Biomaterials 35 (2014) 2401–2410

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Spatial control of adult stem cell fate using nanotopographic cues

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ARTICLE INFO

Article history:
Received 8 October 2013
Accepted 14 November 2013
Available online 31 December 2013

Keywords:
Human mesenchymal stem cells
Differentiation
Nanotopography
Osteogenesis
Adipogenesis
Capillary force lithography

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 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.

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

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0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.11.037

[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]. 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 quots 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 μg/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 various days with changing the media every other day, except the day of cell collection.

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 RNA 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 (18 S rRNA). Taqman gene expression assays used were: LPL (assay ID# Hs00173425_m1); ALPL (# Hs01029144_m1); RUNX2 (# Hs00231692_m1); PPARγ (# Hs01115513_m1); and 18S rRNA (# Hs99999901_s1). Each sample was tested in triplicate, and data was expressed as mean ± 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, permeablized with 0.1% triton X-100, stained with Fast Blue RR/naphthol 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 miscroscope (model# Axiovert 200, Carl Zeiss, Thornwood, NY) equipped with color CCD camera. Cells were maintained on the microscope stage at 37 °C and 5% CO_2 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]. β1 integrin (#sc-18887, Santa Cruz) and β3 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 [27,28]. 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 elastic/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, Kruskall Wallis test (one-way ANOVA) followed by posthoc test/multiple comparison was applied using SigmaPlot 11 (Systat Software Inc.). Differences were considered significant at $p < 0.05$.