expression of collagen III (66.9 ± 4.6).

Discussion

The field of bioengineering may ultimately be able to supply artificially engineered tissues for use in humans; however, substantial challenges must be met before clinical use can be considered. Currently, there is no consensus regarding the ideal cell source, growth matrix, or growth conditions for ligament engineering. This study was undertaken to present the results of a novel technique using a pluripotent cell population, bioabsorbable scaffold, and promising combination of stimuli. Specifically, we wanted to evaluate the ability of cells to adhere to and survive on our 3-D matrix. Furthermore, we wanted to evaluate the influence of mechanical stretch and growth factor stimulation on cellular proliferation, as well as its ability to direct differentiation.

There are a number of limitations to our study that bear mentioning. First, the nature of the cell line selected for this study must be considered. As described below, the bone marrow stromal cell line used in this study is primarily osteogenic, though it has been used in adipogenic applications as well. We are not aware of any studies evaluating the tenocyte or fibroblast potential of this cell line. Indeed, one of the aims of our study is to identify the appropriate conditions for directing ligamentogenesis. Furthermore, the transformed nature of a cell line may limit the general applicability of any conclusions from this study, as a different cell population (such as harvested nonimmortalized cells) may respond differently to the stimuli used in this experiment. Still, the easy availability, relative robustness, and short doubling time of these cells compared to harvested cells makes them ideal for use in these types of investigative studies. Second, the conclusions we are able to draw from the histology are based on qualitative description only. The degree of cell clustering makes quantitative evaluation of cell area, aspect ratio, or cell number impossible. Third, the experimental design does not allow for any insight into the mechanism of action for these stimuli. For example, mechanical stimulation may upregulate growth by improving nutrient diffusion in the surrounding media rather than by direct stimulation of the cells. From the perspective of study design, however, the presence of a treatment effect must be established before the mechanism of action can be investigated; further research into the mechanism of action may be warranted in the future. Fourth, the experiment was limited to 48 hours of culture. Within this time frame, some cellular proliferation and early markers of lineage-specific differentiation may be present. Unfortunately, these early markers of tenocyte differentiation are not well-defined. Conclusive evidence of tenocyte formation and ligamentogenesis, such as the production of an organized collagen matrix, would not be expected at this early time point; longer culture periods are necessary. Naturally, we would like to observe these cells at multiple time points over extended culture; however, an exhaustive multivariable experiment is not technically feasible. Therefore, we chose to examine an early time point to ensure our selected stimuli did indeed have an effect on our cell population before moving to extended culture. Finally, for the purpose of these initial investigations, the number of genes assayed using RT-PCR technique used was limited. A complete panel could include other markers of differentiation (eg, bone specific markers); therefore, the ultimate differentiation pathway of these cells cannot be conclusively determined based on this gene profile. Future areas of investigation should be aimed at addressing these issues, including extended culture periods, evaluations of multiple time and doses, and additional genes of interest.

Because engineering an ACL substitute is one of the goals of ligament- and tendon-engineering projects, the ACL seems like a natural source for cells. However, in comparison to patellar tendon-derived fibroblasts [25], skin-derived fibroblasts [5], and MCL-derived fibroblasts [34], ACL-derived fibroblasts have limited proliferative capacity [5, 25, 34]. In fact, BMSCs may be superior to these candidates as targets for tissue-engineering applications, as BMSCs had higher collagen production and DNA content after seeding on polylactide/glycolide (PLGA) suture material than ACL or skin fibroblasts [47]. Similarly, others [28] have reported bone marrow mesenchymal stem cells had higher proliferation rate, collagen excretion, and durability in the knee when compared to ACL or MCL fibroblasts.

Therefore, most approaches have shifted to the use of mesenchymal stem cells, which can be harvested from the bone marrow. Mesenchymal stem cells have been used in multiple tissue-engineering applications, and they differentiate into multiple lineages, including osteoblasts, chondrocytes, adipocytes, and muscle/tendon forming cells [9–11, 23]. The multipotent, immortalized BMSC line used here is primarily osteogenic [24] and has been used in models of spinal fusion [19] and in the femur [22]. These cells also differentiate into adipocytes under the influence of steroids [20, 21] or alcohol [22]. The immortalized cell line used in this study was selected for its relative ease of use, rapid doubling time, and multilineage potential. We are not aware of any studies describing tenocyte differentiation of this cell line; indeed, one goal of ligament engineering as a field is to identify the necessary conditions to induce ligamentogenesis and tenocytic differentiation.

A multitude of different scaffold technologies have been investigated for use in tissue engineering. Some approaches involve materials currently used as suture material, including processed silk fibers [1, 12] and bioabsorbable polymers [7, 34]. Biologic substrates, including collagen

gels [2, 6, 27], hyaluronan [17], and alginate/chitosan polymers [36] have also been used.

Our current scaffold structure is based on prior work in our laboratory [41, 42], which provides a platform for cell growth using a biodegradable material. Using these fabrication techniques, a wide variety of scaffold structures can be created using appropriately shaped molds. Unlike PLGA, polycaprolactone (PCL) has a low glass transition temperature of −60°C, and it is an amorphous solid at biologic temperatures. Therefore, it is capable of withstanding long-term cyclic strain. Furthermore, it is already an FDA-approved bioabsorbable polymer, sold under the brand name Monocryl® (Ethicon, Somerville, NJ). Our particle leaching technology provides 95% porous scaffold with known pore sizes, providing a high degree of interconnectivity to allow high uniform cell seeding and cell-cell interactions. The high porosity also helps with mass transport of nutrient and waste. Pilot studies have demonstrated our technique has 80% to 90% seeding efficiency using bone marrow stromal cells harvested from rats [41, 42].

Multiple growth factors have been used in tissue-engineering applications to stimulate proliferation and differentiation of cells, including FGF, TGF, platelet-derived growth factor (PDGF), EGF, and GDF [29, 30, 41]. However, it may be that a complicated sequence of growth factor administration is necessary to recapitulate the healing or embryogenesis of tendons and ligaments. One approach to addressing this multivariable problem has been fractional factorial design [39], which helps to identify the ideal media formulations and growth factor combinations for cell growth. Based on these results, a smaller group of growth factor combinations can be targeted for sequential administration to BMSCs; results from silk fiber matrices indicate mitogen/TGF-β-treated groups generally demonstrated both increased proliferation and collagen deposition in extended culture [38, 39].

Of the many proteins known to have mitogenic activity on mesenchymal tissue, the GDF-5, -6, and -7 (also known as BMP-14, -13, and -12, respectively) subfamily of proteins may be the most promising for applications involving tissue and ligament healing, regeneration, and engineering. As members of the TGF- β superfamily, these proteins induce tenocyte differentiation of bone marrow mesenchymal cells [48]. Receptor binding triggers members of the Smad family of nuclear transcription factors, including Smad-1, -5, and -8. Smad-8, in particular, promotes tenocyte differentiation of mesenchymal stem cells [32]. This family of proteins plays a clear role in the embryogenesis and differentiation of collagenous tissue, as GDF-deficient mice have abnormal tissue structure in the Achilles tendon [37] and in the tail [14]. More notably, decreased collagen content and tensile strength [37], as well as delayed healing, have been reported in the Achilles tendon of GDF-deficient mice [13]. Conversely, exogenous GDF protein delivered by carrier [3, 46], suture coating [43], or adenoviral transfection [44] improves tendon healing and tensile strength of transected tendons.

Even more promising, in vivo experiments of ectopic injection of GDF-5, -6, and -7 induces neotendon and ligament formation, suggesting GDFs act as signaling molecules during embryonic tendon and ligament formation [50]. Histologic examination of the induced tissue demonstrated organized collagen with regular periodicity, resembling neonatal tendon and ligament. Similar results have been reported with BMP-13 delivered via adenoviral transfection into athymic nude rats [31].

GDF-5 has been used recently for tissue engineering applications [33]. BMSCs treated with recombinant human TGF-β1 and GDF-5 were cultured on woven 3-D PLGA scaffolds over a period of 12 days. Both growth factors promoted cellular proliferation; however, only TGF-β1 increased collagen production.

Mechanical strain has been evaluated as a potential stimulus for cell proliferation and differentiation [2]. BMSCs seeded on collagen gels subjected to 10% longitudinal strain and 25% rotational strain at 0.0167 Hz for 21 days demonstrated an upregulation of ligament fibroblast markers, including collagen I, collagen III, and tenascin C. In these studies, there was no evidence of osteogenic or chondrogenic differentiation, with no upregulation of bone sialoprotein, collagen II, osteocalcin, or osteopontin. Similarly, dermal fibroblasts seeded on collagen gel constructs subjected to uniaxial cyclic tensile strain (10% strain, 1 Hz) had improved cell viability with cyclic strain; constructs preloaded at 2 mN also had increased collagen production [6].

As true ligament and tendon embryogenesis occurs in a complex environment with an array of external signals and stimuli, it is unlikely ligament engineering will depend on a single external influence. Rather, it will be necessary to identify the appropriate sequence of culture conditions, as well as the timing and nature of growth factors and nutrients. While some have examined growth factor combinations in 2-D systems [6], we are not aware of studies examining the interaction between mechanical stimulation and growth factor administration in a 3-D system. Previous work by our group [41, 42] has demonstrated both stimuli can independently enhance cellular differentiation and these independent effects can be maintained when both stimuli are used in concert.

The field of tissue and ligament engineering is still relatively young, and current techniques have yet to produce a suitable candidate for clinical ACL reconstruction. One problem has been the lack of clear, specific markers of tenocyte differentiation, although scleraxis [18] and tenomodulin appear promising candidates [45]. Future work

must be performed to examine the effects of these stimuli in extended culture conditions, as the production of an organized collagen matrix will likely require longer culture periods.

