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Spatial control of adult stem cell fate using nanotopographic cues

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Abstract

Adult stem cells hold great promise as a source of diverse terminally differentiated cell types for tissue engineering applications. However, due to the complexity of chemical and mechanical cues specifying differentiation outcomes, development of arbitrarily complex geometric and structural arrangements of cells, adopting multiple fates from the same initial stem cell population, has been

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difficult. Here, we show that the topography of the cell adhesion substratum can be an instructive cue to adult stem cells and topographical variations can strongly bias the differentiation outcome of the cells towards adipocyte or osteocyte fates. Switches in cell fate decision from adipogenic to osteogenic lineages were accompanied by changes in cytoskeletal stiffness, spanning a considerable range in the cell softness/rigidity spectrum. Our findings suggest that human mesenchymal stem cells (hMSC) can respond to the varying density of nanotopographical cues by regulating their internal cytoskeletal network and use these mechanical changes to guide them toward making cell fate decisions. We used this finding to design a complex two-dimensional pattern of co-localized cells preferentially adopting two alternative fates, thus paving the road for designing and building more complex tissue constructs with diverse biomedical applications.

Keywords

human mesenchymal stem cells; differentiation; nanotopography; osteogenesis; adipogenesis; capillary force lithography; cytoskeletal stiffness; actin

1. Introduction

Regenerative medicine, including stem cell-based therapy, is already beyond its inception and has gained much attention for its potential therapeutic use in medicine [1]. Robust protocols have been developed, some in clinical trials for treatment of diseases [2-5] to differentiate pluripotent or autologous adult multipotent cells into target cell types. As a part of the stromal system, multipotent human mesenchymal stem cells (hMSC) [6] are known to be capable of differentiating into various cell types [6] including skeletal muscle [7], osteoblasts [8], chondrocytes [9], and adipocytes [10]. hMSC appear to be a promising therapeutic tool owing to their self-renewal capacity, relatively easy isolation, and low immune competence problems [6]. Furthermore, evidence suggests that hMSC can differentiate into ectodermal cell types such as neurons [11] that are beyond the hMSC's innate mesodermal lineage.

Most tissues consist of various cell types that are spatially arranged in a well-defined manner, interacting with each other to create a functional unit [12]. Although the use of biochemical stimulation with hormones [13] and chemokines [14] has been the traditional method of controlling hMSC differentiation, controlling the physical condition of the cells' surrounding environment has been suggested of late as an alternative means to control cell fate [11,15]. However, these methods are still limiting for creating large scale constructs consisting of cells of multiple types arranged in arbitrary geometries, particularly if the constituent differentiated cells are to be derived from a single population of hMSC. The main challenge is in the need for precise control of the availability of distinct differentiation factors in time and space, over long time periods of differentiation [16]. In spite of considerable recent progress, this technical challenge is currently difficult to address [17]. Although mechanical rigidity of the substratum can control the fate and phenotype of hMSC and provide uniform rigidity on a large length scale, arbitrary rigidity profile variables in space in 2D or 3D are still hard to form [18].

As an effective alternative to these methods, recent studies have highlighted the nanotopographical features of the extracellular matrix (ECM) as a new promising differentiation control method [19]. It is important to note that in diverse organs, including the bone marrow, the native niche of hMSC, the ECM is arranged with complex topographic features constituting the mechanical signal to which hMSCs are considerably sensitive [20,21] [ENREF 35](#). Nanotopographical cues that mimic the topographic ECM organization *in vivo* have been shown to regulate cell shape, polarity, migration, proliferation, fate and other phenotypes in various stem cell based systems [22]. However, it is not clear if the local nanotopography can be an instructive cue, driving cells to distinct differentiation outcomes, even though it has been hypothesized that mechanical cues, including substratum rigidity [11] and its local geometry [23] could provide instructive input. The mechanical cues presented by the ECM (rigidity, shear, strain, and topography) can regulate stem cell behavior via overlapping signaling pathways, which modern fabrication techniques allow to unravel through precise control of presentation of combinations of these cues to live cells [24].

