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Increased mechanosensitivity of cells cultured on nanotopographies

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Abstract

Enhancing cellular mechanosensitivity is recognized as a novel tool for successful musculoskeletal tissue engineering. We examined the hypothesis that mechanosensitivity of human mesenchymal stem cells (hMSCs) is enhanced on nanotopographic substrates relative to flat surfaces. hMSCs were cultured on polymer-demixed, randomly distributed nanoisland surfaces with varying island heights and changes in intracellular calcium concentration, $[Ca^{2+}]_i$, in response to fluid flow induced shear stress were quantified. Stem cells cultured on specific scale nanotopographies displayed greater intracellular calcium responses to fluid flow. hMSCs cultured on 10-20 nm high nanoislands displayed a greater percentage of cells responding in calcium relative to cells cultured on flat control, and showed greater average $[Ca^{2+}]_i$ increase relative to cells cultured on other nanoislands (45-80 nm high nanoislands). As $[Ca^{2+}]_i$ is an important regulator of downstream signaling, as well as proliferation and differentiation of hMSCs, this observation suggests that specific scale nanotopographies provide an optimal milieu for promoting stem cell mechanotransduction activity. That mechanical signals and substrate nanotopography may synergistically regulate cell behavior is of significant interest in the development of regenerative medicine protocols.

Keywords

Fluid flow; stem cells; mechanotransduction; nanotopography; regenerative medicine

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Supporting Information Available. Supporting Information is provided to describe details of nanotopography characterization.

Conflict of interest statement. Authors have no conflicts of interest.

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1. Introduction

The use of mechanical signals is one of the key parameters in functional tissue engineering (Guilak, 2002). Stem cells sense and respond to mechanical signals by increasing their proliferation, lineage commitment, and differentiation (Liu et al., 2010). Mechanical signals from stretching or shear stress have been demonstrated to play an important role in determining skeletal and stem cell functions (Arnsdorf et al., 2009; Gurkan and Akkus, 2008; Haasper et al., 2008; Qi et al., 2008). We have demonstrated the stimulatory effects of shear stress on the proliferation and differentiation of osteoblastic and stem cells (Genetos et al., 2007; Riddle et al., 2006; 2007; 2008; Taylor et al., 2007). Enhancing cellular reactivity to mechanical signals is emerging as a novel way to achieve successful regeneration of mechanically functional tissues (bone, cartilage, muscle, etc.). In this study, we demonstrate that culturing cells on nanotopographically modified surfaces is a novel way to promote cellular mechanotransduction activity.

Fabrication of nanotopographies for modulation of cell functions has recently been a key focus in bioengineering (Bettinger et al., 2009). Micro- and nanoscale, ordered and random topographies have been produced for this purpose (Dalby et al., 2006; Graeter et al., 2007). We have shown that osteoblastic and stem cells cultured on nanotopographies display enhanced cell attachment, increased focal adhesion, e.g., greater focal adhesion kinase (FAK) and its phosphorylation at pY397, enhanced integrin α_v , increased cellular modulus, and directed stem cell differentiation (Hansen et al., 2007; Lim et al., 2005a; 2005b; 2007; Salvi et al., 2009). As a proof-of-concept experiment for cell-substrate interaction dependent mechanotransduction, we assessed cellular response to fluid flow on nanotopographies focusing on changes in intracellular calcium concentration, $[Ca^{2+}]_i$, as $[Ca^{2+}]_i$ is a characteristic early-stage mechanosensitive indicator of cells under shear stress cues.

2. Materials and Methods

2.1. Nanotopography

We produced a novel set of nanoisland topographies by modifying the methods used in our previous publication (Lim et al., 2005b). Poly(L-lactic acid) (PLLA, $M_w = 50 \times 10^3$, Polysciences) and polystyrene (PS, $M_w = 289 \times 10^3$, Sigma Aldrich) were used to make polymer-demixed topographies with varying nanoisland height. Details of nanotopography fabrication and topography/chemistry characterization are reported in 'Supporting Information'.

2.2. Cell Culture

Human mesenchymal stem cells (hMSCs, Cambrex PT-2501) were cultured using growth media composed of Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% L-glutamine. For tests, cells were cultured on nanotopography covered quartz slides at 4×10^3 cells/cm². For sterilization, test surfaces were exposed to UV light.