We have developed a novel system allowing for the delivery of mechanical and biochemical stimuli to a 3-D construct seeded with candidate cells. This system has been successfully used to demonstrate the adhesion and proliferation of a multipotent mesenchymal cell line onto a bioabsorbable 3-D construct, with cellular viability throughout the scaffold. Furthermore, both mechanical stimulation and biochemical stimuli can be applied. Preliminary results suggest that, while mechanical stimulation and GDF-5 alone does not increase cellular proliferation, the combination of these factors can increase cell number after 48 hours of culture. Either mechanical stimuli or GDF-5 can increase expression of collagen I, collagen III, and scleraxis, although the combination of these stimuli does not appear to have any increased effect on the pattern of gene expression.

Acknowledgments We thank Dr. Benjamin M. Wu, Dr. Michael Eagan, and Dr. Frank Petrigliano for their assistance and contributions to this work.

References

1. Altman GH, Horan RL, Lu HH, Moreau J, Martin I, Richmond JC, Kaplan DL. Silk matrix for tissue engineered anterior cruciate ligaments. *Biomaterials*. 2002;23:4131–4141.
2. Altman GH, Horan RL, Martin I, Farhadi J, Stark PR, Volloch V, Richmond JC, Vunjak-Novakovic G, Kaplan DL. Cell differentiation by mechanical stress. *Faseb J*. 2002;16:270–272.
3. Aspenberg P, Forslund C. Enhanced tendon healing with GDF 5 and 6. *Acta Orthop Scand*. 1999;70:51–54.
4. Barrack RL, Bruckner JD, Kneisl J, Inman WS, Alexander AH. The outcome of nonoperatively treated complete tears of the anterior cruciate ligament in active young adults. *Clin Orthop Relat Res.* 1990;259:192–199.
5. Bellincampi LD, Closkey RF, Prasad R, Zawadsky JP, Dunn MG. Viability of fibroblast-seeded ligament analogs after autogenous implantation. *J Orthop Res*. 1998;16:414–420.
6. Berry CC, Shelton JC, Bader DL, Lee DA. Influence of external uniaxial cyclic strain on oriented fibroblast-seeded collagen gels. *Tissue Eng*. 2003;9:613–624.
7. Bourke SL, Kohn J, Dunn MG. Preliminary development of a novel resorbable synthetic polymer fiber scaffold for anterior cruciate ligament reconstruction. *Tissue Eng*. 2004;10:43–52.
8. Brown DA, Chou YF, Beygui RE, Dunn JC, Wu BM. Gelatin-embedded cell-polymer constructs for histological cryosectioning. *J Biomed Mater Res B Appl Biomater*. 2005;72:79–85.
9. Bruder SP, Fink DJ, Caplan AI. Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. *J Cell Biochem*. 1994;56:283–294.
10. Caplan AI. Mesenchymal stem cells. *J Orthop Res*. 1991;9: 641–650.
11. Caplan AI, Bruder SP. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. *Trends Mol Med*. 2001;7:259–264.
12. Chen J, Altman GH, Karageorgiou V, Horan R, Collette A, Volloch V, Colabro T, Kaplan DL. Human bone marrow stromal cell and ligament fibroblast responses on RGD-modified silk fibers. *J Biomed Mater Res A*. 2003;67:559–570.
13. Chhabra A, Tsou D, Clark RT, Gaschen V, Hunziker EB, Mikic B. GDF-5 deficiency in mice delays Achilles tendon healing. *J Orthop Res*. 2003;21:826–835.
14. Clark RT, Johnson TL, Schalet BJ, Davis L, Gaschen V, Hunziker EB, Oldberg A, Mikic B. GDF-5 deficiency in mice leads to disruption of tail tendon form and function. *Connect Tissue Res*. 2001;42:175–186.
15. Cooper JA, Lu HH, Ko FK, Freeman JW, Laurencin CT. Fiber-based tissue-engineered scaffold for ligament replacement: design considerations and in vitro evaluation. *Biomaterials*. 2005;26:1523–1532.
16. Crawford C, Kainer M, Jernigan D, Banerjee S, Friedman C, Ahmed F, Archibald LK. Investigation of postoperative allograft-associated infections in patients who underwent musculoskeletal allograft implantation. *Clin Infect Dis*. 2005;41:195–200.
17. Cristino S, Grassi F, Toneguzzi S, Piacentini A, Grigolo B, Santi S, Riccio M, Tognana E, Facchini A, Lisignoli G. Analysis of mesenchymal stem cells grown on a three-dimensional HYAFF 11-based prototype ligament scaffold. *J Biomed Mater Res A*. 2005;73:275–283.
18. Cserjesi P, Brown D, Ligon KL, Lyons GE, Copeland NG, Gilbert DJ, Jenkins NA, Olson EN. Scleraxis: a basic helix-loop-helix protein that prefigures skeletal formation during mouse embryogenesis. *Development*. 1995;121:1099–1110.
19. Cui Q, Ming Xiao Z, Balian G, Wang GJ. Comparison of lumbar spine fusion using mixed and cloned marrow cells. *Spine*. 2001;26:2305–2310.
20. Cui Q, Wang GJ, Balian G. Pluripotential marrow cells produce adipocytes when transplanted into steroid-treated mice. *Connect Tissue Res*. 2000;41:45–56.
21. Cui Q, Wang GJ, Balian G. Steroid-induced adipogenesis in a pluripotential cell line from bone marrow. *J Bone Joint Surg Am*. 1997;79:1054–1063.
22. Cui Q, Wang Y, Saleh KJ, Wang GJ, Balian G. Alcohol-induced adipogenesis in a cloned bone-marrow stem cell. *J Bone Joint Surg Am*. 2006;88(Suppl 3):148–154.
23. Dennis JE, Merriam A, Awadallah A, Yoo JU, Johnstone B, Caplan AI. A quadripotential mesenchymal progenitor cell isolated from the marrow of an adult mouse. *J Bone Miner Res*. 1999;14:700–709.
24. Diduch DR, Coe MR, Joyner C, Owen ME, Balian G. Two cell lines from bone marrow that differ in terms of collagen synthesis, osteogenic characteristics, and matrix mineralization. *J Bone Joint Surg Am*. 1993;75:92–105.
25. Dunn MG, Liesch JB, Tiku ML, Zawadsky JP. Development of fibroblast-seeded ligament analogs for ACL reconstruction. *J Biomed Mater Res*. 1995;29:1363–1371.
26. Fetto JF, Marshall JL. The natural history and diagnosis of anterior cruciate ligament insufficiency. *Clin Orthop Relat Res*. 1980;147:29–38.
27. Garvin J, Qi J, Maloney M, Banes AJ. Novel system for engineering bioartificial tendons and application of mechanical load. *Tissue Eng*. 2003;9:967–979.
28. Ge Z, Goh JC, Lee EH. Selection of cell source for ligament tissue engineering. *Cell Transplant*. 2005;14:573–583.
29. Goh JC, Ouyang HW, Teoh SH, Chan CK, Lee EH. Tissue-engineering approach to the repair and regeneration of tendons and ligaments. *Tissue Eng*. 2003;9:S31–44.
30. Goh JC, Ouyang HW, Toh SL, Lee EH. Tissue engineering techniques in tendon, ligament replacement. *Med J Malaysia*. 2004;59(Suppl B):47–48.

31. Helm GA, Li JZ, Alden TD, Hudson SB, Beres EJ, Cunningham M, Mikkelsen MM, Pittman DD, Kerns KM, Kallmes DF. A light and electron microscopic study of ectopic tendon and ligament formation induced by bone morphogenetic protein-13 adenoviral gene therapy. *J Neurosurg*. 2001;95:298–307.
32. Hoffmann A, Pelled G, Turgeman G, Eberle P, Zilberman Y, Shinar H, Keinan-Adamsky K, Winkel A, Shahab S, Navon G, Gross G, Gazit D. Neotendon formation induced by manipulation of the Smad8 signalling pathway in mesenchymal stem cells. *J Clin Invest*. 2006;116:940–952.
33. Jenner JM, van Eijk F, Saris DB, Willems WJ, Dhert WJ, Creemers LB. Effect of transforming growth factor-beta and growth differentiation factor-5 on proliferation and matrix production by human bone marrow stromal cells cultured on braided poly lactic-co-glycolic acid scaffolds for ligament tissue engineering. *Tissue Eng*. 2007;13:1573–1582.
34. Lin VS, Lee MC, O'Neal S, McKean J, Sung KL. Ligament tissue engineering using synthetic biodegradable fiber scaffolds. *Tissue Eng*. 1999;5:443–452.
35. Lou J, Tu Y, Burns M, Silva MJ, Manske P. BMP-12 gene transfer augmentation of lacerated tendon repair. *J Orthop Res*. 2001;19:1199–1202.
36. Majima T, Funakosi T, Iwasaki N, Yamane ST, Harada K, Nonaka S, Minami A, Nishimura S. Alginate and chitosan polyion complex hybrid fibers for scaffolds in ligament and tendon tissue engineering. *J Orthop Sci*. 2005;10:302–307.
37. Mikic B, Bierwert L, Tsou D. Achilles tendon characterization in GDF-7 deficient mice. *J Orthop Res*. 2006;24:831–841.
38. Moreau J, Chen J, Kaplan D, Altman G. Sequential growth factor stimulation of bone marrow stromal cells in extended culture. *Tissue Eng*. 2006;12:2905–2912.
39. Moreau JE, Chen J, Bramono DS, Volloch V, Chernoff H, Vunjak-Novakovic G, Richmond JC, Kaplan DL, Altman GH. Growth factor induced fibroblast differentiation from human bone marrow stromal cells in vitro. *J Orthop Res*. 2005;23:164–174.
40. Owings MF, Kozak LJ. Ambulatory and inpatient procedures in the United States, 1996. *Vital Health Stat*. 1998;13:1–119.
41. Petrigliano FA, English CS, Barba D, Esmende S, Wu BM, McAllister DR. The effects of local bFGF release and uniaxial strain on cellular adaptation and gene expression in a 3D environment: implications for ligament tissue engineering. *Tissue Eng*. 2007;13:2721–2731.
42. Puk CK, Miller DJ, Gamradt S, Wu BM, McAllister DR. The effects of short-term stimulation on fibroblast spreading in an in vitro 3D system. *J Biomed Mater Res A*. 2006;76:665–673.
43. Rickert M, Jung M, Adiyaman M, Richter W, Simank HG. A growth and differentiation factor-5 (GDF-5)-coated suture stimulates tendon healing in an Achilles tendon model in rats. *Growth Factors*. 2001;19:115–126.
44. Rickert M, Wang H, Wieloch P, Lorenz H, Steck E, Sabo D, Richter W. Adenovirus-mediated gene transfer of growth and differentiation factor-5 into tenocytes and the healing rat Achilles tendon. *Connect Tissue Res*. 2005;46:175–183.
45. Schweitzer R, Chyung JH, Murtaugh LC, Brent AE, Rosen V, Olson EN, Lassar A, Tabin CJ. Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development.* 2001;128:3855–3866.
46. Tashiro T, Hiraoka H, Ikeda Y, Ohnuki T, Suzuki R, Ochi T, Nakamura K, Fukui N. Effect of GDF-5 on ligament healing. *J Orthop Res*. 2006;24:71–79.
47. Van Eijk F, Saris DB, Riesle J, Willems WJ, Van Blitterswijk CA, Verbout AJ, Dhert WJ. Tissue engineering of ligaments: a comparison of bone marrow stromal cells, anterior cruciate ligament, and skin fibroblasts as cell source. *Tissue Eng*. 2004;10:893–903.
48. Wang QW, Chen ZL, Piao YJ. Mesenchymal stem cells differentiate into tenocytes by bone morphogenetic protein (BMP) 12 gene transfer. *J Biosci Bioeng*. 2005;100:418–422.
49. West RV, Harner CD. Graft selection in anterior cruciate ligament reconstruction. *J Am Acad Orthop Surg*. 2005;13:197–207.
50. Wolfman NM, Hattersley G, Cox K, Celeste AJ, Nelson R, Yamaji N, Dube JL, DiBlasio-Smith E, Nove J, Song JJ, Wozney JM, Rosen V. Ectopic induction of tendon and ligament in rats by growth and differentiation factors 5, 6, and 7, members of the TGF-beta gene family. *J Clin Invest*. 1997;100:321–330.