Here, we investigated the role of nanotopographical cues in regulation of differentiation outcomes of hMSC, using capillary force lithography (CFL) a scalable technique used to create large surface area (in multiple cm^2) substrata composed of diverse nanotopographical features with high precision [24]. In particular, we interrogated the role of the density of nanopost arrays in regulating two specific well-studied fates of hMSC: adipocytes and osteocytes. We found that the nanopost density was indeed a powerful instructive differentiation cue.

2. Materials and methods

2.1. Fabrication of nanostructured posts composed of polyurethane acrylate (PUA) using UV-assisted CFL

Nanostructured PUA surfaces with various post-to-post distances (1.2, 2.4, 3.6, and 5.6 μm) were fabricated as described previously [24].

2.2. Culture of human mesenchymal stem cells (hMSC)

hMSC [cat# PT-2501, Lonza, Inc. (Allendale, NJ)] were maintained on regular culture dishes in MSCGM single quote media and then gradually adopted over two weeks by mixing the MSCGM with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS) (HyClone Thermo Scientific, Logan, UT), 1% penicillin:streptomycin (P/S) (Invitrogen) and 1% antibiotic-antimycotic (AM) (Invitrogen). Then, hMSC were maintained in DMEM with 20% FBS, 1% PS, and 1% AM, except for differentiation experimental periods. During differentiation periods, sterilized surfaces without (flat control), and with nanostructured posts were immersed in 50 $\mu\text{g/mL}$ type I collagen (BD bioscience, San Jose, CA or Sigma Aldrich, St. Louis, MO) overnight at CO_2 cell culture incubator. Then, hMSC were seeded on the surfaces without or with nanostructures in DMEM with 20% FBS, 1% PS, and 1% AM, for a day at seeding density of 1600 cells/ cm^2 surface area for flat control; 2400 cells/ cm^2 for 1.2 μm post-to-post distance substratum; 3600 cells/ cm^2 for 5.6 μm post-to-post distance

substratum. These different seeding densities were used due to lower seeding efficiencies of surfaces with increasing densities of nanoposts to achieve similar ultimate densities of attached cells. This generated similar cellular confluence of hMSC cultured on flat control substratum as well as nanopost substratum during differentiation periods. Then, differentiation was induced by culturing the hMSC in the media mixed (1:1, vol; vol) with adipogenesis (#PT-3004 from Lonza; #A10070-01, Invitrogen) and osteogenesis differentiation (#PT-3002 from Lonza; #A10072-01 from Invitrogen, Grand Island, NY) media [described as A/O differentiation media henceforth] for 14 days with changing the media six times.

2.3. RNA extraction and real time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The total RNA of hMSC was extracted using Tri-Reagent (Sigma-Aldrich, St. Louis, MO) and the cDNA was synthesized from total mRNA using Multiscribe reverse transcriptase with random hexamers. Taqman qPCR assay was performed as described [25]. The expression of test genes was normalized to the expression of 18S ribosomal RNA (18S rRNA). Taqman gene expression assays used were: LPL (assay ID# Hs00173425_m1); ALPL (# Hs01029144_m1); RUNX2 (# Hs00231692_m1); PPAR γ (# Hs01115513_m1); and 18S rRNA (# Hs99999901_s1). Each sample was tested in triplicate, and data was expressed as mean $\pm$ SD, where the SD was calculated based on the Delta method for expressing the error for the variance of the ratio of two independent means (a gene of interest; 18S rRNA).

2.4. Staining of Oil red O and alkaline phosphatase

The cells were cultured for appropriate experimental periods and were fixed with 4% formaldehyde for 20 min and then stained with adipogenic and/or osteogenic markers according to the manufacturer's protocol (Sigma-Aldrich). The Oil red O was dissolved in 100% isopropanol and then freshly diluted to 60% isopropanol with water prior to each staining. Alkaline phosphatase of osteogenic cells was fixed with 4% formaldehyde for 20 min, permeabilized with 0.1% triton X-100, stained with Fast Blue RR/napththol mixture for 30 min and washed with water. Both staining kits were purchased from Sigma-Aldrich (St. Louis, MO).

2.5. Immunofluorescence staining of F-actin

The cells were incubated with a primary F-actin antibody, followed by incubating with a secondary antibody and staining with Texas Red®-X Phalloidin (#T7474, Molecular Probe). A primary antibody recognizing vinculin and a secondary antibody conjugated with FITC were obtained from Sigma Aldrich and used at 1:100 dilutions. The stained cells were mounted with SlowFade® antifade reagent (# S26938, Invitrogen) and sealed with nail polish (Sally Hensen Hard as Nails).

