Mechanical stimulation induces morphological and phenotypic changes in bone marrow-derived progenitor cells within a three-dimensional fibrin matrix

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Abstract: One of the major limitations in tissue engineering is cell sourcing. Multipotent progenitor cells appear to have many promising features for that purpose. Mechanical stimulation is known to play an important role in determining cell phenotype. The aim of this work was to investigate the effects of cyclic stretch on rat bone marrow derived progenitor cell (BMPC) morphology and smooth muscle-directed differentiation within a three-dimensional fibrin matrix. BMPCs were suspended in a fibrin gel, pipetted into the trough of FlexcellTM Tissue-TrainTM plates, and stimulated with 10% longitudinal cyclic stretch at 1 Hz for 6 days. Unconstrained (stress- and strain-free) and static anchored (constrained but not stretched) samples were used as controls. Stress filament area per cell was increased in the stretched samples compared to static anchored and free-float controls. Cells in the free float controls were randomly aligned, while they aligned parallel to the direction of the stress or strain in the other groups. Immunofluorescence suggested an increased expression of smooth muscle markers (smooth muscle α actin and h1-calponin) in both stretched and constrained control samples, but not in unconstrained controls. Qualitative assessment suggested that collagen production was increased in both mechanically stimulated samples. Proliferation was inhibited in stretched samples compared to the constrained controls. This work suggests an ability of rat BMPCs to differentiate toward a smooth-muscle-cell-like lineage when exposed to biomechanical stimulation in a three-dimensional model. The observation that the constrained samples induced changes in BMPCs suggests that stress alone may be stimulatory, but addition of cyclic stretch appears to augment the responses. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 81A: 523–530, 2007

Key words: stem cells; mechanical stimulation; three-dimensional; biomechanical stress; fibrin gel; tissue engineering

INTRODUCTION

Despite the fact that organ transplantation has become a common practice worldwide and has saved thousands of lives, there is a shortage of organ donors that prevents further success in this field. The burgeoning field of tissue engineering has recently shown great potential for tissue and organ replacement.1–3 The approaches in this field may vary, but most involve the use of cells incorporated within some type of scaffold or extracellular matrix. Although several cell sources are available and have been used, autologous cells are widely preferred to avoid deleterious effects of rejection and to prevent the shortcomings of immunosuppressive medication.4 A commonly targeted cell type for tissue engineering purposes has been the smooth muscle cell (SMC), which is an essential component of several organs in the body, mainly in the vascular, digestive, or excretory systems.5 However, several rate-limiting steps are inhibiting progress in tissue engineering and regenerative medicine, and it is believed that many of these limitations are related to the use of terminally differentiated cells.5,6 One possible explanation is telomere shortening that occurs after every cell replication and that is thought to be associated with senescence of the cell.7

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An alternative source of autologous cells for tissue engineering applications are multi-potential progenitor cells that have been identified in adult tissues. Bone marrow, in particular, is known to contain primitive and multi-potent progenitor cells. These bone marrow-derived progenitor cells (BMPCs) are capable of differentiating into several different hematopoietic and mesenchymal lineages and have recently been shown to be successful in several clinical applications including cardiovascular, hematopoietic, and osteoarticular disorders. However, the factors that regulate the proliferation and differentiation of the BMPC populations in vivo are largely unknown, despite extensive studies.

Arakawa et al. observed that a bone marrow stromal cell line differentiates into SMCs when grown in the presence of 2-mercaptoethanol and ascorbic acid in vitro, but the cells within many tissues are exposed to several biomechanical forces in vivo (e.g. shear-stress, cyclic stretch, cyclic pressure) that influence their phenotype. Furthermore, it is well known that cells adapt to changes in their mechanical environment by changing morphology and altering gene expression and phenotype. For example, vascular smooth muscle is known to change from a contractile to a synthetic phenotype (which includes a decrease in vasomotor response) when strain is removed.

Our laboratory has recently shown that BMPCs subjected to cyclic stretch in monolayer culture upregulate the expression of smooth muscle markers. This finding is of significant interest when considering guided differentiation of BMPCs for cell therapy or tissue engineering purposes. However, it is well known that cells behave differently in monolayer than in a three-dimensional (3D) environment. The interaction between the cell and the extracellular matrix (ECM) in 3D models has displayed differences, such as the components of the focal adhesions when compared to monolayer culture. Since most tissue engineering approaches use a 3D scaffold to support cell growth, it is important to understand how BMPCs respond to mechanical stimulation in a 3D environment to attempt differentiation in situ.