Tissue Engineering for Anterior Cruciate Ligament Reconstruction: A Review of Current Strategies

Article *in* Arthroscopy The Journal of Arthroscopic and Related Surgery · May 2006

DOI: 10.1016/j.arthro.2006.01.017 · Source: PubMed

3 authors, including:

Frank A Petrigliano
University of California, Los Angeles
97 PUBLICATIONS **979** CITATIONS

SEE PROFILE

TISSUE ENGINEERING
Volume 13, Number 11, 2007

DOI: 10.1089/ten.2006.0434

The Effects of Local bFGF Release and Uniaxial Strain on Cellular Adaptation and Gene Expression in a 3D Environment: Implications for Ligament Tissue Engineering

FRANK A. PETRIGLIANO, M.D.,[1] CHRISTOPHER S. ENGLISH, B.S.,[2] DAVID BARBA, B.S.,[2] SEAN ESMENDE, B.S.,[2] BENJAMIN M. WU, D.D.S., Ph.D.,[3] and DAVID R. McALLISTER, M.D.[1]

ABSTRACT

The objectives of this investigation were (1) to characterize the growth factor release profile of a basic fibroblast growth factor (bFGF)-coated three-dimensional (3D) polymer scaffold under static and cyclically strained conditions, and (2) to delineate the individual and collective contributions of locally released bFGF and mechanical strain on cellular morphology and gene expression in this 3D system. Scaffolds were treated with I^{125}-bFGF and subjected to mechanical strain or maintained in a static environment and the media sampled for factor release over a period of 6 days. Over the first 10 hours, a burst release of 25% of the incorporated growth factor into the surrounding media was noted. At 24 hours, approximately 40% of the bFGF was released into the media, after which steady state was achieved and minimal subsequent release was noted. Mechanical stimulation had no effect on growth factor release from the scaffold in this system. To test the concerted effects of bFGF and mechanical stimulation on bone marrow stromal cells (BMSCs), scaffolds were loaded with 0, 100, or 500 ng of bFGF, seeded with cells, and subjected to mechanical strain or maintained in a static environment. Scaffolds were harvested at 1, 7, and 21 days for RT-PCR and histomorphometry. All scaffolds subjected to growth factor and/or mechanical stimulation demonstrated cellular adherence and spreading at 21 days. Conversely, in the absence of both bFGF and mechanical stimulation, cells demonstrated minimal cytoplasmic spread. Moreover, at 21 days, cells subjected to both mechanical stimulation and bFGF (500 ng) demonstrated the highest upregulation of stress-resistive (collagen I, III) and stress-responsive proteins (tenascin-C). The effect of growth factor may be dose sensitive, however, as unstrained scaffolds treated with 100 ng of bFGF demonstrated upregulation of gene expression comparable to strained scaffolds treated with lower doses of bFGF (0 or 100 ng). In conclusion, results from this study suggest that the stimulatory effects of bFGF are dose sensitive and appear to be influenced by the addition of mechanical strain. The concurrent application of biochemical and mechanical stimuli may be important in promoting the adaptation of BMSCs and driving the transcription of genes essential for synthesis of a functional ligament replacement tissue.

[1]Department of Orthopaedic Surgery, David Geffen School of Medicine, University of California Medical Center, Los Angeles, California.
[2]David Geffen School of Medicine, University of California Medical Center, Los Angeles, California.
[3]Department of Biomedical Engineering, Henry Samueli School of Engineering and Applied Science, University of California Los Angeles, Los Angeles, California.

INTRODUCTION

Rupture of the anterior cruciate ligament (ACL) is one of the most common ligament injuries of the knee. Current surgical treatments for ACL ruptures require the use of grafts for reconstruction as the injured ACL has little capacity for healing. Present graft options include autografts and allografts. Although good clinical results have been obtained with these replacement options, there are also many limitations and disadvantages. Thus, there is a need for a tissue engineering approach to this problem.

The development of a competent tissue-engineered ligament requires a system that incorporates (1) an appropriate reparative cell source with a robust capacity for proliferation and differentiation, (2) a biocompatible scaffold that facilitates cellular adaptation, and (3) regulatory stimuli that drive cellular differentiation down a fibroblast pathway and result in the elaboration of organized collagen. Defining the appropriate constituents of this system and the temporal regulatory cues that initiate and direct ligament synthesis represents the core objective of current research efforts. Under the appropriate stimuli, bone marrow stromal cells (BMSCs) have demonstrated the capacity for *in vitro* proliferation and expression of stress-resistive and stress-responsive proteins essential in soft tissue and ligament development and repair.[1–7] BMSCs are superior in this regard, when compared to mature ligament or skin fibroblasts, which appear to have a diminished capacity for division and collagen synthesis *in vitro*.[7] The work of other investigators has proven instrumental in defining the individual contributions of mechanical and biochemical stimulation in modulating BMSC gene expression. Specifically, Altman *et al.*[4] applied translational and rotational stress to undifferentiated BMSCs seeded in a collagen sponge and demonstrated upregulation of ligament fibroblast-associated gene expression including collagen types I and III and tenascin-C, without the concurrent upregulation of bone- or cartilage-specific genes. Hankemeier *et al.* demonstrated the effects of growth factor on BMSC proliferation and gene expression in a two-dimensional (2D) culture.[8] In that study, low-dose basic fibroblast growth factor (bFGF) triggered a biphasic response: on day 7 cell proliferation reached its maximum, and on days 14 or 28 collagen I, collagen III, fibronectin, and smooth muscle actin mRNA expressions were significantly enhanced in the presence of low-dose bFGF as compared to high-dose bFGF and control groups.[8] More recently, Moreau *et al.*[3] described the influence of multiple growth factors and media constituents on the gene expression of BMSCs on a 2D construct and identified bFGF, epidermal growth factor, and transforming growth factor-beta as effective mediators of fibroblast differentiation. Further, the sequential addition of different combinations of these growth factors to the media of BMSCs seeded on 3D silk matrices resulted in a greater mitogenic and synthetic response as compared to the application of a single growth factor or mechanical stimulation alone.[1] Collectively, these data suggest that either mechanical stimulation alone or the application of growth factor alone may upregulate the expression of stress-resistive (collagen I and III) and stress-responsive genes (tenascin-C) in BMSCs. These findings represent the most elemental steps toward the realization of a tissue-engineered ligament replacement.

While the aforementioned studies have evaluated the sole influence of either biochemical or mechanical stimuli on the behavior of BMSCs *in vitro*, we are unaware of any published investigations describing their combined effects on promoting cellular adaptation or gene expression for the purpose of ligament engineering. The aim of the present investigation is twofold: (1) to describe the release profile of noncovalently bound bFGF from a collagen-coated 3D porous scaffold in the absence and presence of mechanical stimulation, and (2) to determine the collective contributions of locally released bFGF and uniaxial cyclical strain on BMSC gene expression and morphological characteristics in a 3D system. We hypothesized that locally released bFGF would act in concert with mechanical strain to augment upregulation of fibroblast-related collagen I, collagen III, and tenascin-C gene expression in BMSCs and enhance cellular adaptation on a 3D scaffold as compared to untreated scaffolds or scaffolds treated with growth factor or mechanical stimulation alone.

MATERIALS AND METHODS

Experimental design

To describe the release profile of bFGF from 3D ε-polycaprolactone (PCL) scaffolds, two experimental groups were established. Scaffolds were treated with I^{125}-labeled bFGF and seeded with BMSCs. Scaffolds were transferred into a bioreactor chamber and either subjected to uniaxial cyclical mechanical strain ($n = 4$) or maintained in a static culture environment ($n = 4$) in a custom bioreactor. Media was sampled from the bioreactor at specified intervals for quantification of radioactivity and subsequent determination of growth factor release rate and bFGF retention on the polymer scaffold.