2.6. Optical and immunofluorescence confocal microscopy and cell image analysis

Bright field/phase contrast and color images and epifluorescence images of fixed cells were taken on an inverted microscope/confocal microscope (model# Axiovert 200, Carl Zeiss,

Thornwood, NY) equipped with color CCD camera. Cells were maintained on the microscope stage at 37 °C and 5% CO₂ while taking images of cells. Slidebook software (Intelligent Imaging Innovations) was used for the cell image analysis. The cell area, circularity and boundary are manually detected using Mask Processing function in the Slidebook software program.

2.7. Flow cytometry

The cells were collected for flow cytometry and analyzed via FACS Diva (BD Biosciences) as described previously [26]. $\beta 1$ integrin (#sc-18887, Santa Cruz) and $\beta 3$ integrin (#sc-52685, Santa Cruz) were used as primary antibodies.

2.8. Magnetic twisting cytometry (MTC)

The material property of living adherent cells was quantified using MTC as described previously. A ferrimagnetic microbead anchored to the cytoskeleton through cell surface integrin receptors was magnetized horizontally and then twisted in a vertically aligned homogenous magnetic field that varied sinusoidally in time. The sinusoidal twisting field causes both a rotation and a pivoting displacement of the bead. As the bead moves, the cell develops internal stresses which in turn resist bead motions [27]. Lateral bead displacements in response to the resulting oscillatory torque were detected via a CCD camera (Orca II-ER, Hamamatsu, Japan) attached to an inverted optical microscope (Leica Microsystems, Bannockburn, IL), and with an accuracy of 5 nm using an intensity-weighted center-of-mass algorithm. We defined the ratio of specific applied torque to lateral bead displacements as the complex elastic modulus (g^*) of the cell, $g^*(f) = g'(f) + ig''(f)$, where g' is the storage modulus (cell stiffness), g'' is the loss modulus (cell friction), and $i^2 = -1$ [27,28]. Cell stiffness g' and friction g'' are expressed in units of Pascal per nm (Pa/nm).

2.9. Statistical analysis

The Analysis of Variance (ANOVA) was used for single cell mechanics analysis. To satisfy the normal distribution assumptions associated with ANOVA, cell stiffness data was converted to log scale prior to analyses. Unless otherwise stated, all analyses were performed in SAS V.9.2 (SAS Institute Inc., Cary, NC) and the two-sided P -values less than 0.05 were considered significant. For other data, Kruskal Wallis test (one-way ANOVA) followed by posthoc test/multiple comparison was applied using SigmaPlot 11 (Systat Software Inc.). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Fabrication of biomimetic scalable nanopost patterns with variable densities

Although substratum topography has been shown to enhance osteogenic differentiation of hMSC [21,29], it is not clear whether it can also be an instructive cue allowing for specification of alternative cell fates. To examine this, we created a scalable cell culture substratum made of poly urethane (PUA) via CFL [24] with controlled distances between adjacent nanoposts (Fig. 1A). Each nanopost was 700 nm wide (Fig. 1A) with post-to-post distances of 1.2, 2.4, 3.6, and 5.6 μm , respectively. The PUA surface without topographic patterning was used as the 'flat' control (Flat). These scalable nanopatterns can be combined

on a single coverslip, allowing the analysis of the effects of varying nanopost density in one experiment, thus reducing experimental variability and difference in cell batches and medium composition (Fig. 1B).

3.2. Regulation of hMSC phenotype and fate by nanopost density

To investigate the role of nanotopography and its effect on hMSC phenotypes, we cultured hMSC on surfaces with variable nanopost densities. hMSC were cultured for 2 days from the cell seeding in regular (non-differentiation) media (DMEM+20%FBS) and the cells were observed using phase-contrast microscopy (Fig. S1A-E). We found that the surface contact area of hMSC decreased as the post-to-post distance decreased (Fig. S1F). Cell shapes were more rounded in morphology occupying a smaller surface area with higher nanopost density (i.e. shorter post-to-post distance), whereas cells were more spread and covered a larger area on the surface with lower nanopost density (i.e. longer post-to-post distance) (Fig. S1F). However, after 14 days of culture in a mixture of osteogenesis and adipogenesis differentiation induction media (1:1, vol:vol), the cell area showed a biphasic dependence on post-to-post distance, with cells on 2.4 μm post-to-post distance substrata exhibiting the lowest cellular circularity (Fig. 1C) and spreading area (Fig. 1D).