2.3. Fluid flow stimulation

After culturing hMSCs on test surfaces, the medium was changed to low serum (0.5%) flow media on day 2. Before applying fluid flow on day 3, cells were exposed to calcium-sensitive Fura Red-AM dye (Invitrogen, F3021) at 10 μ M for 30 min. By using oscillatory fluid flow within a parallel plate flow chamber, we applied shear stresses of 5, 10, and 20 dyne/cm², which fall within the physiologically relevant shear stress levels (Weinbaum et al., 1994). The wall shear stress (τ) was adjusted by changing the volumetric flow rate ($\tau = 6Q\mu/bh^2$, where Q is the volume flow rate, μ is the media viscosity of 0.01 dyne·s·cm⁻², and

b and h are the width and height of the chamber). Volume flow rates of 4.5, 9, and 18 ml/min, monitored in real-time with an ultrasonic flowmeter, correspond to shear stresses of 5, 10, and 20 dyne/cm². Oscillatory flow with a physiologically relevant frequency (1 Hz) was used to mimic the loading and unloading of long bones (Weinbaum et al., 1994).

2.4. [Ca²⁺]_i quantification under fluid flow

Fluid flow was applied for 180 s after a 180 s static period, and during the entire period [Ca²⁺]_i in hMSCs was quantified using fluorescence intensity measurement with the flow chamber placed on an inverted fluorescence microscope. Consecutive images at 420 and 480 nm were recorded once every 2 s. For Fura Red-AM dye, the fluorescence intensity increases at 420 nm with increasing [Ca²⁺]_i and decreases at 480 nm with decreasing [Ca²⁺]_i. We used Fura Red-AM dye instead of Fura-2 dye, a widely used calcium indicator, to avoid the auto-fluorescence of polymer film and its interference with Fura-2. The intensity reading was analyzed by using MetaFluor image software (Universal Imaging), where the [Ca²⁺]_i in each individual cell was obtained using the ratiometric method. A total of 7-9 slides were flowed and for each slide 20-40 cells were analyzed, giving to a total of 150-300 cell assessments per condition.

2.5. Statistics

One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post-hoc tests was used at *: $p < 0.05$ and **: $p < 0.01$ when compared with flat control and #: $p < 0.05$ and ##: $p < 0.01$ when compared among nanoislands.

3. Results

We produced PLLA/PS demixed films covered with islands with varying island heights (12, 21, 45, and 80 nm, Figure 1). Polymer-demixing produces random topographic features, which is in contrast to uniform topographies produced by lithographic techniques. The average island height was almost linearly controlled as a function of the total polymer concentration in the spin-casting solution. Polymer-demixing is advantageous because of its low cost and ability to fabricate large surface areas. Producing large area (75×25 mm² used in this study) substrates via lithography is expensive. Also, the uniformity across the large area is usually not guaranteed even with the lithographies. The increase of surface area in polymer-demixed nanotextured films was less than 1.37% (see 'Supporting Information'). Another advantage of our polymer-demixing is that surface chemistry is maintained the same as PLLA, and thus the topography effect could be assessed under the same surface chemistry (also see 'Supporting Information').

Figure 2 (top) shows an example of hMSC calcium response to fluid flow on nanotopographies. Each colored line represents an individual cell response. During the static period, [Ca²⁺]_i remained at a baseline level and after induction of fluid flow a rapid and transient increase in [Ca²⁺]_i occurred. With time, [Ca²⁺]_i tends to return toward baseline levels. Each cell's fluorescent trace was analyzed as in Figure 2 (bottom). The mean and standard deviation (SD) of fluorescence for the static period were obtained, and if a cell displayed a [Ca²⁺]_i peak greater than 'mean+5SD' during the flow period the cell was defined as a responding cell. This was repeated for each cell trace. We obtained two parameters, percentage of cells responding to fluid flow with an increase in [Ca²⁺]_i and average increase in [Ca²⁺]_i among responding cells.

hMSCs on all test surfaces displayed a distinct intracellular calcium response to fluid flow when compared with cells under static conditions (Figure 3). Interestingly, at a shear stress of 5 dyne/cm², hMSCs cultured on 12 and 21 nm high nanoislands displayed a significantly greater percentage of cells responding to fluid flow compared with cells cultured on flat

substrata (almost double the number of cells displayed $[Ca^{2+}]_i$ increase). Cells cultured on larger nanoislands (45 and 80 nm) displayed a similar trend but this did not reach statistical significance. At relatively higher shear stresses of 10 and 20 dyne/cm², similar trends were found but there were no significant differences among test surfaces.

