Original Article

Analysis of Alterations in Morphologic Characteristics of Mesenchymal Stem Cells by Mechanical Stimulation during Differentiation into Smooth Muscle Cells

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Abstract

Objective: Mesenchymal stem cells (MSCs) can be expanded and differentiated into many mature cell types including smooth muscle cells (SMCs). In addition to growth factor, cyclic stretch contributes to differentiation of stem cells. Mechanical stimuli are critical to morphological changes, development, regeneration, differentiation and pathology of mesenchymal tissues. The aim of this study is to investigate effects of cyclic stretch with differing amplitudes on morphology and differentiation of mesenchymal stem cells.

Materials and Methods: Mesenchymal stem cells are extracted from human bone marrow. Cells are cultured on silicone membrane and exposed to cyclic stretch by a custom made device. Cellular images are captured before and after tests. Effects of 5% and 15% uniaxial strain with 1Hz frequency and 1-8 hour durations on morphology of human mesenchymal stem cells are investigated. It is assumed that environmental factors such as mechanical loading regulate MSCs differentiation to SMCs. Fractal analysis is used to quantify alterations in cellular morphology. An image processing method with a designed code is used for evaluation of fractal dimension parameter.

Results: Results demonstrate statistically significant change in cell morphology due to mechanical stretch. By elevation of strain amplitude and number of load cycles, fractal dimensions of cell images decrease. Such decrease is equivalent to alignment of cells by mechanical stimulus. Cells are differentiated to SMCs purely by cyclic stretch. The initiation and rate of differentiation depend on mechanical conditions.

Conclusion: To produce functional SMCs for engineered tissues, MSCs can be exposed to uniaxial cyclic stretch. The functionality of differentiated SMCs depends on loading conditions.

Keywords: Mesenchymal Stem Cells,Differentiation, Smooth Muscle Cells

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Introduction

Bone marrow mesenchymal stem cells (MSCs) are capable of self-renewal and can differentiate from unspecialized cells into multiple non-hematopoietic cell lineages, under appropriate conditions (1-3). Studies have shown that MSCs are able to differentiate to cells of various connective tissues such as adipocytes, osteocytes, skeletal myocytes, cardiomyocytes, fibroblasts, chondrocytes, neurons, tenocytes and bone marrow stromal cells (4-8).

Cells and tissues are subjected to micro-environmental factors such as mechanical strain in vivo; hence it is essential to understand effects of mechanical strain on regulation of cell differentiation and functions (9, 10). The most important aim of tissue engineering is to construct tissues in vitro and maintain their functions after implantation. This is achieved by mimic the biophysical conditions that a tissue experiences in vivo including mechanical loadings (11, 12).

Mechanical loading can affect cell phenotype (9), morphology (9, 13), gene expression (11), mechanical properties (12), signal transduction (14), migration (15), receptor activation and regulation of cell functions (16-19). Cyclic uniaxial strain applied on elastic substrates causes change in cell orientation and alignment (20). Cells show a tendency to align perpendicularly to the strain axis to minimize the amount of stress applied on cell bodies (21). Previous results have shown that cyclic uniaxial strain can cause activation of mechanotrasduction cascades involved in the differentiation of mesenchymal stem cells, generation of smooth muscle cells (SMCs), and regulation of matrix molecule expression without changing the expression of cartilage and bone differentia-

tion markers such as collagen II and alkaline phosphatase (16, 22). Such loading can be used to construct tissue engineered vascular grafts for blood vessel replacement. It has been reported that by transplantation of MSCs into the heart, they may differentiate into SMCs and contribute to vascular remodeling (23, 24).

It has been shown that application of uniaxial cyclic stretch causes differentiation of MSCs to SMCs (16, 21). Such effect has been done without introducing growth factors to culture medium. Different loading parameters have been used for differentiation of MSCs such as number of cycles and strain amplitude. Uniaxial strain amplitudes in the range of 5%-20% and test durations up to seven days have been used for application of cyclic stretch on mesenchymal cells (16, 21, 25). Results have shown significant differentiation of MSCs to SMCs; however, differentiated cells have not been quantitatively compared based on their characteristics.