Cell shape, in particular, the degree of cell spreading reflected in the cell area, is known to influence cell fate decisions of hMSC [30]. Since cell area was shown to be regulated by the density of nanoposts, we explored whether the nanopost density could also influence the cell fate decision of hMSC. hMSC were cultured in a mixture of osteogenesis and adipogenesis differentiation induction media (1:1, vol:vol) for 14 days and stained with an adipocyte specific marker Oil red O and an osteocyte specific marker alkaline phosphatase (ALP) (Fig. 2A-2E). Consistent with the previous finding of McBeath *et al* [31], on the flat control substrata, hMSC displayed greater commitment to the osteogenic lineage. We also found that, while the rounded cells on nanoposts with shorter post-to-post distances (Fig. 2A, 1.2 μm) favored the adipogenic lineage, the stretched cells on nanoposts with longer post-to-post distances (Fig. 2D, 5.6 μm) favored the osteogenic lineage commitment. The substrata with 3.6 μm post-to-post distance appeared to represent the borderline in the change of the hMSC cell fate decision. Interestingly, substrata with the post-to-post distance of 2.4 μm were optimal for adipogenic differentiation (Fig. 2F). These findings, together with the biphasic relationship of cell spreading area on nanopost densities (Fig. 1D), suggest a close association between adipogenic cell fate decision and regulation of cell spreading area.

The lineage commitment was further analyzed by assaying for expression of adipogenic and osteogenic markers using qRT-PCR in hMSC cultured on substrata with 1.2 μm and 5.6 μm post-to-post distances. In the cells cultured on the 5.6 μm post-to-post distance nanopatterns, we found an increase in the expression of alkaline phosphatase (ALP), an early marker of osteogenesis (Fig. 2G) as well as an osteoblast-specific transcription factor runt-related transcription factor 2 (RUNX2) (Fig. 2H). In contrast, hMSC cultured on the 1.2 μm post-to-post distance nanopatterns displayed increased expression of a mature adipogenesis marker, the lipoprotein lipase (LPL) (Fig. 2I) and a major regulator of adipogenesis, the peroxisome proliferator-activated receptor gamma (PPAR γ) (Fig. 2J). These data, in combination, show that a relatively longer post-to-post distance favors the osteogenic lineage, whereas

adipogenesis is regulated by the post-post distance in biphasic fashion with 2.4 μm post-to-post distance demonstrating the smallest cell area and maximal adipogenesis.

3.3. Regulation of cellular cytoskeletal organization by nanopost density

The correlation between the cell shape (Fig. 1) and the differentiation outcome (Fig. 2) suggest the importance of controlling cell shape by the nano-post density in defining cell fate decisions. The cell shape is controlled by the balance of cell-matrix interactions (e.g., the degree of cell adhesion) and the state of the cytoskeleton [32,33]. These two important factors are not independent, as the molecules responsible for cell adhesion, such as those in the integrin family of receptors, also engage in cell signaling, which might affect the cytoskeletal organization [34]. Thus, we investigated whether the signaling pathways contributing to changes in cell shape and cell fate can be affected by nanotopography cues. We first conducted a meta-analysis of published microarray data [35, 36] analyzing hMSC differentiation into osteocytes and adipocytes. The nine most significant canonical processes that are differentially regulated in osteogenesis and adipogenesis during hMSC differentiation and the corresponding differentially expressed genes in each pathway are summarized in Fig. 3A and supplementary Table 1A;1B. Genes whose expression levels are up/down-regulated during osteogenesis in comparison to adipogenesis of hMSC are shown in Fig. S2. Out of the nine signaling pathways identified, we closely examined molecules that play important roles in the most significant two pathways: actin cytoskeleton and integrin signaling (Fig. 3A).