In this article, we report the effect of 10% cyclic stretch and static load on the proliferation, morphology, differentiation, and collagen production of BMPCs within a 3D fibrin gel. The extracellular matrix material was chosen based on recent reports on fibrin gel as a suitable alternative to develop tissue engineering constructs, in particular for vascular applications. The Tulane Center for Gene Therapy at University of Tulane has extensively characterized a line of bone marrow mesenchymal stem cells that are considered a Comparative Medicine Resource for The National Center for Research Resources and those cells were used for this study (www.ncrr.nih.gov/ncrrprog/cmpdir/cmdirectory.asp).

MATERIALS AND METHODS

Cell source and culture

Rat BMPCs were obtained from the Tulane Center for Gene Therapy. The cells were plated at low density (200 cells/cm²) on a 175-cm² flask and cultured at 37°C and 3.5% CO₂ with complete α-modified Eagle’s medium (α-MEM) containing 20% fetal bovine serum (lot selected for promoting rapid expansion of mesenchymal stem cells; Atlanta Biologicals; Norcross, GA), 2 mM l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were expanded to the desired number and were only used between passages 3–5. Media changes were performed every 48 h before experiments. BMPC monolayers were washed three times in phosphate-buffered saline (PBS) and then incubated with 0.1% trypsin for 5 min to remove them from the flasks. Afterward, BMPCs were centrifuged at 1200 rpm for 5 min to form a pellet. The BMPCs were then resuspended in α-MEM to the desired concentration. The cells, as suggested by the Tulane Center for Gene Therapy, are subjected to a colony-forming unit test to prove the absence of fibroblasts. All the cells used in the study, from different original vials provided by Tulane University, were free of fibroblast contamination.

Fabrication of 3D constructs

To obtain 3D constructs that could be exposed to cyclic stretch, a solution of cells and fibrin gel was molded within the troughs of Flexcell™ Tissue-Train plates (Flexcell, Hillsborough, NC) based on established protocols. Briefly, a 5 mg/mL solution of bovine fibrinogen type I (Sigma–Aldrich, St. Louis, MO) and α-MEM at 4°C was mixed with 80 x 10⁷ cells/mL and supplemented with 1 mg/mL of 6-aminohexanoic acid (Sigma–Aldrich, St. Louis, MO). The enzymatic reaction was started with 0.66 IU of bovine thrombin (Sigma–Aldrich, St. Louis, MO). Each trough was loaded with 180 μL of the final solution and incubated at 37°C and 3.5% CO₂ to allow gelling of the samples [Fig. 1(A)]. After 45 min, 5 mL of complete media was added to each construct and incubated for 48 h to allow compaction of the samples.

Mechanical stimulation

Following compaction, the plates were mounted on the FX-4000T strain unit (Flexcell, Hillsborough, NC), and cyclic stretch was applied by deformation of the plate through regulated air vacuum supplied to the bottom of the plate causing the membrane to stretch across a loading post. This creates a uniaxial cyclic strain to the 3D constructs anchored to both edges of the wells with negligible shear stress [Fig. 1(B)]. The stimulation regimen used was 10% strain at 1 Hz for 6 days (following the 2-day static incubation step).

Controls

Two different control groups were included in these experiments. The first control group were 3D constructs pre-
pared in identical manner to the "cyclic stretch" samples, but statically cultured within the same plates for the entire 8-day duration. We refer to this group as the "constrained" controls because the act of anchoring the constructs at their ends would cause an axial stress load in response to the cellular traction forces [Fig. 1(B)]. To examine the effects of this stress load, a second group control was created by loading samples of the same solution and same volume on plastic 12-well plates (Costar, Acton, MA). The resulting gelled constructs were detached from the bottom of the well after 45 min and allowed to compact freely in all directions. We refer to this group as "unconstrained" controls and assume that these constructs are free of both stress and strain [Fig. 1(C)].

F-actin labeling and morphology analysis

Strained and unstrained samples were rinsed with PBS, fixed in 4% paraformaldehyde for 60 min, and then incubated for another 60 min with 0.1% Triton-X 100 in PBS to permeabilize the cell membranes. Next, the samples were incubated with Alexa 488-conjugated phalloidin (Sigma–Aldrich, St. Louis, MO) (dilution 1:250) for 60 min in a moist chamber to prevent drying of the samples. Unbound phalloidin was removed by subsequent washes in PBS. For nuclear visualization, cells were counter-stained with DAPI. Samples were mounted in gelvatol and viewed at 400× using an Olympus F1000 confocal microscope.

