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Research Paper

Controlling cell geometry on substrates of variable stiffness can tune the degree of osteogenesis in human mesenchymal stem cells

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ARTICLE INFO

Article history:
Received 19 June 2013
Received in revised form
12 December 2013
Accepted 14 January 2014

Keywords:
Substrate stiffness
Microcontact printing
Microenvironment
Polyacrylamide hydrogels
Stem cell differentiation

ABSTRACT

The physical properties of the extracellular matrix (ECM) play an important role in regulating tissue-specific human mesenchymal stem cell (MSC) differentiation. Protein-coated hydrogels with tunable stiffness have been shown to influence lineage specific gene expression in MSCs. In addition, the control of cell shape – either through changing substrate stiffness or restricting spreading with micropatterning – has proved to be important in guiding the differentiation of MSCs. However, few studies have explored the interplay between these physical cues during MSC lineage specification. Here, we demonstrate geometric control of osteogenesis in MSCs cultured on micropatterned polyacrylamide gels. Cells cultured on fibronectin-coated gels express markers associated with osteogenesis in a stiffness dependent fashion with a maximum at ~30 kPa. Controlling the geometry of single cells across the substrate demonstrates elevated osteogenesis when cells are confined to shapes that promote increased cytoskeletal tension. Patterning MSCs across hydrogels of variable stiffness will enable the exploration of the interplay between these physical cues and their relationship with the mechanochemical signals that guide stem cell fate decisions.

1. Introduction

Mesenchymal stem cells (MSCs) isolated from bone marrow, which are a promising source of cells for regenerative medicine and tissue engineering, have the capacity for self-renewal and can differentiate into multiple lineages such as osteogenic, myogenic, adipogenic, chondrogenic, and neurogenic (Crisan et al., 2008; Engler et al., 2006; Pittenger, 1999). The relationship between MSC fate and the structure and properties of the extracellular matrix (ECM) has attracted great attention because the ECM is a key factor in mediating cell apoptosis, proliferation, motility and morphology. Early work by Engler et al. (2006) demonstrated that MSCs could sense the mechanical properties of the ECM to guide differentiation towards neurogenic (<1 kPa), myogenic (~10 kPa), and osteogenic (~30 kPa) lineages. Subsequently, there have been many research efforts to study the mechano-sensitive signal transduction pathways associated with ECM properties in a host of cellular systems (Gilbert et al., 2010; Guvendiren and Burdick, 2012; Keung et al., 2012; Rowlands et al., 2008; Saha et al., 2008; Winer et al., 2009).

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http://dx.doi.org/10.1016/j.jmbbm.2014.01.009

Another important parameter that has been shown to guide MSC differentiation is cell shape which can be controlled by cell seeding density or through micropatterned matrix proteins (Zhang and Kilian, 2013; Gao et al., 2010; McBeath et al., 2004; Théry, 2010; Peng et al., 2012). Mrksich and colleagues used soft lithography to pattern MSCs in an array of different shapes. Cells cultured in geometries with increasing aspect ratio and in ones that present subcellular concave regions beneath the cell led to enhanced non-muscle myosin and rho-associated protein kinase (ROCK) activity which directed initiation of osteogenesis gene expression (Kilian et al., 2010). In addition to these geometric cues, increased cytoskeletal tension through cell spreading has been shown to play a decisive role during MSC differentiation. For example, Chen and colleagues used a micropatterning technique to demonstrate that spread cells are more prone to commit to an osteogenic (McBeath et al., 2004) or smooth muscle myogenic (Gao et al., 2010) lineage compared to adipogenesis or chondrogenesis when exposed to mixed induction cocktails. Both of these cell spreading-related outcomes were shown to be regulated by the small GTPases RhoA (osteogenesis) and Rac (myogenesis), respectively. Other materials-based platforms that influence cell shape, size, and degree of spreading have also been shown to promote the osteogenic differentiation of MSCs (Dalby et al., 2007; Ni et al., 2008; Oh et al., 2009; Yang et al., 1999). Taken together, these studies demonstrate an intimate connection between cell geometry, adhesion architecture and the degree of cytoskeletal tension during MSC osteogenesis. However, the interplay between these physical cues and their respective roles in guiding differentiation remains to be explored.