The expression of $\beta 1$ and $\beta 3$ integrins was analyzed by flow cytometry (Fig. S3A - S3D). hMSC cultured on the 5.6 μm post-to-post distance substrata showed higher protein expression levels of $\beta 1$ integrin vs. hMSC cultured on the substratum with 1.2 μm post-to-post distances (Fig. S3A, S3B). In contrast, the average expression levels of $\beta 3$ integrin were similar on average in hMSC cultured on both 5.6 μm and 1.2 μm post-to-post distance substrata (Fig. S3C), although a sub-population of hMSC did exhibit a higher $\beta 3$ integrin protein expression level (Fig. S3D). We recently reported that $\beta 1$ integrin, but not $\beta 3$ integrin, modulates levels of p190RhoGAP, a GAP protein for RhoA, in cardiac stem cells [19]. RhoA, in turn, can strongly affect the cytoskeletal state and cell shape through its downstream effect on the myosin light chain (MLC) [31]. These data suggest that integrin expression is correlated with hMSC fate as determined by nanopost density, which may also be a causal factor in determining cell fate.

To investigate the influence of nanopost density on actin cytoskeleton signaling, we determined the F-actin structure in hMSC cultured on different nanopost density surfaces. On the surface with higher nanopost densities (i.e., 1.2 μm post-to-post distance), F-actin staining was mostly localized near the cell periphery (Fig. 3B). As the post-to-post distance increased up to 5.6 μm , formation of actin stress fibers within the cell body became much more pronounced (Fig. 3B-3E). Stress fiber formations on surfaces with 5.6 μm post-to-post distance (Fig. 3E) were similar to that on the flat control substrata (Fig. 3F). Interestingly, the total F-actin levels also manifested a biphasic dependence on nanopost arrays, with 2.4 μm post-to-post distance showing the lowest actin staining level per cell (Fig. 3G). These

observations support the role of integrin mediated cell-to-nanopost interaction in controlling cytoskeleton and cell shape.

3.4. Regulation of cytoskeletal stiffness of hMSC by nanopost density

Since we found that the density of nanoposts regulates the cytoskeletal organization of hMSC, we further investigated the mechanical changes in the cells. We cultured hMSC on flat control substrata in either adipogenic medium, osteogenic medium, or a mixture of both (adipogenic/osteogenic, 1:1), for various periods (days 1, 7, and 14). Next we performed a microrheology analysis of the cells, using magnetic twisting cytometry (MTC) [27,28]. In this technique, the resistance of magnetic microbeads tethered to the cytoskeleton through integrin receptors to externally applied torque is used to evaluate the mechanical cell properties, such as the stiffness (the elastic/storage index, g') and the friction (the loss modulus, g'') within the living cells.

Over five decades of the twisting frequency, the stiffness g' of individual cells increased with frequency according to a weak power law (Fig. 4). The internal friction g'' also followed a weak power law at low frequency (below ~ 10 Hz), but showed stronger frequency dependence at higher frequencies (Fig. 4). Strikingly, when measured on days 1, 7 and 14, on the flat control substrata, hMSC cultured in the adipogenic medium exhibited a temporal decrease in cell stiffness, whereas cells cultured in osteogenic media exhibited an increase in cell stiffness over the same time period (Fig. 4A-4C). On day 14 (Fig. 4C), we found striking divergence of stiffness changes between cells cultured in adipogenic and osteogenic media. We also noted that the power-law frequency dependence of stiffness g' and friction g'' differed appreciably between the two cell cultures (adipogenic, $f^{0.149}$ and osteogenic, $f^{0.129}$). As described previously [27,28], these power laws can be used to define the effective 'temperature' of the cells and their similarity to fluid or solid like media. Our data indicate that cells cultured in adipogenic media became more fluid-like, while cells cultured in osteogenic media became more solid-like during their differentiation, indicating important changes in the cytoskeletal organization.

hMSC were also cultured on nanopost arrays of variable post-to-post distances for two weeks. At each probing frequency, cell stiffness monotonically changed with the post-to-post distance (Fig. 5D-5F). We observed, in particular, that the hMSC cultured on the $5.6 \mu\text{m}$ post-to-post distance arrays exhibited the stiffest cytoskeleton, whereas the hMSC cultured on the $2.4 \mu\text{m}$ post-to-post distance showed the least stiff cytoskeleton. There were no appreciable changes in the effective cell temperature. These findings suggest that the nanopost density may be capable of directly regulating cytoskeletal stiffness and that dynamic changes in stiffness correlate with the differentiation of hMSC into osteogenic (Fig. 2F-2H) or adipogenic (Fig. 2F-2J) lineages.