The aim of this study is to quantify morphological changes and consequent differentiation of mesenchymal stem cells caused by cyclic tensile stretch. It is assumed that environmental factors such as mechanical loading regulate differentiation of MSCs to SMCs. This leads to achievement of different differentiated SMCs depending on loading conditions. Functionally, proper differentiated SMCs are essential in tissue engineering. The cyclic loading is applied utilizing a custom-made device to impose uniform tensile strain on cultured cells in a specific range of load amplitude and number of cycles. A new digital image analysis method is described based on fractal parameters to evaluate morphological changes of mesenchymal stem cells before and after cyclic loading. The resultant alterations of morphology of MSCs and differentiated SMCs are quantified by fractal analysis to present possibility of achievement of differentiated cells with different functionalities. Functional differentiated cells can contribute to reconstruction of damaged tissues.

Materials and Methods

MSCs isolation and analysis of smooth muscle differentiation by immunostaining

Human bone marrow mesenchymal stem cells were isolated from bone marrow aspirates of 10-20 ml taken from the iliac crest of patients. Ethical approval had been obtained for this study by Iranian Blood Transfusion Organization. The Ficoll-Paque technique of density gradient centrifugation was used by first diluting the bone marrow sample with cell culture medium. Then, the resulting solution was poured on the top of the separation medium (Ficoll-Paque solution of 1.077 g/ml density) in a 50 ml centrifuge tube and centrifuged at room temperature, at 445×g, for 35 minutes. The majority of the mononucleated cells accumulated on the Ficoll-Plasma interface and was resuspended in complete culture medium.

The capability of hMSCs to differentiate under test conditions was studied by surface markers. For immunofluorescence staining, cells were washed in PBS twice and then fixed in 4% paraformaldehyde for 15 minutes. Then, cells were permeabilized with 0.5% Triton X-100 for 10 minutes, followed by further washings with PBS. For SM α-actin staining, each sample was incubated for 2 hours with mouse a-actin antibody at 1:100 dilutions in PBS, followed by incubation for 1 hour with FITC-conjugated secondary antibody at 1:50 dilution. Preparations were examined using fluorescence microscopy.

MSCs and application of cyclic strain

Stem cells from passages three to seven were used for exposure to cyclic stretch tests with 80-90% confluency. The culture medium used was Dulbecco's modified Eagle's (Gibco, USA) supplemented with 10% fetal bovine serum (sigma, USA), 100 IU/mL penicillin (Sigma, USA), and 100 µg/mL streptomycin (Sigma, USA) at 37 °C in a humidified incubator containing 5% CO_2. The culture medium was replaced with fresh medium twice a week. Medical grade silicon elastic membranes (0.25mm thick) obtained from Iran Polymer and Petrochemical Institute were used as the substrate of cultured cells. Before cell seeding, the central 6×6 mm region of a 50×15 mm silicone membrane was coated with 0.1 ml of 0.5 mg/ml collagen type I (Sigma, USA) for the required cell attachment (26-28). Membranes were washed with phosphate buffer saline (PBS) and exposed to UV for 30 minutes to sterilize the coated surface, then MSCs were cultured on the membranes. After 24 hours of culture, membranes were attached to the uniaxial strain device and stretched according to the loading protocol.

To apply axial cyclic load on the membrane, a tensile device was designed and manufactured for operating in a wide range of load amplitude and frequency. The device contained mechanical and electrical units and was capable of operation in an incubator (Fig 1). The electrical unit contained a PLC + HMI, a stepper motor driver, and a power supply with a designed program for data acquisition. A special program was designed for manual and automatic operation modes. For initial adjust-

ment, the manual mode was used. The operational conditions were set by the designed software after setting zero point of movement. The conditions include initial length of sample, applied strain, loading frequency and number of load cycles. The operational mode was then shifted to automatic mode for the entire experiments.
The mechanical unit included a step motor, a ballscrew, a connector rod, an encoder, and mobile and fixed grippers. When the specimen was placed in the fixed gripper, it was stretched by the moving gripper according to the required test conditions, programmed by the designed software.

