



Relative impact of form-induced stress vs. uniaxial alignment on multipotent stem cell myogenesis

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ARTICLE INFO

Article history:

Received 15 September 2011

Received in revised form 7 April 2012

Accepted 21 June 2012

Available online 11 July 2012

Keywords:

Smooth muscle lineage progression

Multipotent stem cells

Uniaxial alignment

Form-induced stress

Hydrogel patterning

ABSTRACT

Tissue engineering strategies based on multipotent stem cells (MSCs) hold significant promise for the repair or replacement of damaged smooth muscle tissue. To design scaffolds which specifically induce MSC smooth muscle lineage progression requires a deeper understanding of the relative influence of various microenvironmental signals on myogenesis. For instance, MSC myogenic differentiation has been shown to be promoted by increases in active RhoA and FAK, both of which can be induced via increased cell–substrate stress. Separate studies have demonstrated MSC myogenesis to be enhanced by uniaxial cell alignment. The goal of the present study was to compare the impact of increased peak cell–substrate stresses vs. increased uniaxial cell alignment on MSC myogenic differentiation. To this end, MSC fate decisions were compared within two distinct multicellular “forms”. A “stripe” multicellular pattern was designed to induce uniaxial cell alignment. In contrast, a second multicellular pattern was designed with “loops” or curves, which altered cell directionality while simultaneously generating regional peak stresses significantly above that intrinsic to the “stripe” form. As anticipated, the higher peak stress levels of the “loop” pattern were associated with increased fractions of active RhoA and active FAK. In contrast, two markers of early smooth muscle lineage progression, myocardin and SM- α -actin, were significantly elevated in the “stripe” pattern relative to the “loop” pattern. These results indicate that scaffolds which promote uniaxial MSC alignment may be more inductive of myogenic differentiation than those associated with increased peak, cell–substrate stress but in which cell directionality varies.

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1. Introduction

Regenerative medicine is a potential approach to repairing or replacing diseased or damaged smooth muscle tissue when current treatment methods fail. In particular, tissue engineering strategies based on multipotent stem cells (MSCs) hold significant promise owing to the greater regenerative capacity of MSCs relative to adult, differentiated muscle cells. However, the design of scaffolds with properties which “optimally” promote MSC lineage progression toward smooth muscle fates requires a deeper understanding of the relative influence of various microenvironmental signals on myogenesis than currently exists.

Recent studies have shown that multicellular organization or form is a critical determinant of MSC fate decisions [1]. Different multicellular forms appear to influence cell behavior in part via the distinct cell–substrate stress patterns supported by particular

forms [1,2]. Indeed, increased cell–substrate stress has been associated with higher levels of active RhoA [2] and activated focal adhesion kinase (FAK) [3], both of which have been linked to increases in muscle-specific gene expression [4–6]. Separate studies have demonstrated that multicellular forms that promote uniaxial cell alignment induce MSC expression of muscle-specific genes [7]. The present study was therefore designed to compare the impact of increased form-induced stress with that of increased uniaxial cell alignment on MSC smooth muscle lineage progression.

To this end, the fate decisions of mouse NIH/3T3 MSCs were compared within two distinct multicellular forms. Although NIH/3T3 cells are commonly referred to as “fibroblasts”, they were derived from the murine embryonic mesoderm and have the capacity to differentiate into a range of cell types, including adipocytes [8,9], muscle cells [10,11] and osteoblasts [12]. These cells were seeded onto either “stripe” or “loop” patterns formed on substrates of “myogenic” stiffness. The “stripe” pattern was designed to induce uniaxial cell alignment. In contrast, the “loop” pattern was designed with curves intended to alter cell directionality while simultaneously generating peak, cell–substrate stresses significantly above

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that intrinsic to the “stripe” form. In fabricating these “stripe” and “loop” patterns, poly(ethylene glycol) (PEG)-based hydrogels were employed. Diacrylate-derivatized PEG (PEGDA) hydrogels intrinsically resist protein adsorption and cell adhesion while enabling regional patterning of cell adhesive ligands using transparency-based [13] or projection-based [14] photolithography. In addition, in contrast to the glass surfaces often used in analyzing pattern influence on cell behavior, PEGDA hydrogels can be readily prepared with moduli within the “myogenic” range (≈ 15 – 45 kPa) identified for mouse progenitor cells [15].

Confluent MSCs were cultured in the absence of growth factors within the “stripe” and “loop” patterns. After 24 h, cells were analyzed for differences in early lineage markers as well as for fractional levels of active RhoA and active FAK (pFAK). Results suggest that scaffolds that promote uniaxial MSC alignment may be more inductive of myogenic differentiation than those associated with increased peak, cell–substrate stress but in which cell directionality varies.

2. Materials and methods

2.1. Synthesis of PEGDA

PEGDA was prepared as previously described by combining 0.1 mmol ml^{-1} dry PEG (3.4 kDa; Fluka), 0.4 mmol ml^{-1} acryloyl chloride and 0.2 mmol ml^{-1} triethylamine in anhydrous dichloromethane and stirring under argon overnight [16]. The resulting solution was washed with $2 \text{ M K}_2\text{CO}_3$ and separated into aqueous and organic phases to remove HCl. The organic phase was subsequently dried with anhydrous MgSO_4 , and PEGDA was precipitated in diethyl ether, filtered and dried under vacuum. The extent of PEG diacrylation was determined by $^1\text{H-NMR}$ as $\approx 90\%$.