Remarkably, cell stiffness and actin cytoskeleton levels showed a biphasic dependence on underlying nanopost densities similar to dependencies found for the cell circularity (Fig. 1C) and area (Fig. 1D), in addition to their adipogenic potential (Fig. 2F-2J).

3.5. Spatial control of hMSC fate by nanopost architecture

Our results suggest that the topographic mechanical cues, as expressed in the density of the nanopost arrays, can be powerful instructive cues in directing hMSC differentiation. Moreover, the nanopost density can be relatively easily varied within the same array, allowing for the opportunity to form graded spatial differential profiles of arbitrary 2D organization and complexity. We examined therefore, whether it is possible to create a multicellular ensemble consisting of cells adopting two possible fates in a spatially complex, pre-defined pattern. Since nanoposts are readily scalable, we created a large surface area (5cm x 4cm) of nanopost arrays with square sub-domains containing different post-to-post distance arrays placed adjacent to each other in a checkerboard pattern (Fig. 5A). We found that sparse (5.6 μm) nanopost arrays induced osteogenic differentiation of hMSC (shown in blue of Fig. 5B, 5C), whereas dense nanoposts induced adipogenic differentiation (shown in red of Fig. 5B, 5C), with the overall patterns of differentiation strongly biased by the underlying checkerboard cue, as revealed by ALP (an osteogenic marker) and Oil red O (an adipogenic marker) staining (Fig. 5D). Since the nanopost density is controllable in cell culture, our results suggest a simple and effective method for creation of multicellular ensembles of cells derived from a single stem cell type, potentially instrumental in creating multicellular tissue constructs.

4. Discussion

Fate selection in adult stem cell differentiation is exquisitely sensitive to various mechanical cues. These cues can be both static and dynamic in nature, frequently influencing cell behavior both in short term and long term [37]. These cues include nanotopographic definitions of the cell adhesion substrata. Indeed, the ECM structure surrounding stem cells *in vivo* can be quite complex, and its organization can vary among different organs or even within a single organ as a function of time and space. This variable environment can provide an important cue regulating cell differentiation, as suggested e.g., for osteogenic cell fate [ENREF 39](#). To date, however, it has not been clear if the nanostructure of the cell adhesion substrata can be an instructive cue selectively biasing for the adoption of two alternative stem cell fates. Beyond the fundamental implications for stem cell biology, this question has practical implications, as the substratum nanotopography is the aspect of the mechanical cell microenvironment that is much easier to define with very high spatial resolution than other differentiation cues, such as the substratum rigidity or the chemical composition of the extracellular medium.

The results presented in this report argue that the topography of the cell adhesion substratum can indeed control cellular fates of differentiating hMSC when presented in the form of arrays of nano-sized posts separated by micro-scale sized distances. Greater distance between nanoposts favored osteogenic differentiation, whereas smaller post-to-post distances were associated with adipogenesis. Interestingly, we found that adipogenesis was maximized at 2.4 μm post-to-post distance, decreasing as the density of nanoposts further increased. This result suggested that the sensitivity to topography can be ultimately limited, as the density of nanoposts increases and the surface is perceived as progressively similar to the flat control substratum by the cells cultured on it. Overall, the results suggest that the

bias in selection between two alternative fates can indeed be instructed by the density of the topographic features in the cell microenvironment.

What can be the mediators of the fate selection? Previously, it has been suggested that mechanical cues can influence adult stem cell differentiation through activation of integrin dependent signaling networks [38] [ENREF 61](#). We indeed found by analyzing the published microarray data sets [35, 36] that a variety of genes implicated in the activity of these networks, including actin cytoskeleton and integrin signaling pathways, are differentially regulated. Furthermore, we confirmed that expression of the $\beta 1$ and $\beta 3$ integrins and F-actin were differentially regulated. The signaling pathways are expected to differentially trigger cytoskeleton re-organization, which can affect cell stiffness and thus cell shape. This in turn could be a correlative or causal change accompanying cell differentiation. We found that a change in the cell stiffness has occurred during differentiation into alternative lineages even on the flat control substrata, with a gradual increase of stiffness over time in cells undergoing osteogenic differentiation.