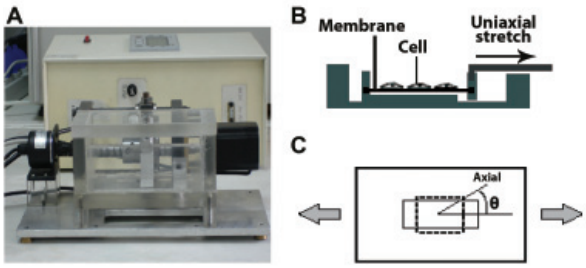

Fig 1: (A) Stretch device (B) Schematic representation of uniaxial stretch device (C) Schematic representation of uniaxial stretch of the membrane

Quantified analysis of cell morphology

Alterations in the morphology of cells were quantified using images of cultured cells captured by an invert microscope and a digital camera before and after tensile tests. The resultant images were processed by design and development of an image analysis code in MATLAB 7.4 image processing toolbox. The output data of the program were quantities describing morphology of cellular images.
The image processing algorithm initially included conversion of images to a grayscale format. Then, the resultant image was filtered to reduce excessive noise. After application of a gradient detection algorithm, binary thresholding was performed on the gradient image by selecting its mean gray value as the threshold quantity (29). Finally, a geometric filter was applied to remove remaining artifacts.
To quantify morphological alignment of cells, fractal dimensions of cellular images were calculated. Fractal dimension (D) is a quantitative descriptor of complexity of an image (30, 31). In this study, the fractal dimension (D) was obtained using "box-counting" method; a length related approach which measured the distances between points on the border of the skeleton of the binary image (32, 33). Sets of square boxes with differing box size were used to cover the entire image border and for each set, the number of boxes was counted. The fractal dimension (D) was defined as the slope of the line, relating the log of box size and the log of box count (33, 34). Previous studies reported fractal dimension values in the range of 1.2-1.4 for images of cellular networks (31, 33, 34).

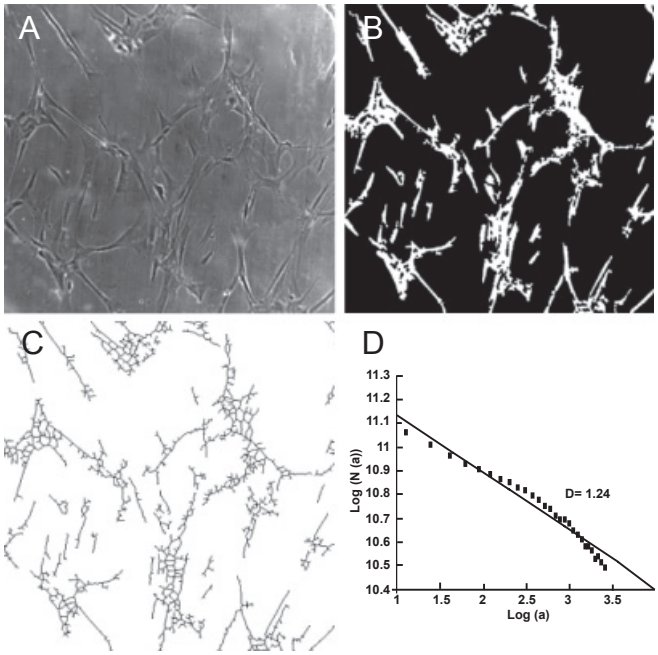

Fig 2: Image processing and fractal analysis procedure of human MSCs: (A) grayscale of original image, (B) binary threshold image, (C) skeleton of cell network, (D) calculation of fractal dimension as the slope of the curve.

To generate the skeleton of the image, binary thinning was used. The remaining artifactual branches of the skeleton were removed by pruning the image (29). The image processing code was used for the consequent image generation and calculation of fractal dimension. Fig 2 shows typical image processing steps for calculation of fractal dimension of images of mesenchymal stem cells.

Quantified analysis of cell differentiation

Alterations of alignment-differentiation of cells induced by application of cyclic load were modeled by polynomial curve fitting of resultant morphological data. Such curves are produced for tests with two load amplitudes. The resultant curves quantify trend of cell differentiation with test duration.

The human mesenchymal stem cells were cultured on the medical grade silicon membrane. The membrane was placed into test grippers and the test conditions are set by the software. The tank was filled with culture medium before the test. Then the loading apparatus was placed into the incubator for application of cyclic stretch on the membrane and cultured cells. Cyclic tensile tests were performed for different stretch amplitudes and test durations as the variables of this study. Before and after tests, images of cells on the membrane were captured and processed by the designed image processing code to calculate fractal dimension parameter.