The development of systems to study the relationships between cell shape and substrate stiffness in the cellular microenvironment is an area of significant interest (Rape et al., 2011; Tang et al., 2012; Tee et al., 2011; Tseng et al., 2011). Recently, we used micropatterned hydrogels to demonstrate how cell spreading and adhesion protein composition can regulate the degree of adipogenesis and neurogenesis on soft substrates (<1 kPa) (Lee et al., 2013). In the present work, we control geometric cues at the single cell level across hydrogels of different stiffness to explore how cell geometry influences osteogenesis on lineage-matched substrates. Using microcontact printing of adhesion proteins on polyacrylamide (PA) gels of variable stiffness, we show that cell shape combined with matrix stiffness can direct the osteogenic differentiation of mesenchymal stem cells. The influence of geometric cues (subcellular curvature and aspect ratio) across the substrate on cell fate decisions is investigated and we show that osteogenesis marker expression can be elevated when cells are confined to shapes that promote increased cytoskeletal tension.

2. Materials and methods

2.1. Materials

Laboratory chemicals and reagents were purchased from Sigma Aldrich unless otherwise noted. Tissue culture plastic ware was purchased from Fisher Scientific. Cell culture media and reagents were purchased from Gibco. Human MSCs and differentiation media were purchased from Lonza. Rabbit anti-Runx2 and anti-Osteopontin were purchased from Abcam. Rabbit anti-Myosin IIb was purchased from Cell signaling Technologies. Tetramethylrhodamine-conjugated anti-rabbit IgG antibody, Alexa488-phalloidin and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen. BCIP/NBT solution was purchased from Amresco. Glass coverslips (18-mm circular) for surface preparation were purchased from Fisher Scientific.

2.2. Surface preparation

Polyacrylamide gels were fabricated on a glass cover slip (15 mm) according to established conventional methods (Tse and Engler, 2010). We used the protocol of making hydrogels with varying stiffness by applying a mixture of acrylamide and bis-acrylamide according to the desired stiffness, and for the polymerization, 0.1% ammonium persulfate (APS) and 0.1% of tetramethylethylenediamine (TEMED). 20 μL of the mixture was pipetted onto the hydrophobic treated glass slides, and the amino-silanized coverslips were added with the treated side down (Aratyn-Schaus et al., 2010). After appropriate polymerization time for each stiffness condition (see Fig. S1), the gel-coated cover slips were gently detached. Hydrazine hydrate 55% (Fisher Scientific) was utilized for 1 h to convert amide groups in polyacrylamide to reactive hydrazide groups (Damljanović et al., 2005). Sodium periodate (Sigma-aldrich) was incubated with the glycoproteins to yield free aldehydes. The gels were washed for 1 h in 5% glacial acetic acid (Fluka/Sigma) and for 1 h in distilled water. To create patterned surfaces, polydimethylsiloxane (PDMS, Polysciences, Inc.) stamps were fabricated by polymerization upon a patterned master of photoresist (SU-8, MicroChem) created using UV photolithography through a laser printed mask. 25 μg/mL of fibronectin in PBS was applied for 30 min to the top of patterned or unpatterned PDMS, and then dried under air, and applied to the surface.

2.3. Cell source and culture

Human mesenchymal stem cells (MSCs) from bone marrow were thawed from cryopreservation (10% DMSO) and cultured in Dulbecco's modified Eagle's medium (DMEM) low glucose (1 g/mL) supplemented with 10% fetal bovine serum (MSC approved FBS; Invitrogen), and 1% penicillin/streptomycin (p/s). Media was changed every 4 days and cells were passaged at nearly 80% confluency using 0.25% Trypsin:EDTA (Gibco). Passage 4–7 MSCs were seeded on patterned and non-patterned surfaces at a cell density of ~5000 cells/cm^2.

2.4. Immunocytochemistry

After incubation for 10 days, surfaces were fixed with 4% formaldehyde (Alfa Aesar) for 20 min, and cells were permeablized in 0.1% Triton X-100 in PBS for 30 min and blocked with 1% bovine serum albumin (BSA) for 15 min. Primary antibody labeling was performed in 1% BSA in PBS for 1 h at room temperature (20 °C) with rabbit anti-Runx2, anti-osteopontin or myosin IIb (1:200 dilution). Secondary antibody labeling was performed using the same procedure with tetramethylrhodamine-conjugated anti-rabbit IgG antibody along with Alexa Fluor

Please cite this article as: Lee, J., et al., Controlling cell geometry on substrates of variable stiffness can tune the degree of osteogenesis in human mesenchymal stem cells. Journal of the Mechanical Behavior of Biomedical Materials (2014), http://dx.doi.org/10.1016/j.jmbbm.2014.01.009