2.2. Synthesis of fluorescently labeled, acrylate-derivatized cell adhesion ligand

Cell adhesion peptide $\text{NH}_2\text{-Arg-Gly-Asp-Ser-COOH}$ (RGDS; American Peptide) was conjugated to PEG (3.4 kDa) by reaction with acryloyl-PEG-*N*-hydroxysuccinimide (ACRL-PEG-NHS; Nektar) at a 1:1 M ratio for 2 h in 50 mM sodium bicarbonate buffer, pH 8.5. Alexa Fluor 488 carboxylic acid, tetrafluorophenyl ester (Invitrogen) was then added to the ACRL-PEG-RGDS reaction mixture at $\approx 5 \text{ mol dye per mol RGDS}$ and allowed to react for 1 h at room temperature [17]. The desired product was purified by dialysis and then lyophilized. The resulting fluorescently labeled, photoactive RGDS moieties were subsequently covalently linked to specific regions of PEGDA hydrogel surfaces using photopatterning.

2.3. Pattern design and finite element modeling of form-induced stress

The desired “stripe” and “loop” patterns were prepared using Photoshop (Fig. 1A and B), and the cell–substrate stress distributions induced by the two patterns were modeled using COMSOL 4.0a per Nelson et al. [2]. In brief, three-dimensional finite element models of the patterned multicellular “forms” were constructed with two components (a contractile layer and a passive layer) and using previously reported physical parameters [2]. Both the contractile and passive layers had lateral dimensions set by the pattern form (Fig. 1A and B) and heights of $20 \mu\text{m}$ and $4 \mu\text{m}$, respectively [2]. The contractile “cell mimetic” layer was treated as an isotropic elastic material with a Young’s modulus of 500 Pa , a Poisson’s ratio of 0.499 (incompressible), a thermal conductivity of $10 \text{ Wm}^{-1} \text{ K}^{-1}$, and a coefficient of expansion of 0.05 K^{-1} . The passive layer was treated as an isotropic elastic material with a modulus of 100 Pa and a Poisson’s ratio of 0.499 . The passive layer was intended to represent the properties of the layer of pendant PEG-RGDS chains patterned onto the hydrogel surface (Supplementary Fig. S1A). In both the “stripe” and “loop” models, the bottom surface of the passive layer was mechanically fixed in order to approximate the greater rigidity of the hydrogel surface ($\approx 35 \text{ kPa}$) relative to patterned PEG-RGDS chain layer.

For each geometry, a free quadrilateral mesh network was formed for the upper surface, with a mesh density corresponding to a spacing of 4 – $10 \mu\text{m}$ per node. This mesh was then mapped to the passive and contractile layers (Supplementary Fig. 1B). To simulate cell monolayer contraction, a thermal strain was prescribed by imposing a temperature drop of 5 K , i.e. the top surface of the contractile layer was set to 305 K , while the remaining structure was set to 310 K (37°C). Stress and strain tensors were calculated throughout the structures via steady-state heat and structural dynamics balances. The maximum principal stress at each of the bottom, fixed surface of the passive layer was reported [2]. Convergence of results was confirmed using multiple mesh densities and a range of physical parameters for model layers.

To calculate the average maximum principal stress associated with each pattern, the mean of the stresses at the nodes of a uniform grid was calculated for each of the geometries. To accomplish this, the solution for the maximum principal stress at each of the quadrilateral mesh points of the bottom surface of the passive layer was imported into MATLAB. The MATLAB “griddata” function was used to interpolate the values of the COMSOL solution at grid points of a uniform mesh with a grid spacing of $0.125 \mu\text{m}$.

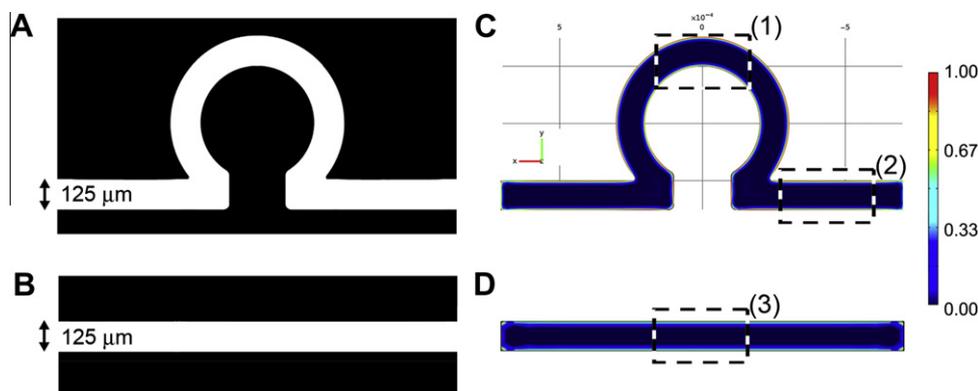


Fig. 1. (A) “Loop” and (B) “stripe” patterns designed in Photoshop, and the corresponding maximum principal stress distributions associated with the (C) “loop” and (D) “stripe” forms per finite element modeling. The regions of (C) and (D) labeled (1), (2) and (3) are examined further in Fig. 2. The color scale applies to images (C) and (D), with red-to-blue indicating a transition from high stress to relatively low stress.

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