To characterize the effects of bFGF and uniaxial stimulation on BMSC cellular morphology and fibroblast-related gene expression, six experimental groups were established (Table 1). Scaffolds were treated with 0, 100, or 500 ng of bFGF, and seeded with BMSCs. All scaffolds were

Table 1. Experimental Group Designations

Group	*Designation*	*Mechanical stimulation*	*bFGF*
I	S0	None	0 ng
II	S100	None	100 ng
III	S500	None	500 ng
IV	D0	0.6% strain, 0.125 Hz	0 ng
V	D100	0.6% strain, 0.125 Hz	100 ng
VI	D500	0.6% strain, 0.125 Hz	500 ng

maintained in static culture conditions for 24 hours. Subsequently, media from each group was collected for evaluating cell seeding efficiency ($n = 4$) and scaffolds were transferred into a custom bioreactor chamber and either subjected to uniaxial cyclical mechanical strain or maintained in a static culture environment. Scaffold arms were harvested at 24 hours, 7 days, and 21 days for histomorphometric evaluation ($n = 4$) or RT-PCR ($n = 4$).

Porous scaffold preparation

Scaffolds were prepared by mixing PCL (100,000 MW; i.v. 1.1 dL/g in chloroform ($CHCl_3$) at 30°C; Lactel, Pelham, AL) in $CHCl_3$ (w/w) and adding crystalline sucrose with a grain size of 212–300 μm diameter to achieve 95% porosity (v/v) (Fig. 1). Forty percent methanol (w/w) was added to the polymer solution to promote phase separation between the sugar and polymer following extraction of the solvents. This mixture was added to a machined Teflon mold (26.5×15×5 mm) with a removable center (16.5×5×5 mm) as described previously[9] and allowed to freeze dry overnight. Once removed from the molds, the scaffolds were stored in a desiccator for further use. Pore size and interconnectivity were confirmed by scanning electron microscopy. Average pore size was measured as 262 ± 59 μm. Before cell seeding, scaffolds were placed in deionized, distilled water for 24 hours to leach the crystalline sucrose, disinfected with 70% ethanol for 20 minutes, and finally rinsed with 1× phosphate buffered saline (PBS) prior to collagen coating.

Growth factor incorporation

A noncovalent collagen and growth factor coating method was used for treating porous PCL scaffolds prior to cell seeding. Purified bovine dermal collagen (Cohesion Technologies, Palo Alto, CA) was neutralized to pH 7.4 with 10× PBS and sodium hydroxide in an 8:1:1 ratio at 4°C. This solution was further diluted with sterile water to a final concentration of 0.25 mg/mL. The bFGF (Sigma, St. Louis, MO) was then added to establish the final concentrations that would yield 0, 100, or 500 ng of bFGF per scaffold arm. Aliquots of this solution were then serially added to each arm of the scaffold, with 30 minutes of drying time between applications to allow for collagen and growth factor adhesion. A total of 500 μL of solution, or 0.125 mg collagen, was added to each scaffold arm. A final drying period of 30 minutes was allowed to elapse prior to cell seeding.

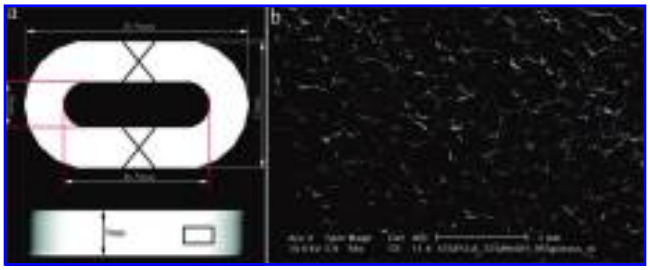

FIG. 1. (**A**) Racetrack-shaped PCL scaffold (26.5×15×5 mm) with a removable center (16.5×5×5 mm). X indicates the top two cell-seeding surfaces. Black rectangle represents a small sample that was examined under scanning electron microscope (SEM). (**B**) SEM of the internal structure of the PCL scaffold matrix, demonstrating extensive porosity. Reproduced with permission from Puk *et al.*[9] Color images available online at www.liebertpub.com/ten.

BMSC culture expansion

BMSCs were obtained from the iliac crest and hind limbs of 6-week-old Lewis rats sacrificed for other medical research purposes. Cells were cultured in Iscove's modified Dulbecco's media (IMDM; Invitrogen, Carlsbad, CA) containing 15% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 IU/mL streptomycin, and 0.25 μg/mL Fungizone (antibiotic-antimycotic, ABAM) at 37°C in an atmosphere of 5% carbon dioxide (CO_2). Cells were maintained for 5 days prior to the first media change, at which time nonadherent cells were removed with culture media. Subsequently, the media was changed every 2–3 days until cells were ~ 80–90% confluent, at which time they were detached with 0.5% trypsin–1 mM ethylenediaminetetraacetate (Invitrogen) and plated at a density of 5×10^6 cells per plate. Second passage (P2) BMSCs near 90% confluence were trypsinized and used for all experiments described hereafter.

Cell seeding

Porous PCL scaffolds were seeded with a total of 4×10^6 cells. Specifically, the top and bottom surfaces of both long arms of the scaffold (a total of four surfaces) were seeded with ~1 million cells per 50 mL IMDM with 15% FBS + ABAM. Scaffolds were first seeded with 1 million cells on both long arms and placed in an incubator at 37°C and 5% CO_2 for 4 hours. The scaffolds were then inverted and an additional 1 million cells were seeded onto the opposing surfaces of both long arms. We attempted to minimize the influence of strain gradients by seeding cells along the midportion of the scaffolds, which is linear in geometry and likely to be subjected to the most uniform strain. After 1 hour of incubation, fresh IMDM with 15% FBS + ABAM was added until the scaffolds were completely submerged. After incubating the cell-seeded scaffolds for 24 hours, the culture medium was collected from the scaffolds and cell count performed using a hemocytometer. The cell seeding efficiency was expressed as the number of cells attached to the scaffolds as a percentage of the number of cells seeded. This was determined by subtracting the number of cells counted in the culture medium from 4×10^6 and dividing by 4×10^6.

Application of uniaxial cyclical strain

Using a strict sterile technique, scaffolds were loaded into a custom bioreactor as previously described. Details of our

bioreactor can be found in a previous publication from our group.[9] Cell-seeded scaffolds were secured in a media chamber and submerged in fresh IMDM with 15% FBS + ABAM. Scaffolds designated to undergo mechanical stimulation were subjected to 6% uniaxial cyclical strain at a frequency of 0.125 Hz for 23 hours/day. These parameters were selected based on the results of previous work demonstrating cellular phenotypic adaptation and upregulation of our genes of interest at this strain magnitude and frequency.[9] Strain was initiated 24 hours following initial seeding and was applied and modulated via a MD-2 stepper motor with a customized motion program (Arrick Robotics, Tyler, TX). Those scaffolds not designated to receive strain were loaded into identical media chambers but were not attached to the motor. The media was changed every third day.

bFGF release kinetics

The bFGF was labeled with the radioisotope I^{125} (MP Biomedicals, Irvine, CA). The iodination procedure was performed using IODO-BEADS (Pierce, Rockford, IL), yielding I^{125}-labeled bFGF with a specific activity of 137,989 cpm/ng at a concentration of 6.45 ng/μL. I^{125}-labeled bFGF was added to 3D porous scaffolds to yield a concentration of 100 ng per scaffold arm. Scaffolds ($n = 4$) were then seeded with 1×10^6 BMSCs on each side of each scaffold arm (a total of 4×10^6 cells/scaffold) and either subjected to uniaxial cyclical strain as previously described or maintained at static culture conditions under a humidified atmosphere of 37°C and 5% CO_2. The culture media was sampled using a syringe attached to a siphon port on the lid of the sample chamber. The radioactivity of the collected sample was measured with a Packard Cobra Auto Gamma Model B5002 (GMI, Ramsey, MN) to determine the burst release of bFGF into the surrounding media. During the first 24 hours, samples were collected every 15 to 60 minutes. Samples were collected every 4 to 6 hours thereafter. After 3 days, the culture media was changed and media was interrogated every hour for 10 hours and then 4–6 hours thereafter. The release profile of bFGF was assessed over time by measuring the radioactivity of the peptide released into the surrounding medium. At the final time point, the scaffolds were morselized and rinsed, and the total growth factor retained on the scaffolds was measured using the aforementioned radioactivity assay.

Histology

At 24 hours, 7 days, or 21 days, scaffolds were removed from the bioreactor and prepared for histological analysis as previously described.[9] Briefly, scaffolds were rinsed gently in 1× PBS and immersed in 3.7% paraformaldehyde overnight. After fixation, the scaffold arms were gently rinsed in 1×PBS and then immersed in a solution of 5% (w/w) porcine-derived gelatin (Sigma) and 5% (w/w) sucrose in deionized water and stored in a convection shaker at 45°C for 24 hours to facilitate gelatin infiltration.[10] Prior to cryosectioning, embedded samples were frozen in an acetone-dry ice bath and stored at −80°C. Samples were sectioned at 10 μm and mounted on acid-treated Superfrost Plus glass slides (VWR Scientific, West Chester, PA) and allowed to air dry. Slides were rinsed with 1× PBS and stained with hematoxylin and eosin (H&E) for further analysis.