To calculate the stress filament area, five representative images of each construct were captured and imported into Metamorph Image analysis software (v 6.3, Molecular Devices, USA). The stress filaments were then identified via gray-scale thresholding, and the measured area of stress filaments per number of cells analyzed was calculated.

Proliferation and viability (MTT assay)

To evaluate BMPC viability and calculate proliferation rates, constructs were placed in the wells of a 96-well plate with 200 μL of serum-free α-MEM and 20 μL of Thiazolyl Blue Tetrazolium Bromide (Sigma–Aldrich, St. Louis, MO). Samples were then incubated at 37°C for 4 h to allow crystal formation. Supernatant volume was then carefully removed and 200 μL of 0.04N HCl in 2-propanol solution were added to dissolve the crystals. Samples were kept in the dark at 4°C for 24 h. Finally, absorbance readings were taken for 100 μL of the solution for each condition at a wavelength of 550 nm using a microplate reader (Bio Rad, Hercules, CA). The final number of cells was calculated using a standard curve generated for previously known cell concentrations and transforming absorbance to cell number using the equation generated by the slope of the curve.

Phenotypic markers

To assess the SMC-guided differentiation of the BMPCs within the constructs, samples were rinsed with PBS, fixed in 4% paraformaldehyde for 60 min, and then incubated for another 60 min with 0.1% Triton-X 100 in PBS to permeabilize the cell membranes. Nonspecific binding of antibodies was blocked by incubating the samples for 45 min with 5%
normal donkey serum in PBS with 0.5% bovine serum albumin (Fraction V, Sigma–Aldrich, St. Louis, MO) and 0.15% glycine. Following this, the samples were incubated at room temperature with the primary antibodies (smooth muscle α-actin (α-SMA) (1:500) [Chemicon, Temecula, CA], h1-calponin (1:400) [Dako, Carpinteria, CA], Myosin Heavy Chain (MHC) (1:500) [Dako, Carpinteria, CA], and CD31 (1:200) [Chemicon, Temecula, CA]) diluted in blocking solution for 60 min in a moist chamber to prevent drying of the samples. Unbound primary antibody was removed by subsequent washes in PBS. Next, the samples were incubated with a Cy3-conjugated (Sigma–Aldrich, St. Louis, MO) secondary antibody (1:500) for 1 h at room temperature and then rinsed three times for 15 min with PBS. For nuclear visualization, cells were counter-stained with DAPI. The samples were then mounted in gelvatol and viewed under confocal microscopy. Differentiation was evaluated qualitatively through inspection of immunofluorescent images using Adobe Photoshop (v. 7.0, Adobe Systems, USA).

Collagen production

To assess collagen production by the cells within the constructs, samples were rinsed with PBS and fixed in 10% neutral buffered formalin for 1 h. They were then embedded in paraffin blocks and 5-μm sections were cut using a Shandon.

Figure 2. Stress filament analysis. Cells within the free float control group (A) are randomly organized, while the cells within the static stress group (B) and cyclic strain group (C) are aligned parallel to direction of the stress or strain (arrow) providing an organized structure with the surrounding matrix. Quantitative analysis of the F-actin stain (D) indicates an increase in the stress filament area per cell in the constrained compared to the unconstrained samples and constrained/stretched samples significantly increased this response (n = 4). For (A–C): Green = F-actin filaments, Blue = Nuclei, Magnification = ×40, Insets = ×100. Scale bar (white) = 10 μm.
Finesse microtome (Thermo Shandon, Pittsburgh, PA). Sections were mounted on slides, stained with Mason’s trichrome, and viewed under bright light optics using a Nikon Eclipse E600 microscope (Nikon, Melville, NY). Collagen production was qualitatively assessed on acquired images using Adobe Photoshop.

Degree of compaction

To assess the degree of compaction, samples were submerged in a microcentrifuge tube containing a 0.01% solution of saline with Triton X 100 to reduce surface tension and avoid meniscus formation. The fluid column displacement was measured with a Vernier caliper, and the volume of the sample estimated by the equation: \( \pi r^2 \times \text{column height} \). Degree of compaction was expressed in percent of the original volume.