The resultant fractal parameters for each variable were calculated as mean values obtained from individual tests. Data are expressed as mean ± standard deviation (SD). For each data point of each variable, four tests were performed and from each test, an image of mesenchymal stem cell network was captured and divided into four zones. For each zone, four randomly chosen fields of the images were analyzed and the average values and standard deviations of resultant parameters were calculated. Therefore, for every value of each variable, four tests were performed and for each test, sixteen sub-images are analyzed and D values were obtained, accordingly.

Statistical differences in mean values of fractal dimension between before and after test samples were analyzed for different values of load amplitude and number of cycles (test duration). The fractal dimensions of samples were compared utilizing one-way analysis of variances (ANOVA) among test groups. To analyze significance of differences among mean values of resultant parameters, probability values were calculated by alteration of variables. The significance of differences between mean values was justified at p-value less than 0.05.

Results

MSCs isolation and analysis of smooth muscle differentiation by immunostaining

Human bone marrow mesenchymal stem cells were isolated from bone marrow and its capability to differentiate under test conditions was studied by surface markers (data not shown).

The differentiation of MSCs is examined by fluorescence staining and fluorescent microscopy. Uniaxial strain significantly up-regulates the gene expression of α-actin (Fig 3A), but no α-actin expression is observed in control cells (Fig 3B). This response depends on strain magnitude since α-actin expression is reduced in samples exposed to lower strain amplitude.

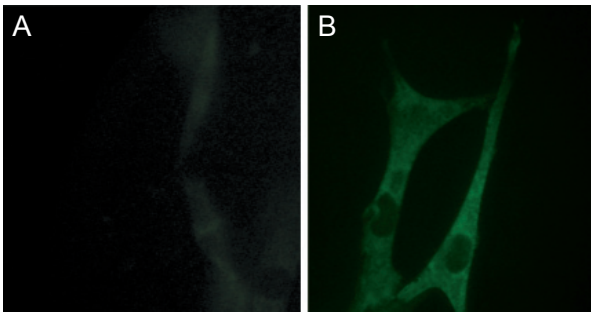

Fig 3: Immunofluorescent evidence of α-smooth muscle actin (ASMA) expression upon cyclic mechanical tensile loading of 10% at 1 Hz: (A). ASMA expression in control cells after 24 hours (B). ASMA expression in cells exposed to 24 hours uniaxial strain (original magnification ×63).

MSCs and application of cyclic strain

Comparison between images of MSCs before and after tests indicates alignment, rearrangement and elongation of cells after cyclic stretch before differentiation. Figure 4 shows the elongation and reorientation of typical samples of MSCs after two hour cyclic stretch using binary images. As can be seen, application of cyclic stretch causes mesenchymal cells to align in such way that the average value of orientation angle increases from 34° before test to almost 50° after application of cyclic stretch. Such angle indicates the average value of angles between cells axis and the axis perpendicular to the loading axis (13).

Results show that during application of cyclic stretch, stem cells undergo differentiation after certain time intervals, depending on the loading conditions. Such differentiation occurs without addition of growth factor; and its rate and magnitude depend on the load duration and amplitude.

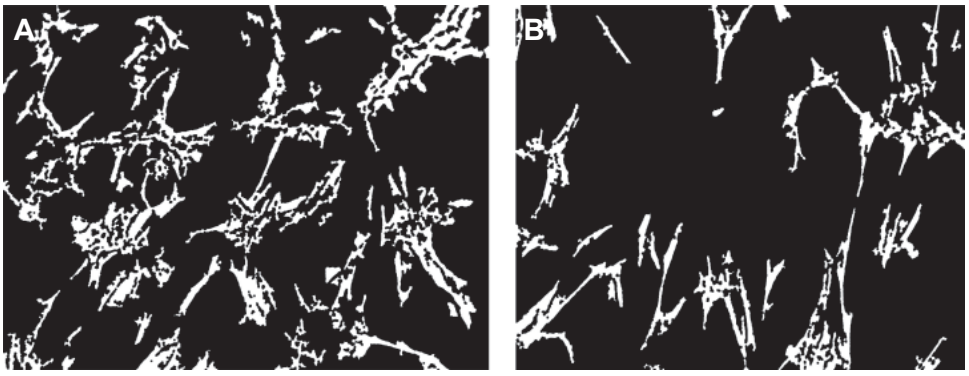

Fig 4: Orientation angle of cells (A) Before test (Θ=33.8) (B) After test (Θ=49.8)

Quantified analysis of cell morphology

Figures 5 and 6 show resultant average and standard deviation values of fractal dimension for different test durations, frequency of 1Hz, and strain amplitudes of 5% and 15%, respectively. Results show initial reduction of fractal dimension by application of cyclic load. However, after preliminary alignment of cells by cyclic loading, stem cells undergo differentiation to smooth muscle cells demonstrated by immunofluorescence staining. After this stage, the fractal dimension increases describing the generation of new cells which are not aligned, initially. Further loading aligns the newly generated smooth muscle cells.