We observed a similar trend during the fate selection induced by the nano-topographic cues, i.e. different nanopost densities. Supporting the connection between the cytoskeleton organization controlling the cell shape, cell area, and the differentiation outcome, we found that the cells had the lowest stiffness at post-to-post distances of 1.2 and 2.4 μm , and more rounded shapes at the same post-to-post distances (1.2, and 2.4 μm) were found for higher adipogenesis outcomes. We therefore conclude that these topographic cues can affect the differentiation outcome by engaging integrin-dependent signaling networks defining the cell shape and tension.

The bias in the differentiation outcomes introduced by the cell substratum topography can provide a very convenient tool to locally control cell differentiation, in an addressable fashion. This, in turn, can pave the way to construction of synthetic tissues composed of multiple cell types, each emerging from the same precursor cell population. To provide a proof of concept, we designed a checkerboard pattern of two alternative fates alternating in 2D. Although the individual differentiation outcome is always stochastic, there was a clearly detectable bias defining the outcome along the predefined geometric pattern.

5. Conclusion

In this study, we have found that the adipogenic fate of hMSC followed a biphasic dependence on the nanopost density, whereas osteogenesis of hMSC was defined by nanopost density in a monotonic fashion. This fate selection was correlated with the changes in the intracellular signaling apparatus regulating cytoskeleton and cellular tension and shape. Using the ability of CFL to produce substrata with arbitrarily arranged topographical features in two-dimensional space, we were able to bias differentiation of hMSC according to a predefined 2D pattern. We have shown that nanotopography-induced regulation of the balance of osteogenic and adipogenic differentiation in human mesenchymal stem cells allows complex spatial control of cell fate. Our report provides an important new insight into the biology of differentiation of hMSC. In the human body, the cell adhesion substrata are typically topographically complex, with the texture defined on the nano- or micro-scale.

Therefore, our observation that the substratum nano-topography can control the differentiation outcome has important implications for the understanding of the differentiation patterns *in vivo*. Furthermore, our nanotopographical approach provides an important practical tool for engineering synthetic tissues with complex geometric organizations from the same stem cell source.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A: Supplementary data

Supplementary data related to this article can be found online.

Abbreviations

hMSC	human mesenchymal stem cells
MSC	mesenchymal stem cells
CFL	capillary force lithography
ECM	extracellular matrix
PUA	polyurethane acrylate
LPL	lipoprotein lipase
ALPL	alkaline phosphatase liver/bone/kidney
RUNX2	runt-related transcription factor 2
PPARγ	peroxisome proliferator-activated receptor gamma
MLC	myosin light chain

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Fig. 1.

Fabrication of scalable nanopatterns with defined densities of nanoposts. (A) A schematic showing nanopatterns with varying nanopost densities fabricated using capillary force lithography (CFL). Nanoposts were created at mutual post distances (d) of 1.2, 2.4, 3.6, and 5.6 μm . (B) Scanning electron microscopy (SEM) images of nanopatterned arrays with varying post densities. All the above patterns, with flat control, were fabricated on a single glass coverslip to reduce experimental variation. A photo of the coverslip is shown in the middle. The diameter of the posts is 700 nm with the varying distances between two adjacent posts. The post-to-post distances were 1.2 (top left), 2.4 (top right), 3.6 (bottom left) and 5.6 μm (bottom right). (C; D) hMSC were cultured for 14 days in a mixture (1:1, vol: vol) of adipogenesis and osteogenesis differentiation media on flat control (without nanoposts) and substrata with varying post-to-post distances. Cell circularity (C) and cell spreading area (D) of hMSC were analyzed.

Fig. 2.

Nanopost density regulates hMSC fate. (A-F) hMSC were cultured for 14 days in a mixture (1:1, vol: vol) of adipogenesis and osteogenesis differentiation media on 1.2 μm (A), 2.4 μm (B), 3.6 μm (C), 5.6 μm (D) and flat control (E) surfaces and then stained with Oil Red O (adipocyte marker) and alkaline phosphatase (osteocyte marker). (F) Percentage of hMSC stained with osteogenic (Grey bars) and adipogenic markers (Black bars) are shown. (G-J) The expression of adipocyte (LPL, PPARγ) and osteocyte (ALP, RUNX2)-specific markers were determined using qRT-PCR assays in hMSC cultured on flat control (without nanoposts) and substrata with nanoposts at post-to-post distances of 1.2 μm (dense) and 5.6 μm (sparse). The mRNA expression levels of ALP, RUNX2, LPL, and PPARγ were normalized to the expression of 18S rRNA (mean ± S.D., n=3).