Image analysis and aspect ratio

The aspect ratio of a cell is used as a surrogate measure of cellular adaptation.[9,11] To quantify changes in cellular morphology and adaptation to experimental variables in this system, six to twelve digital images were captured per sample section with a color digital camera (Optronics, Goleta, CA) with a Leica DM IRB inverted microscope (McBain Instruments, Chatsworth, CA). From the central portion of each H&E section, two images were obtained using the digital setup as described above. Thirty sections were obtained from each scaffold, yielding approximately 180 sample images. The cell area and the length of the longest cytoplasmic process were measured for two randomly selected cells in each of these images using Bioquant Nova v. 5.01.8 (R&M Biometrics, Nashville, TN). Using the cell area (A) and the longitudinal length (L) of each cell and the ellipse equation ($A = \pi \times r_1 \times r_1$), the relative height of the cell (H) could be determined. Using both the height and length of the cell, the aspect ratio, $\frac{H}{L}$, could be calculated. A lower aspect ratio was indicative of greater cell spreading and suggested greater morphologic cellular adaptation to the 3D environment.

Real-time polymerase chain reaction

At 24 hours, 7 days, or 21 days, scaffolds were harvested and snap frozen in liquid nitrogen. A tissue homogenizer was then used to pulverize the scaffolds, and total RNA was extracted using an RNEasy Mini Prep Kit (Qiagen, Valencia, CA). RNase-Free DNase was used to eliminate DNA contamination in RNA samples. RNA samples were eluted in 100 μL of water and, using a standard spectrophotometer, concentration and purity were determined at 260 and 280 nm, respectively. Purified RNA was stored at −80°C in water prior to RT-PCR. cDNA was synthesized from ∼0.03 ng of total RNA for a 18.75 mL reverse transcriptase reaction volume using QuantiTect Probe RT-PCR reagents. cDNA was amplified in the presence of primer probe oligonucleotide sets (Applied Biosystems, Foster City, CA) for collagen type I (Assay ID: Rn01428781_m1), collagen type III (Assay ID: Rn01437683_m1), tenascin-C (Assay ID: Rn01454950_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rodent endogenous control in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). After an initial denaturation step at 95°C for 15 minutes, cDNA products were amplified through 40 cycles, each consisting of a denaturation step at 94°C for 15 seconds and an extension step at 60°C for 60 seconds. All samples were assessed at a cycle threshold of 1.0. Expression levels of collagen I, collagen III,

and tenascin-C mRNA were quantified using the comparative threshold cycle method, with relative expression of each target gene normalized to GAPDH.

Statistical analyses

Comparisons of independent variables were made using a two-way ANOVA. Univariate comparisons between mechanical stimulation, growth factor stimulation, and time points were performed with a Bonferroni correction. For pair-wise comparisons, two-tailed, unpaired Student's *t*-tests were used and $p < 0.05$ was accepted as significant. Data was presented as the mean ± standard deviation.

RESULTS

Growth factor release

Release of bFGF was quantified by measuring radioactivity from I^{125}-labeled bFGF on either strained or static scaffolds. The initial loading efficiency of bFGF was approximately 60% (60 ng bFGF) across all experimental groups. After 10 hours, approximately 30% (18 ng) of the bFGF that had been successfully loaded onto the scaffold was burst released, and an additional 10% (6 ng) was released after 6 days, with approximately 60% (36 ng) remaining on the scaffolds at steady state. Mechanical stimulation did not affect the release of bFGF within this complex biological environment (Fig. 2).

Cell seeding efficiency

Cell seeding efficiency was evaluated for each experimental group at 24 hours. The mean seeding efficiency was $81.2 \pm 13.2\%$ for control scaffolds, $83.8 \pm 9.2\%$ for scaffolds treated with 100 ng of bFGF, and $85.0 \pm 6.9\%$ for scaffolds treated with 500 ng bFGF. Uniform cell seeding was noted throughout the experimental scaffolds on bright field microscopy as assessed on multiple sections obtained through the peripheral and central portions of the scaffold. No significant difference in seeding efficiency was noted between experimental controls and treatment groups.

Effects of uniaxial cyclical strain and local growth factor release on cellular morphology

Histomorphometry was used to assess cellular morphology in control and experimental groups and was quantified via aspect ratio at 24 hours, 7 days, and 21 days. At 24 hours following seeding, cells showed uniform adherence to the PCL scaffold in all groups, with a high aspect ratio (Fig. 3) and minimal cytoplasmic spread noted on H&E sections (Fig. 4A–C). At 7 days, cells subjected to growth factor stimulation in the absence of mechanical stimulation demonstrated cellular spreading with a significant decrease in aspect ratio ($p < 0.05$) from the 24-hour time point (Fig. 3). Moreover, at 7 days, cells subjected to mechanical stimulation in the presence or absence of growth factor stimulation demonstrated cellular spreading (Fig. 3), with a significant decrease in aspect ratio ($p < 0.05$) from the initial 24-hour time point. It is important to note that at the 7-day time point, phenotypic change was noted in all treatment groups despite the mode of stimulation and that there was no significant change in aspect ratio among treatment groups between 7 and 21 days (Fig. 3). This phenotypic adaptation persisted at 21 days (Figs. 3 and 4E–I), and the aspect ratio for each of the treated groups remained significantly lower than that of untreated controls at this time point ($p < 0.05$). Cells not treated with mechanical

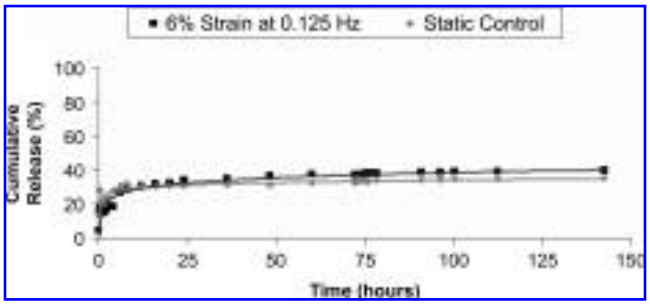

FIG. 2. bFGF release kinetics from a 3D PCL porous scaffold in the presence or absence of mechanical strain. bFGF release was monitored by assaying the bioreactor culture medium for the tracer I^{125}-bFGF with a gamma counter. Each point represents a collection period. During the first 24 hours, samples were collected every 15–60 minutes and 4–6 hours thereafter. After 3 days, the culture media were changed and interrogated every hour for 10 hours and then 4–6 hours thereafter. After 10 hours, approximately 30% of the bFGF that had been successfully loaded onto the scaffold was burst released, and an additional 10% was released after 6 days, with approximately 60% remaining on the scaffolds at steady state.

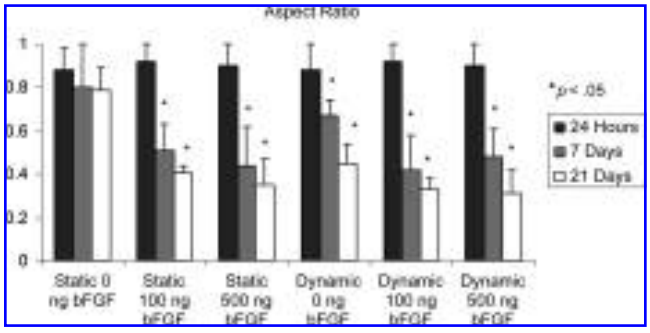

FIG. 3. Aspect ratio (cell spreading) of BMSCs on PCL scaffolds at 24 hours, 7 days, and 21 days as assessed via histomorphometry. A significant decrease in aspect ratio is noted over time in all groups that received growth factor and/or dynamic stimulation (*$p < 0.05$). Most of this change is noted in the time interval from 24 hours to 7 days. A minimal decrease in aspect ratio is noted in all groups between days 7 and 21. Importantly, there was minimal decrease in aspect ratio over time in the absence of both mechanical and growth factor stimulation (Static, 0 ng bFGF).

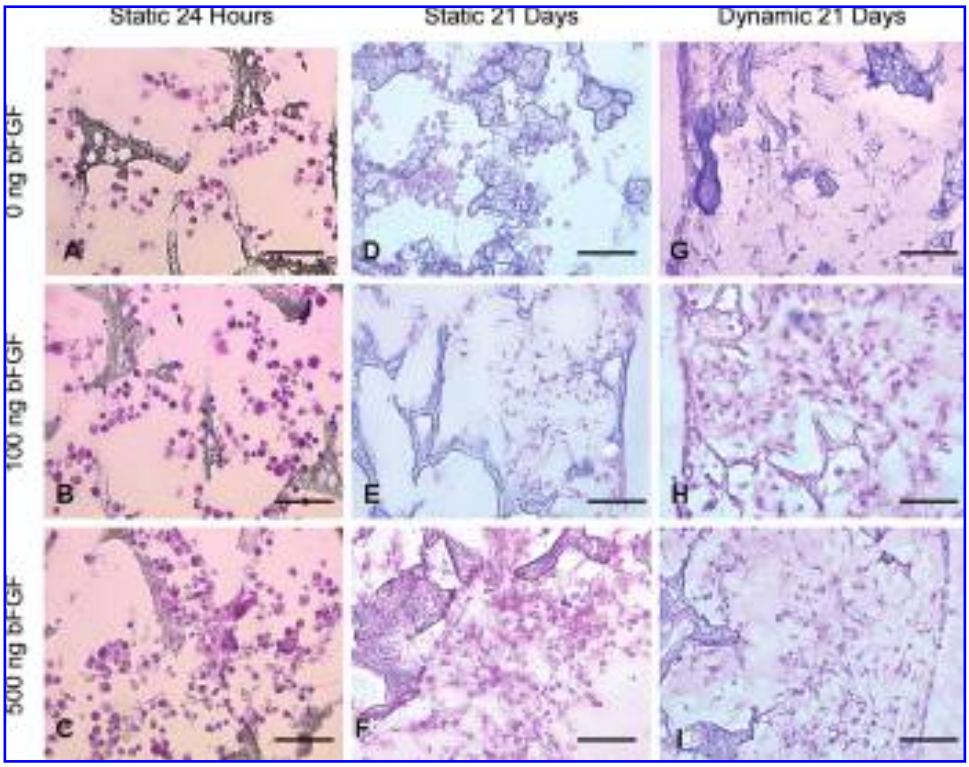

FIG. 4. Histological evaluation of PCL scaffolds seeded with BMSCs. The left column (**A**, **B**, **C**) represents scaffolds with 0, 100, and 500 ng bFGF at 24 hours prior to mechanical stimulation. Note the round shape of the cells with minimal cytoplasmic spreading. The middle column (**D**, **E**, **F**) represents scaffolds at 21 days in the absence of mechanical stimulation. Note that in panel (**D**) (0 ng bFGF, no stimulation), there is minimal change in the morphology of the cells as compared to 24 hours. However, those cells subjected to bFGF (100 or 500 ng) demonstrate cellular spread. The right column (**G**, **H**, **I**) represents scaffolds at 21 days in the presence of mechanical stimulation. Note that there is cytoplasmic spread noted in all groups (0, 100, and 500 ng bFGF) (Bar = 100 μm). Color images available online at www.liebertpub.com/ten.

or biochemical stimulation demonstrated little cellular adaptation to the scaffold (Figs. 3 and 4D).