The topographic scale preference of cellular mechanosensitivity is more evident in average $[Ca^{2+}]_i$ increase (Figure 4). At a lower shear stress of 5 dyne/cm² hMSCs on 12 nm nanoislands displayed a significantly greater $[Ca^{2+}]_i$ increase compared with cells on 45 and 80 nm nanoislands (note, comparison with flat control did not reach statistical significance). Again, no significant differences were observed in $[Ca^{2+}]_i$ increase at 10 and 20 dyne/cm².

4. Discussion

Calcium signaling has been demonstrated to be critically important upstream event mediating the cellular response to mechanical stimuli including stretching or shear stress, although the exact source of the $[Ca^{2+}]_i$ increase has not been fully revealed (Duncan et al., 1994; Li et al., 2002; Riddle et al., 2007). One thing that is firmly established is that $[Ca^{2+}]_i$ increases under fluid flow and this increase mediates many important downstream signaling pathways including activation of mitogen-activated protein (MAP) kinases. Fluid flow induced calcium signaling via MAP kinase has been demonstrated to regulate cell proliferation and differentiation. Specifically, we have shown that fluid flow induced $[Ca^{2+}]_i$ upregulation triggers one of the MAP kinases, extracellular signal-regulated kinase (ERK), in hMSCs and the fluid flow triggered ERK phosphorylation promotes hMSC proliferation (Riddle et al., 2006; 2007). Keeping in mind that calcium signaling is one of the characteristic early mechanotransduction events, and as a pilot examination of cell mechanosensitivity on topographies, we chose to quantify changes in $[Ca^{2+}]_i$ as a function of the nanotopographies on which cells are cultured.

Our data (Figure 3, 4) clearly demonstrate that cells display altered sensitivity to mechanical stimuli when cultured on particular nanotopographies. The observation that approximately 10-20 nm high nanotopographies enhance $[Ca^{2+}]_i$ mechanosensitivity is particularly relevant when coupled with previous findings. Our previous data showed that cells cultured on similar scale nanotopographies (10-20 nm scale nanoisland or nanopit) exhibit increased cell attachment number and greater cell spreading relative to cells cultured on flat control and larger scale nanotopographies (~ 100 nm) (Lim et al., 2005a; 2005b). We also observed that human osteoblastic cells cultured on approximately 10-20 nm scale nanopits display enhanced FAK phosphorylation and integrin α_v expression (Lim et al., 2007). Furthermore, similar scale nanotopographies increased cellular mechanical properties, e.g., cell stiffness. We observed that murine osteoblastic cells display greater cellular elastic modulus, as assessed by atomic force microscopy (AFM) indentation, when cultured on 11 and 38 nm high nanoislands relative to cells on flat control (Hansen et al., 2007). Additionally, the comparison with the previous data partly explains the differences among the nanotopographies. Decreased cell attachment, less-well spread cell morphology, and less-well developed focal adhesion assembly observed on larger sized (~ 100 nm) nanotopographies (Lim et al., 2005a; 2005b) suggest a clue for decreased cellular mechanosensitivity (as in Fig. 4). Taken together, these data suggest that there is a positive correlation among specific scale nanotopographies, cell adhesion and cell stiffness, and mechanosensitivity. We speculate that increased cell spreading, enhanced focal adhesion, and greater cellular stiffness on specific nanotopographies induce greater cellular reactivity to mechanical signals.

The less pronounced differences on varying test surfaces at higher stress levels (10 and 20 dyne/cm²) is most likely a result of shear stresses being so large and thus effects from the

underlying nanotopography on cells may become marginal under dominating mechanical cues (“wash-out” effect). This observation, on the other hand, suggests that under circumstances where it is difficult to apply higher shear stresses, e.g., fluid flow through less porous three-dimensional scaffolds where it is difficult to achieve high volume flow rate, nanotopographic surface modification may allow even lower shear stresses to serve as a potent stimulator for enhancing cellular mechanosensitivity.