Statistics

Since all experiments were paired with their controls, a paired \( t \)-test was used to compare measures between groups. Probability of null hypothesis <5% (\( p < 0.05 \)) was considered statistically significant.

RESULTS

BMPCs were successfully incorporated within the fibrin matrix and were viable after 6 days. The unconstrained controls were able to compact radially into a spherical shape, while the constrained samples compacted perpendicular to their long axis [Fig. 1(D)]

Both the constrained and constrained/stretched groups portrayed a markedly altered morphology of BMPCs (Fig. 2). Specifically, cells within unconstrained controls did not appear to have spread and exhibited random cellular orientation [Fig. 2(A)]. BMPCs under constrained and constrained/stretched conditions were elongated, and arranged longitudinally with their F-actin filaments aligned parallel to the direction of the stress [Fig. 2(B,C)]. Constrained controls had an increased area of stress filaments per cell compared to unconstrained controls (9585 ± 1525 pixels per cell vs. 4682 ± 2251 pixels per cell; \( p = 0.01 \)). Cyclic stretch significantly increased that response compared to both constrained and unconstrained controls (15752 ± 4278 pixels per cell; \( p = 0.05 \) and 0.01, respectively) [Fig. 2(D)].

All groups contained viable cells and showed proliferation within the fibrin matrix with respect to the initial seeding density (Fig. 3). Both the constrained and constrained/stretched groups had a greater fold increase in cell number (56.8 ± 17.6 and 48.4 ± 16.3, respectively) than the unconstrained controls (36.3 ± 16.8; \( p < 0.01 \) and \( p = 0.01 \), respectively). However, constrained/stretched constructs had significantly fewer cells than the constrained samples (\( p = 0.01 \)).

All samples from the three groups compacted to a (20 ± 3.7)% of their original volume showing no differences in gross thickness.

Constrained and constrained/stretched samples qualitatively exhibited an increase in collagen deposition compared to the unconstrained controls (Fig. 4).

Phenotypic differentiation analysis revealed that both constrained and constrained/stretched samples stained positive for \( \alpha \)-SMA and h1-calponin, while neither protein was detected in the unconstrained controls (Fig. 5). All samples were negative for MHC and CD31.

DISCUSSION

In this article, we have shown that the static stress load generated by fibrin gel compaction induced morphological changes and upregulation of early and intermediate SMC markers in BMPCs. This response appears to be augmented by 10% cyclic strain. While our previous work demonstrated that monolayers of BMPCs tend to align perpendicular to the direction of applied strain,20 our current analysis shows that the long axis of the BMPCs aligns parallel to the direction of applied stress in 3D culture. The cells did not express MHC suggesting they are not fully contractile, probably due to the short period of stimulation or the lack of any biochemical stimulus. The fact that the cells did not express the endothelial marker CD31 suggests the specificity of the differentiation pathway. We also found that BMPCs increased their collagen production under constrained and cyclic stretch conditions. Consistent with our previous monolayer work,20 cyclic stretch induced a decrease in BMPC proliferation. However, this and other studies investigating the mechanosensitivity of BMPCs have been done in 2D,30 but the composition and function of adhesions in 3D matrices affects the way that cells respond. For example, it has been shown that cell-ECM interactions in 3D culture display enhanced biological activity with narrower integrin usage com-
pared to 2D culture. \(^{23,31,32}\) 3D models and dynamic culture have also shown the ability to switch SMCs from a synthetic to a contractile phenotype. \(^{33,34}\)

The advent of tissue engineering has generated particular interests in the different pathways and conditions that control cellular phenotype and function. Although many tissue engineering approaches use terminally-differentiated cells, it has been demonstrated that they possess several disadvantages, especially when considering clinical viability. The need for long-term culture, the difficult isolation, and the short lifespan, among other issues, may hinder success with terminally-differentiated cells.\(^{4}\)

The use of adult, autologous BMPCs appears as a promising solution and has been utilized in several tissue engineering approaches.\(^{14,35-37}\) However, it is still unclear how these cells contribute to the final development of the tissue engineered constructs. Understanding how the chemical and mechanical environment affects their morphology and differentiation is likely an important consideration in optimizing the fabrication of the constructs. BMPCs have been shown to differentiate to SMCs under defined chemical stimuli, including ascorbic acid, 2-mercaptoethanol, transforming growth factor-\(\beta\), and platelet-derived growth factor.\(^{18,21,38}\) However, the observation that mechani-

![Image](528.jpg)

**Figure 4.** Collagen production. A: Unconstrained control, B: Constrained control, and C: Constrained/Stretched. Trichrome stain (longitudinal sections) shows increased collagen production in constrained and constrained/stretched samples compared to unconstrained controls. Arrows indicate direction of stress/strain, Red = fibrin, Black = nuclei, Blue = Collagen. 8-\(\mu\)m sections, \(\times 20\). Scale bar (black) = 100 \(\mu\)m.