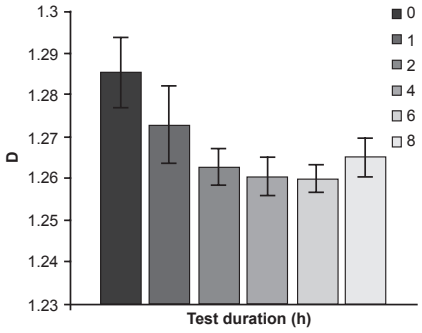

Fig 5: Effects of differing number of cycles (test duration) on fractal dimension with frequency of 1 Hz and 5% strain.

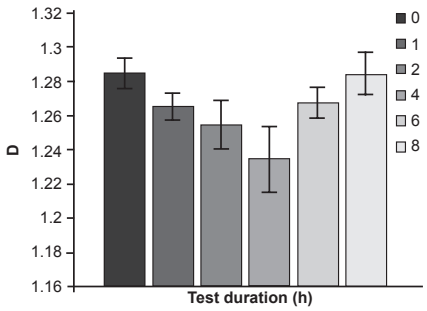

Fig 6: Effects of differing number of cycles (test duration) on fractal dimension with frequency of 1 Hz and 15% strain.

Effect of load duration parameter on cell morphology is statistically significant. The calculated p values for comparison of mean values of fractal dimension before and after test groups are 0.001.

Quantified analysis of cell differentiation

Different cells are under various loading conditions in vivo and application of mechanical loading with variables such as load amplitude facilitates production of such cells. Figure 7 shows mesenchymal stem cells and two samples of differentiated smooth muscle cells with strain amplitude of 5% and 15%, respectively.

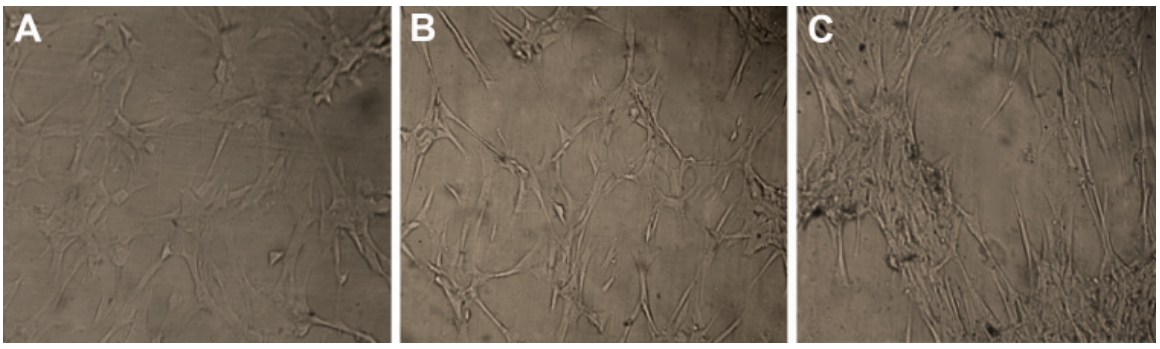

Fig 7: (A) Mesenchymal stem cells on silicon membrane (B) Differentiated smooth muscle cells with strain amplitude of 5% (C) Differentiated smooth muscle cells with strain amplitude of 15%.

The elevated strain amplitude leads to acceleration of cell differentiation and generation of SMC colony compared to isolated SMCs differentiated by lower strain amplitude.
Figure 8 indicates comparison of alignment-differentiation for test results with two load amplitudes by polyominal curve fitting. The goodness of fitting is determined by R^2 value. Polynomial curves with R^2 values of 0.937 and 0.905 are best fitted for 5% and 15% strain groups, respectively. Immunofluorescence staining indicates that minimum points on the curves demonstrate the initiation of differentiation of MSCs. Results emphasize the importance of strain amplitude on stem cell differentiation. Data indicate that for constant number of cycles, differentiation occurs earlier in higher strain amplitude. The final points on graphs indicate different states of differentiated cells based on different values of fractal dimension.