Effects of uniaxial cyclical strain and local growth factor release on mRNA expression

With regard to collagen I, dynamic scaffolds treated with 500 ng of bFGF demonstrated significantly higher mRNA expression than all other groups at the 21-day time point ($p < 0.05$) (Fig. 5A). Static scaffolds treated with 100 ng bFGF and dynamic scaffolds treated with either 0 or 100 ng of bFGF demonstrated comparable collagen I mRNA expression, which was significantly higher than for static scaffolds treated with either 0 or 500 ng of bFGF. This appears to demonstrate nominal downregulation of collagen I at the final time point. At 21 days, dynamic scaffolds treated with either 0 or 500 ng of bFGF demonstrated a statistically greater expression of collagen III mRNA than all other groups ($p < 0.05$) (Fig. 5B). Again, static scaffolds treated with either 0 or 500 ng of bFGF appeared to demonstrate minimal downregulation of collagen III. With regard to tenascin-C, dynamic scaffolds treated with 0 or 500 ng of bFGF demonstrated significantly higher mRNA expression than all other groups at the 21-day time point ($p < 0.05$) (Fig. 5C). Strikingly, static 500 ng bFGF scaffolds yielded very low levels for all three genes, while mechanical stimulation of the 500 ng bFGF scaffolds produced the highest levels for all three genes among all experimental groups at day 21. (Fig. 5A–C).

DISCUSSION

Current investigations addressing the development of an *ex vivo* ligament replacement have focused on defining the optimal constituents of a ligament tissue engineering platform. BMSCs have demonstrated considerable potential in this regard, as these cells have been shown to adhere and proliferate on a variety of synthetic and natural scaffolds, providing great flexibility in construct design.[3,5,6,12–20] Moreover, they represent a cell source that is responsive to both mechanical and biochemical stimuli and can be induced to express

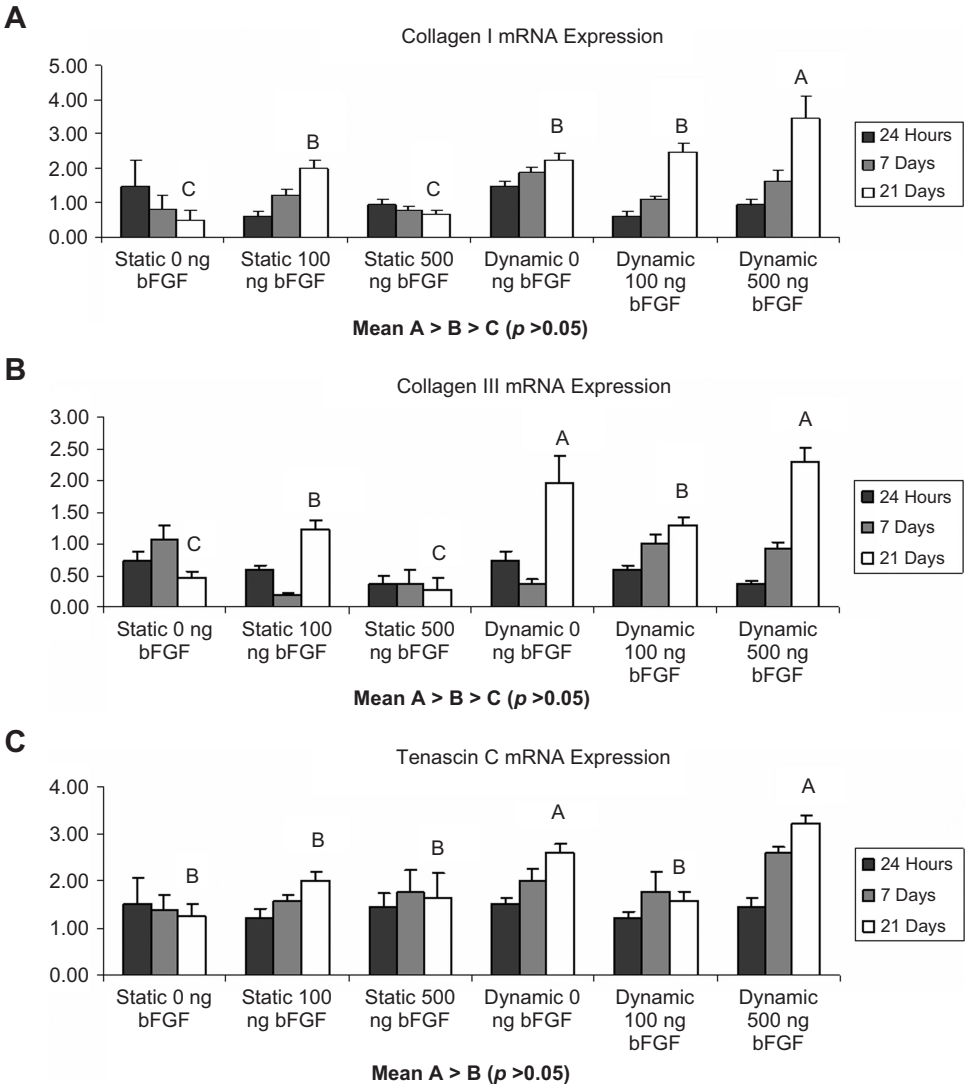

FIG. 5. Relative mRNA expression of collagen type I (**A**), collagen type III (**B**), and tenascin-C (**C**) at 24 hours, 7 days, and 21 days. All mRNA expression is normalized to GAPDH transcript levels. The mean comparison for the 21-day time point is annotated on each figure.

upregulation of stress-resistive and stress-responsive proteins.[1,4,5,7,8,13,17,21,22] Our current investigation sought to describe the individual and collective contributions of mechanical and chemical stimulation to BMSC morphology and mRNA expression on a 3D porous PCL scaffold capable of providing local release of bFGF.

Release profile of bFGF from a collagen-coated PCL scaffold

Most contemporary tissue engineering platforms necessitate the addition of growth factor directly to culture media, and subsequently depend on diffusion to deliver the growth factor to cells. Our team has developed a protocol to provide direct local delivery of growth factor to cells seeded on 3D scaffolds for various tissue engineering applications. We have used a noncovalent collagen and growth factor coating method for priming our PCL scaffolds prior to cellular seeding. This method allows for an initial burst of growth factor directly into the cellular environment, followed by gradual growth factor release from the collagen framework that is established within the scaffold pores. Cells can use the bFGF as it is released from the scaffold or it may diffuse into the surrounding media. Our technique relies on protein adsorption onto natural polymers or biodegradable synthetic polymers; therefore, protein-polymer interactions (electrostatic, hydrophobic, hydrophilic, and Van der Waals) play a critical role in protein release kinetics. Accordingly, protein adsorption can depend somewhat on the net charges of the proteins themselves. Proteins with isoelectric points above 7

are positively charged at pH 7.4, and are therefore expected to interact electrostatically and bind strongly to collagen, which is negatively charged at pH 7.4. In contrast, proteins with isoelectric points below 7 are negatively charged at pH 7.4, and therefore bind loosely to negatively charged collagen at pH 7.4. The current investigation studied the release of bFGF (isoelectric point 9.6) and its influence on cellular phenotype and gene expression. Our results demonstrate that bFGF exhibits a burst release in the first 10 hours, and that at steady state approximately 60% of the bFGF remains bound to the scaffold with minimal release noted for the remaining 6 days. This impressive retention of bFGF to the scaffold can be attributed to the strong electrostatic bond formed between the positively charged bFGF and the negatively charged collagen. Heparin-immobilized biodegradable poly(lactic-*co*-glycolic acid) scaffolds and photoresponsive hydrogel scaffolds have demonstrated similar bFGF release profiles.[23,24] We believe that this early burst exposure may be important in initiating the cellular pathways that lead to cellular adaptation and upregulation of collagen I, III, and tenascin-C. It is well described that appropriate temporal exposure of progenitor cells to bone morphogenetic protein is crucial in driving cells down an osteoblast pathway.[25,26] It may be the case that early temporal exposure of bFGF plays an important role in driving the cellular upregulation of the genes of interest. A recent investigation by Moreau *et al.*[1] demonstrated the importance of appropriate temporal application of growth factors, including bFGF, in promoting collagen I production and cellular ingrowth in a 3D system. Future experiments may be designed to test the short- and long-term influences of bFGF by modulating its release profile by altering the pH of the media and its constituents. It should be mentioned that the bFGF that escapes the scaffold and diffuses into the media may also remain available to cells albeit at a much lower concentration. It may also prove useful to examine the effects of the retained surface-bound bFGF on gene expression or cellular adaptation in this system.

Finally, it was noted that mechanical stimulation did not affect the release of bFGF within this complex biological environment. Given that the release kinetics of bFGF is not influenced by uniaxial cyclical strain, this system allows us to uncouple the effects of mechanical stimulation and growth factor delivery in the current experimental design.