**Figure 5.** Phenotype characterization via immunofluorescence. Constrained (B,E) and constrained/stretched (C,F) samples express \(\alpha\)-SMA (top row) and h-calponin (bottom row) suggesting a differentiation toward the SMC phenotype (\(n = 4\)). None of the markers were found in unconstrained controls (A,D) or in cells prior to stimulation (insets). Arrows indicate direction of stress/strain, Blue = nuclei, Red = \(\alpha\)-SMA/h-Calponin, Confocal images on intact constructs, Magnification = \(\times 40\). Scale bar (white) = 10 \(\mu\)m.
cal stimulation is needed to maintain the contractile phenotype in SMCs suggests that biochemical stimulation alone might not be enough to differentiate BMPCs to functional SMCs.

Our observation that BMPCs play an active role in compacting and aligning the ECM is consistent with similar studies using SMCs, which changed their morphology from polygonal to elongated when cultured under constrained and constrained/stretch conditions within 3D collagen lattices. It has also been shown that SMCs in 3D culture respond to mechanical stimulation by aligning parallel to the direction of the strain, by increasing collagen production and by a reduction in proliferation. Furthermore, the static stress induced by compaction has been observed to increase functionality in 3D cultured SMCs. Several studies have used collagen gels for studying cells in 3D cultures. Recently, fibrin gel has shown promising features for tissue engineering applications exhibiting better mechanical properties and increasing matrix production by entrapped SMCs.

A limitation of the present study is that our assessment of collagen production and the expression of SMC markers were only qualitative in nature, and therefore we were unable to observe the possible significant differences of these between the experimental groups. Studies that include gene and protein expression analysis to quantify the effects of different strain magnitudes and the synergistic effect of biochemical and chemical stimulation would overcome this limitation. The fact that these cells require a high serum content in the media for their culture prevents us to conclude whether the differentiation is due to the mechanical forces alone or to a synergistic effect with the serum. However, the paired nature of this study, where all samples were treated with the same culture conditions, allows us to conclude that the mechanical forces play a significant role in the differentiation pathway. Although our findings suggest that mechanical stimulation may induce differentiation toward a SMC phenotype in 3D culture, it is likely that a combination of biochemical and mechanical factors will be necessary to achieve a fully contractile SMC phenotype from BMPCs, where full expression of contractile markers such as MHC and functional properties such as contraction upon chemical stimulation would be desired. The effect of mechanical forces on fibrin degradation was not assessed in this study, nor its correlation to the final cell phenotype, but the use of aminohexanoic acid as a fibrinolysis inhibitor to reduce that phenomenon and the fact that final volumes were similar in all samples suggests that there were no dramatic differences in degradation rates. The strain rate was chosen based on the physiologic strain that blood vessels undergo during the cardiac cycle and on our previous observations in monoculture studies. This physiologic strain is thought to play an important role in the homeostasis and phenotype of vascular SMCs. The mechanism by which cells respond and adapt to mechanical forces is still under investigation. Several mechano-transduction studies have recently shown how the cells can sense different mechanical stimuli (through ion channels and tyrosine kinase receptors) to activate second messenger proteins (cAMP, cGMP, ATP, GTP, NO, and PGE2) through integrin linkage thus translating a mechanical stimulus into a biochemical signal that exerts effects on DNA and phenotype expression.

In summary, we have shown here that the mechanical stimulation of BMPCs within a 3D matrix, either with static-stress or 10% cyclic stretch, may contribute to the differentiation of BMPCs toward a SMC phenotype. This is, to our knowledge, the first investigation incorporating BMPCs within a fibrin matrix, showing their ability to arrange, compact, and yield a tissue-like structure. Furthermore, this is the first evidence suggesting that mechanical stimulation induces morphological and phenotypical changes toward SMCs in BMPCs within a 3D model. These findings are of significant relevance for tissue engineering, particularly in the design of bioreactors that should provide the adequate mechanical environment to help differentiate and maintain the desired phenotype in situ.

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