It is not clear why data on the number of cells responding (Figure 3) and average $[Ca^{2+}]_i$ increase (Figure 4) display similar but not exactly matching trends. However, both sets of data indicate that cellular mechanosensitivity may be enhanced on specific nanotopographies. Furthermore, this may provide a clue for future studies examining which factor, percent cells responding in $[Ca^{2+}]_i$ or average $[Ca^{2+}]_i$ increase among responding cells, is more influential in downstream signaling and cell fate and terminal differentiation.

In conclusion, we showed that stem cells display enhanced mechanosensitivity when cultured on specific nanotopographies (Figure 5). Surface nanotopography has the potential to promote cellular responsiveness to mechanical signals. Cells not only prefer nanotopographies but the size of nanoislands play a significant role, with enhanced mechanosensitivity on specific scale nanoislands (approximately 10 nm). Although the mechanism by which this occurs is not known, our data, coupled with published data that focal adhesion and cellular stiffness are increased on nanotopographies of similar sizes, suggest that nanotopography induced differences in cell adhesion and stiffness are positively correlated with cell mechanosensitivity and signaling. The use of nanotopographies would thus be a novel way to modulate cellular reactivity to mechanical cues and provide new functional tissue engineering protocols.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Atomic force microscopy (AFM) of polymer-demixed nanotopographic films. PLLA/PS (70/30 w/w) demixed films spin-casted from solutions having a total polymer concentration of 0.5%, 1.0%, 2.0%, and 3.0% w/w in chloroform corresponded to 12 nm, 21 nm, 45 nm, and 80 nm average high nanoislands, respectively. A flat PLLA film is also shown.

Figure 2.

Analysis of $[Ca^{2+}]_i$ responsiveness to fluid flow by calcium-sensitive Fura Red-AM dye. hMSCs were stained with $10\ \mu M$ Fura Red-AM and exposed to oscillating fluid flow that generates physiological level shear stresses. After a 180 s static period fluid flow was applied and $[Ca^{2+}]_i$ was quantified using a fluorescent ratiometric method. The mean and standard deviation (SD) of $[Ca^{2+}]_i$ for the static period were obtained, and if a cell displayed a $[Ca^{2+}]_i$ peak greater than 'mean+5SD' during the flow period the cell was defined as a responding cell. One can see distinct calcium responses from many cells and non-responding cell traces (green and light green) in the top figure.

Figure 3.

Intracellular calcium response in hMSCs on nanotopographies exposed to fluid flow induced shear stresses as assessed by percentage of cells responding with an increase in $[Ca^{2+}]_i$. hMSCs cultured on each of the four nanotopographies and the flat control were exposed to shear stresses of 5, 10, and 20 dyne/cm². At 5 dyne/cm², hMSCs cultured on 12 and 21 nm nanoislands displayed significantly greater percentage of cells responding to oscillatory fluid flow with an increase in $[Ca^{2+}]_i$ compared with those cells cultured on flat controls (*: $p < 0.05$, **: $p < 0.01$; $n = 7-9$ slides, ANOVA with post-hoc test). At higher shear stresses of 10 and 20 dyne/cm², similar trends were observed but did not reach statistical significance.

Figure 4.

Intracellular calcium response in hMSCs on nanotopographies exposed to fluid flow induced shear stresses as assessed by average $[Ca^{2+}]_i$ increase. Increase in $[Ca^{2+}]_i$ for each responding cell was determined as peak value during the flow period minus mean of the baseline fluorescence during the static period. The increases for each responding cells were averaged and plotted. At 5 dyne/cm², cells cultured on 12 nm nanoislands exhibited significantly greater $[Ca^{2+}]_i$ increase compared with those on 45 and 80 nm nanoislands (##: $p < 0.01$, $n = 7-9$ slides, ANOVA with post-hoc test).

Figure 5. Schematic of nanotopography induced increase in stem cell mechanosensitivity. We demonstrated that human mesenchymal stem cells display enhanced $[Ca^{2+}]_i$ mechanosensitivity to fluid flow stimulation when cultured on specific nanoscale topographies.