Effect of local bFGF release and cyclic uniaxial strain on cellular morphology

In a previous study, our laboratory characterized the effects of uniaxial cyclical strain on fibroblast morphology in a 3D system, demonstrating that this mode of mechanical strain induced fibroblast spreading as measured by aspect ratio.[9] In the current investigation, locally released bFGF and uniaxial mechanical strain, alone and together, resulted in BMSC elongation and spreading on a 3D porous scaffold. No significant difference was noted between low-dose and high-dose bFGF groups, nor was there an additive or synergistic response noted in those scaffolds subjected to simultaneous mechanical and chemical stimulation. Of note, BMSCs seeded on control scaffolds, which were not subjected to either method of stimulation, demonstrated little change in cellular morphology, remaining round, with minimal scaffold conformity noted at 21 days. These findings may indicate that, in this 3D system, macroscopic changes in cellular morphology as assessed via bright field microscopy do not necessarily correlate with cellular expression of collagen I, III, and tenascin-C mRNA. However, it is clear that in the absence of chemical or mechanical stimulation BMSCs show minimal macroscopic adaptation to our 3D porous scaffold. Further investigations evaluating the deposition of cell-derived extracellular matrix (ECM) via histology at later time points are needed to delineate the functional implications of these findings.

Effect of local bFGF release and cyclic uniaxial strain on mRNA expression

At 21 days, scaffolds either treated with 100 ng of bFGF in the absence of mechanical stimulation or subjected to uniaxial cyclic strain alone demonstrated greater stress-resistive (collagen types I and III) and stress-responsive (tenascin-C) mRNA expression than control scaffolds, which did not receive chemical or mechanical stimulation. These findings are consistent with previous studies evaluating the individual contributions of mechanical and chemical factors, in which low-dose bFGF or uniaxial strain alone resulted in upregulation of these genes.[4,8,21] Interestingly, scaffolds subjected to both uniaxial cyclic strain and 500 ng of bFGF demonstrated the greatest expression of collagen types I and III and tenascin-C mRNA. However, scaffolds treated with 0 or 500 ng of bFGF in the absence of mechanical strain demonstrated a nominal downregulation of collagen I and III mRNA.

We believe that the fluctuations in gene expression demonstrated in the static group are a result of the inherent fluctuations in gene expression that are manifest during cellular maturation and adaptation to the scaffold. In the static situation, treatment with 100 ng of bFGF appears to promote a dose-sensitive upregulation of the genes of interest, which is not seen at other concentrations (0 and 500 ng). In the dynamic situation, the combination of bFGF (500 ng) and mechanical stimulation leads to a robust dose-sensitive upregulation of the genes of interest, which is greater than that observed in any of the static groups. Another important question is, why did stimulation with 100 ng bFGF under dynamic conditions enhance mRNA levels of collagen III and tenascin-C to an amount less than that by 0 or 500 ng bFGF at the final time point? This is more difficult to fully explain, but, as stated previously, the response of cells in the dynamic situation may be altered by the concomitant application of strain. Accordingly, in the dynamic situation,

low-dose bFGF (100 ng) may have a dose-sensitive inhibitory effect on collagen III and tenascin-C expression, while high-dose bFGF (500 ng) gives a profound upregulation of collagen I, III, and tenascin-C. It is this complex modulation of the cellular response to bFGF by mechanical stimulation that, in our opinion, makes this data provocative.

Previous investigations have sought to describe the relationship between mechanotransduction and bFGF-mediated cell proliferation and activation of signal transduction cascades.[27–31] Sudhir *et al*.[29] examined the effect of cyclic mechanical strain and growth factor addition on mitogenic response in ovine coronary artery smooth muscle cells, and observed an increase in tritiated thymidine incorporation and DNA synthesis in response to strain with a temporal response identical to that observed in response to bFGF. This mitogenic response was abolished by monoclonal antibodies to bFGF. Shin *et al*.[31] investigated bFGF signaling as a putative mechanotransduction pathway involved in the proliferative responses of human umbilical vein endothelial cells (HUVECs) to cyclic pressure. Under dynamic conditions, the enhanced proliferative response of these HUVECs was not associated with an increased synthesis of bFGF, but involved rapid tyrosine phosphorylation of the bFGF receptor, fibroblast growth factor receptor 2 (FGFR-2). Further, monoclonal antibodies to either bFGF or FGFR-2 attenuated the increased proliferation of HUVECs exposed to cyclic pressure. Vincent *et al*.[28] demonstrated rapid activation of the extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase pathway with cyclical loading of articular cartilage. Activation of this pathway was restricted by introduction of a bFGF receptor inhibitor.

Strain parameters may also have a substantial effect on cellular response in this system. Our parameters (6% strain, 0.125 Hz) were predicated on the results of previous investigations performed both in our laboratory and by other authors evaluating the effects of mechanical stimulation on cell differentiation. Thus, the rationale behind our selection of this range of strain and frequency was twofold: (1) previous authors have demonstrated upregulation of stress-resistive proteins such as collagen in response to similar parameters,[4,21,32,33] and (2) these strain parameters have been shown to induce phenotypic cell changes in our system.[9] However, it should be emphasized that the optimal parameters (onset of strain, and its magnitude, frequency, and duration) in regard to upregulation of stress-responsive and stress-resistive proteins have yet to be defined. These parameters will likely reflect the physiologic development or repair mechanism of native ligament. In a recent study by Chen *et al*.,[6] the authors attempted to characterize the appropriate time frame for applying mechanical stimuli to induce BMSC differentiation for ligament engineering by monitoring developmental cell phenotypes during a period of *in vitro* culture. At day 9, when levels of cell metabolic activity, ECM, integrin, and heat shock protein-70 transcription peaked, mechanical stimulation increased MSC metabolic activity, alignment, and collagen production, while stimulation at days 1 and 3 demonstrated detrimental effects on these parameters. Perhaps a more timely initiation of mechanical strain in this system would further augment the cellular response to the bFGF and further upregulate stress-resistive proteins. It has also been argued that systems such as ours, which are designed to produce longitudinal strain in 3D, may possess gradients that may influence the overall behavior of cells within the scaffold. Strain gradients are expected to exist across the scaffold due to scaffold geometry, and the control groups in this experiment are designed to account for this variability. We attempted to minimize the strain gradient by seeding cells along the midportion of the linear aspect of the scaffold. The direct contributions to the observed results due to heterogeneity of strain vectors and the presence of strain gradients in this study are not known, but are not expected to alter the conclusions of this study. Scaffolds are manufactured and cells are seeded onto the scaffolds in a standardized manner. It is reasonable to presume that, on average, cells within and between experimental groups are subjected to strain gradients of similar magnitude and direction.

Lastly, it is important to emphasize that the stress-resistive (collagen I and III) and stress-responsive (tenascin-C) genes that we have chosen to investigate in this study are not fibroblast specific. Bone and connective tissue are rich in collagen I and III, while tenascin-C is found in cartilage and dermis.[34] Nonetheless, the presence of these proteins is essential in the development and repair of native ligament and tendon. Consequently, the capacity to induce upregulation of these critical genes may be viewed as a fundamental step in driving cells toward a fibroblast lineage. Further descriptive studies are needed to determine reliable protein markers of fibroblast differentiation.

CONCLUSION

Porous 3D PCL scaffolds treated with collagen and bFGF and seeded with BMSCs have the potential to act as a useful platform for evaluating cellular response to growth factor and mechanical strain. In this system, locally released bFGF has a dose-sensitive influence on the regulation of stress-responsive and stress-resistive genes, which are important in the synthesis and repair of ligament and other connective tissues. Moreover, these effects are influenced by the application of uniaxial mechanical strain. The combined interaction of these regulatory stimuli may be useful in driving the differentiation of BMSCs and may aid in the development of a tissue-engineered ligament construct.

ACKNOWLEDGMENT

This research has been supported by a grant from the Musculoskeletal Transplant Foundation and the Orthopaedic Hospital Foundation.