Fig. 3.

Differentially regulated pathways in osteogenesis and adipogenesis during hMSC differentiation (A) and the immunofluorescence staining of F-actin in hMSC (B-G). (A) Canonical processes that are differentially regulated in osteogenesis and adipogenesis during hMSC differentiation were analyzed using meta-analysis of published microarray data (as mentioned in *Supplementary methods*). Differentially expressed genes were analyzed to locate canonical biological themes that regulate cytoskeletal changes or adhesion mediated pathways. Orange line indicates the threshold (with q-value of 0.05) based on Fischer exact test. All mentioned processes are differentially expressed in datasets, suggesting that adhesion/cytoskeletal regulators are important during osteogenesis and adipogenesis. (B-G) Cytoskeletal organization of hMSC is influenced by nanopost density. hMSC were cultured for 2 days from the cell seeding on substrata of nanoposts with post-to-post distances of 1.2 μm, 2.4 μm, 3.6 μm, 5.6 μm, and flat control. (B-F) Representative images of F-actin immunofluorescence staining of hMSC exhibit a higher cortical actin organization on smaller post-to-post distance nanopatterns, while actin stress fibers are more abundant in cells cultured on larger post-to-post distance nanopatterns. A part of each photo of cells cultured on each substratum was magnified 2.5 times and shown individually in the inset. (G) The mean intensity of F-actin per cell was quantitated. Data are mean ± S.E.M [1.2 μm (n=21), 2.4 μm (n=30), 3.6 μm (n=32), 5.6 μm (n=44), and flat (n=24)].

Fig. 4.

Nanopost density regulates hMSC rheology correlated with observed rheological alterations in differentiation. (A-C) Time course magnetic twisting cytometry analyses showing elastic modulus and loss modulus of hMSC cultured in the presence of osteogenic (blue circles), adipogenic (red triangles), or a 1:1 mixture (vol:vol) of osteogenic and adipogenic media (green squares) for (A) 1 day, (B) 7 days, and (C) 14 days on flat control substrata. (D-F) hMSC were cultured for 14 days in a mixed differentiation media (adipogenesis: osteogenesis, 1:1) on substrata with various post-to-post distances. Representative images (D) and elastic (E) and loss (F) moduli of hMSC on substrata with post-to-post distances of 1.2 μm (red), 2.4 μm (green), 3.6 μm (purple), and 5.6 μm (blue squares).

Fig. 5.

Creation of large surface area nanopost arrays to control spatial differentiation of hMSC. (A) A schematic showing the conceptual design of large surface area nanopost arrays with dense (D; 1.2 μm) and sparse (S; 5.6 μm) arrangements in a chessboard like fashion. Nanopost densities can be controlled in arbitrary fashion in two-dimensions. (B; C) hMSC were cultured for three weeks in a mixture (1:1, vol: vol) of adipogenesis and osteogenesis differentiation media on substrata with various post-to-post distances (1.2 μm ; 5.6 μm) and then the cells were stained with Oil red O and alkaline phosphatase. (B) Staining images of hMSC cultured for three weeks on substrata with various post-to-post distances (1.2 μm ; 5.6 μm) are shown with scanning electron microscopy (SEM) images of nanopatterns. (C) Stitched staining images of hMSC cultured for three weeks on substrata with variable post-to-post distances (1.2 μm ; 5.6 μm). A higher level of Oil red O staining (an adipogenesis marker) was shown on smaller post-to-post distance areas (dense arrays), while a higher level of alkaline phosphatase staining (an osteogenesis marker) was shown on larger post-to-post distance areas (sparse arrays). The white bar indicates 50 μm . (D) Kymographic staining analysis for the percentage of adipogenic and osteogenic fate of hMSC cultured on a large surface area substratum with various post-to-post distances. Differential degrees of osteogenesis (blue) and adipogenesis (red) are shown in variations of the two colors, blue and red.