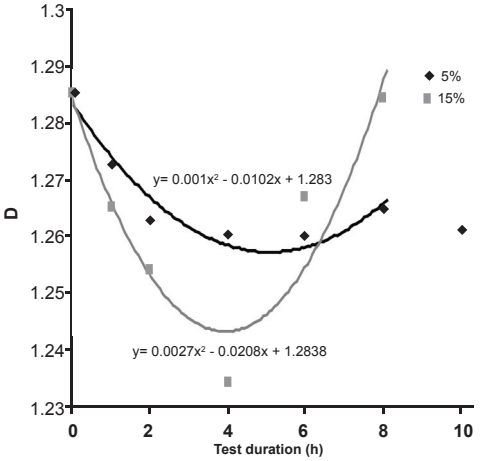

Fig 8: Dependency of stem cell alignment and differentiation on stretch amplitude.

Discussion

Effects of mechanical loading on MSCs and their differentiation to SMCs were studied. Results showed that application of cyclic stretch with different loading conditions leads to SMCs with different morphologies, and consequently with different functionalities. This indicates ability of achievement of different engineered SMCs by mimic physiological environment in which cells are exposed. In tissue engineering, it is required that produced cells are functionally similar to those *in vivo*.
Since smooth muscle cells of arterial wall media are functionally under physiological stretch caused by pulsatile pressure, it is hypothesized that such loading is essential for production of engineered SMCs *in vitro*. To obtain functional SMCs, mesencymal stem cells were differentiated to SMCs by exposing to uniaxial cyclic stretch. The morphology of MSCs and differentiated SMCs, as indicator of their functionality, was analyzed using fractal analysis.
MSCs were aligned after loading. Such alignment results in decrease of complexity of the cell network and consequent reduction of fractal dimension. Such effect has been extensively shown in other cell types under cyclic stretch (35, 36). Elevation of number of cycles (test duration) and load amplitude lead to further alignment of cells, similar to the trends reported by studies on other cell types (13). The difference in morphology of cells was shown to be statistically significant when the applied cyclic load is different in amplitude and duration. Such process leads to achievement of MSCs with differing morphology. Since in biological processes, the form of cells follows their functions, the functionality of stem cells differs with alteration of loading conditions. This process gives the possibility of production of differentiated SMCs with optimized function.
MSCs exposed to cyclic stretch were differentiated in the absence of growth factors. Such result has also been reported by previous studies with different strain amplitudes and durations (16, 21, 25). However, the differences among differentiated cell have not been quantified. The differentiation time depends on loading duration and amplitude. Such differentiation is merely due to cyclic loading. Since loading conditions can be altered according to experimental protocol, different muscle cells can be obtained from identical stem cells. Such phenomenon has been shown in this study by quantification of morphology of differentiated cells (Fig 8). In future research, the differences among functionality of differentiated cells can be quantified utilizing biological tests.
When cyclic stretch is not applied, cells show no particular orientation. After applying uniaxial loading, cells show a tendency to align perpendicularly to the direction of strain axis. This is an adaptation process of the cells to minimize the amount of stresses applied on cell bodies, and to reduce the stretch of actin fibers and other skeletal networks (25, 37). By generation of stress fibers and alignment of structural filaments, cells become elongated and aligned with respect to neighboring cells. Such process affects cell to cell contact and ability of the cell colony to generate a functional tissue. To obtain optimized engineered cells and consequent tissues, consideration of the physico-mechanical environment is essential.

Conclusion

Blood vessels are constantly exposed to hemodynamic forces and pulsatile pressure. Such pulsatile

pressure causes wall stretch and cyclic loading on arterial smooth muscle cells. Since cyclic loading is essential to the function of smooth muscle cells, mechanical stimuli might be of great importance in differentiation of stem cells into smooth muscle cells. Results show that cyclic tensile stretch influences cell morphology and alignment as a mechanism for matrix remodeling, functional adaptation, and differentiation to smooth muscle cells (SMCs). Inclusion of cyclic stretch in differentiation of stem cells to smooth muscle cells simulates the biophysical environment in vitro that SMCs are likely to experience in vivo. The results of this study can be applied in generation of engineered functional tissues from development of stem cells.

Acknowledgments

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