REFERENCES

1. Moreau, J.E., Chen, J., Horan, R.L., Kaplan, D.L., and Altman, G.H. Sequential growth factor application in bone marrow stromal cell ligament engineering. Tissue Eng **11,** 1887–1897, 2005.
2. Sahoo, S., Ouyang, H., Goh, J.C., Tay, T.E., and Toh, S.L. Characterization of a novel polymeric scaffold for potential application in tendon/ligament tissue engineering. Tissue Eng **12,** 91–99, 2006.
3. Moreau, J.E., Chen, J., Bramono, D.S., Volloch, V., Chernoff, H., Vunjak-Novakovic, G., Richmond, J.C., Kaplan, D.L., and Altman, G.H. Growth factor induced fibroblast differentiation from human bone marrow stromal cells *in vitro*. J Orthop Res **23,** 164–174, 2005.
4. Altman, G.H., Horan, R.L., Martin, I., Farhadi, J., Stark, P.R., Volloch, V., Richmond, J.C., Vunjak-Novakovic, G., and Kaplan, D.L. Cell differentiation by mechanical stress. FASEB J **16,** 270–272, 2002.
5. Chen, J., Altman, G.H., Karageorgiou, V., Horan, R., Collette, A., Volloch, V., Colabro, T., and Kaplan, D.L. Human bone marrow stromal cell and ligament fibroblast responses on RGD-modified silk fibers. J Biomed Mater Res A **67,** 559–570, 2003.
6. Chen, J., Horan, R.L., Bramono, D., Moreau, J.E., Wang, Y., Geuss, L.R., Collette, A.L., Volloch, V., and Altman, G.H. Monitoring mesenchymal stromal cell developmental stage to apply on-time mechanical stimulation for ligament tissue engineering. Tissue Eng **12,** 3085–3095, 2006.
7. Van Eijk, F., Saris, D.B., Riesle, J., Willems, W.J., Van Blitterswijk, C.A., Verbout, A.J., and Dhert, W.J. Tissue engineering of ligaments: a comparison of bone marrow stromal cells, anterior cruciate ligament, and skin fibroblasts as cell source. Tissue Eng **10,** 893–903, 2004.
8. Hankemeier, S., Keus, M., Zeichen, J., Jagodzinski, M., Barkhausen, T., Bosch, U., Krettek, C., and Van Griensven, M. Modulation of proliferation and differentiation of human bone marrow stromal cells by fibroblast growth factor 2: potential implications for tissue engineering of tendons and ligaments. Tissue Eng **11,** 41–49, 2005.
9. Puk, C.K., Miller, D.J., Gamradt, S., Wu, B.M., and McAllister, D.R. The effects of short-term stimulation on fibroblast spreading in an *in vitro* 3D system. J Biomed Mater Res A **76,** 665–673, 2006.
10. Brown, D.A., Chou, Y.F., Beygui, R.E., Dunn, J.C., and Wu, B.M. Gelatin-embedded cell-polymer constructs for histological cryosectioning. J Biomed Mater Res B Appl Biomater **72,** 79–85, 2005.
11. Disatnik, M.H., and Rando, T.A. Integrin-mediated muscle cell spreading. The role of protein kinase c in outside-in and inside-out signaling and evidence of integrin cross-talk. J Biol Chem **274,** 32486–32492, 1999.
12. Laurencin, C.T., Khan, Y., Kofron, M., El-Amin, S., Botchwey, E., Yu, X., and Cooper, J.A., Jr. The ABJS Nicolas Andry Award: tissue engineering of bone and ligament: a 15-year perspective. Clin Orthop Relat Res **447,** 221–236, 2006.
13. Cristino, S., Grassi, F., Toneguzzi, S., Piacentini, A., Grigolo, B., Santi, S., Riccio, M., Tognana, E., Facchini, A., and Lisignoli, G. Analysis of mesenchymal stem cells grown on a three-dimensional HYAFF 11-based prototype ligament scaffold. J Biomed Mater Res A **73,** 275–283, 2005.
14. Fujita, M., Kinoshita, Y., Sato, E., Maeda, H., Ozono, S., Negishi, H., Kawase, T., Hiraoka, Y., Takamoto, T., Tabatta, Y., and Kameyama, Y. Proliferation and differentiation of rat bone marrow stromal cells on poly(glycolic acid)-collagen sponge. Tissue Eng **11,** 1346–1355, 2005.
15. Ge, Z., Yang, F., Goh, J.C., Ramakrishna, S., and Lee, E.H. Biomaterials and scaffolds for ligament tissue engineering. J Biomed Mater Res A **77,** 639–652, 2006.
16. Gunatillake, P.A., and Adhikari, R. Biodegradable synthetic polymers for tissue engineering. Eur Cell Mater **5,** 1–16, 2003; discussion 16.
17. Heckmann, L., Schlenker, H.J., Fiedler, J., Brenner, R., Dauner, M., Bergenthal, G., Mattes, T., Claes, L., and Ignatius, A. Human mesenchymal progenitor cell responses to a novel textured poly(L-lactide) scaffold for ligament tissue engineering. J Biomed Mater Res B Appl Biomater **81B,** 82–90, 2006.
18. Laurencin, C.T., and Freeman, J.W. Ligament tissue engineering: an evolutionary materials science approach. Biomaterials **26,** 7530–7536, 2005.
19. Hori, Y., Inoue, S., Hirano, Y., and Tabata, Y. Effect of culture substrates and fibroblast growth factor addition on the proliferation and differentiation of rat bone marrow stromal cells. Tissue Eng **10,** 995–1005, 2004.
20. Liu, G., Hu, Y.Y., Zhao, J.N., Wu, S.J., Xiong, Z., and Lu, R. Effect of type I collagen on the adhesion, proliferation, and osteoblastic gene expression of bone marrow-derived mesenchymal stem cells. Chin J Traumatol **7,** 358–362, 2004.
21. Altman, G.H., Lu, H.H., Horan, R.L., Calabro, T., Ryder, D., Kaplan, D.L., Stark, P., Martin, I., Richmond, J.C., and Vunjak-Novakovic, G. Advanced bioreactor with controlled application of multi-dimensional strain for tissue engineering. J Biomech Eng **124,** 742–749, 2002.
22. Noth, U., Schupp, K., Heymer, A., Kall, S., Jakob, F., Schutze, N., Baumann, B., Barthel, T., Eulert, J., and Hendrich, C. Anterior cruciate ligament constructs fabricated from human mesenchymal stem cells in a collagen type I hydrogel. Cytotherapy **7,** 447–455, 2005.
23. Andreopoulos, F.M., and Persaud, I. Delivery of basic fibroblast growth factor (bFGF) from photoresponsive hydrogel scaffolds. Biomaterials **27,** 2468–2476, 2006.
24. Yoon, J.J., Chung, H.J., Lee, H.J., and Park, T.G. Heparin-immobilized biodegradable scaffolds for local and sustained release of angiogenic growth factor. J Biomed Mater Res A **79,** 934–942, 2006.
25. Beck, C.W., Christen, B., Barker, D., and Slack, J.M. Temporal requirement for bone morphogenetic proteins in regeneration of the tail and limb of Xenopus tadpoles. Mech Dev **123,** 674–688, 2006.
26. Marom, K., Levy, V., Pillemer, G., and Fainsod, A. Temporal analysis of the early BMP functions identifies distinct anti-organizer and mesoderm patterning phases. Dev Biol **282,** 442–454, 2005.
27. Vincent, T., and Saklatvala, J. Basic fibroblast growth factor: an extracellular mechanotransducer in articular cartilage? Biochem Soc Trans **34,** 456–457, 2006.
28. Vincent, T.L., Hermansson, M.A., Hansen, U.N., Amis, A.A., and Saklatvala, J. Basic fibroblast growth factor mediates transduction of mechanical signals when articular cartilage is loaded. Arthritis Rheum **50,** 526–533, 2004.

29. Sudhir, K., Hashimura, K., Bobik, A., Dilley, R.J., Jennings, G.L., and Little, P.J. Mechanical strain stimulates a mitogenic response in coronary vascular smooth muscle cells via release of basic fibroblast growth factor. Am J Hypertens **14,** 1128–1134, 2001.
30. Yu, J.C., Lucas, J.H., Fryberg, K., and Borke, J.L. Extrinsic tension results in FGF-2 release, membrane permeability change, and intracellular Ca++ increase in immature cranial sutures. J Craniofac Surg **12,** 391–398, 2001.
31. Shin, H.Y., Schwartz, E.A., Bizios, R., and Gerritsen, M.E. Receptor-mediated basic fibroblast growth factor signaling regulates cyclic pressure-induced human endothelial cell proliferation. Endothelium **11,** 285–291, 2004.
32. Berry, C.C., Cacou, C., Lee, D.A., Bader, D.L., and Shelton, J.C. Dermal fibroblasts respond to mechanical conditioning in a strain profile dependent manner. Biorheology **40,** 337–345, 2003.
33. Berry, C.C., Shelton, J.C., Bader, D.L., and Lee, D.A. Influence of external uniaxial cyclic strain on oriented fibroblast-seeded collagen gels. Tissue Eng **9,** 613–624, 2003.
34. Jenner, J.M., van Eijk, F., Saris, D.B., Willems, W.J., Dhert, W.J., and Creemers, L.B. Effect of transforming growth factor-beta and growth differentiation factor-5 on proliferation and matrix production by human bone marrow stromal cells cultured on braided poly lactic-co-glycolic acid scaffolds for ligament tissue engineering. Tissue Eng **13,** 1573–1582, 2007.

Address reprint requests to:
Frank A. Petrigliano, M.D.
Department of Orthopaedic Surgery
David Geffen School of Medicine
University of California Medical Center
10833 Le Conte Ave., Room 16-155 CHS
Los Angeles, CA 90095

E-mail: fpetrigliano@mednet.ucla.edu

W2007_523.doc

LIGAMENT ENGINEERING: CHARACTERISTICS OF BETA FIBROBLAST GROWTH FACTOR RELEASE FROM A BIOENGINEERED SCAFFOLD

D. Barba, V. Sun, S. Esmende, F. Petrigliano, D. Kamei, D. McAllister, B. Wu, David Geffen School of Medicine at UCLA, Los Angeles, CA.

The anterior cruciate ligament is a commonly injured ligament of the knee, in which ligament reconstruction with a graft is necessary, due to its limited healing capacity. With limitations on the existing graft alternatives, we aim to engineer a ligament utilizing a donor's bone marrow stromal cells (BMSCs). Recently, our lab reported synergism between biomechanical and biochemical stimulation (bFGF) resulting in up-regulation of gene expression of stress-resistant Collagen I, and stress-responsive Collagen III and Tenascin- C. However, it was unclear if this observation was due to true synergism between mechanical and biochemical signal transduction pathways, or due to enhanced release kinetics of the bFGF. The objective of this project is to determine the effects of mechanical stimulation on the release kinetics of bFGF in the presence of a complex biological environment (cells, media, etc.). Microporous scaffolds were constructed from biodegradable polycaprolactone polymers, loaded with 400 ng I-125 labeled bFGF per scaffold, and the seeded with BMSCs harvested from rats. Release of bFGF was quantified by measuring radioactivity from I-125 labeled bFGF on either strained (6%) or unstrained scaffolds. After 10 hours, approximately 30% of the bFGF was burst-released from the scaffolds, 10% of bFGF was released after 6 days, and approximately 60% remained on the scaffolds. Mechanical stimulation did not affect the release of bFGF within this complex biological environment. In summary, we have established and characterized a controlled release system to deliver bFGF to cells seeded within microporous scaffolds and subjected to biomechanical stimulation. Given that the release kinetics are not influenced by mechanical stimulation, this system allows us to uncouple the effects of mechanical stimulation and growth factor delivery in future experiments. This finding also encourages the investigation of common signal transduction pathways that may be involved in the observed synergism between biomechanical and biochemical stimulation.