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Directing stem cell fate on hydrogel substrates by controlling cell geometry, matrix mechanics and adhesion ligand composition

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ABSTRACT

There is a dynamic relationship between physical and biochemical signals presented in the stem cell microenvironment to guide cell fate determination. Model systems that modulate cell geometry, substrate stiffness or matrix composition have proved useful in exploring how these signals influence stem cell fate. However, the interplay between these physical and biochemical cues during differentiation remains unclear. Here, we demonstrate a microengineering strategy to vary single cell geometry and the composition of adhesion ligands — on substrates that approximate the mechanical properties of soft tissues — to study adipogenesis and neurogenesis in adherent mesenchymal stem cells. Cells cultured in small circular islands show elevated expression of adipogenesis markers while cells that spread in anisotropic geometries tend to express elevated neurogenic markers. Arraying different combinations of matrix protein in a myriad of 2D and pseudo-3D geometries reveals optimal microenvironments for controlling the differentiation of stem cells to these "soft" lineages without the use of media supplements.

1. Introduction

Cells adhering to the extracellular matrix (ECM) can sense the mechanical properties through specific interactions of cell surface integrins with adhesion ligands [1–5]. Traction forces exerted by the cell through these interactions influence cytoskeletal tension and lead to changes in cell shape and associated signaling cascades that ultimately regulate gene expression [6–10]. This process of mechanotransduction has emerged as an important aspect of stem cell differentiation and is dependent on both the mechanics and the composition of the microenvironment. For example, Datta et al. revealed the importance of the mechanical and biochemical microenvironment by culturing osteoprogenitor cells on a decellularized osteoblast matrix leading to increased expression of osteogenic markers [11]. Work in the Schaffer and Healey groups has demonstrated that mechanical properties can guide neurogenesis in neural stem cells where softer matrices promote dendritic process extension [12]. A study by Engler, Discher and colleagues demonstrated the importance of matrix mechanics in guiding MSC fate by studying cells adherent to collagen-coated polyacrylamide hydrogels of variable stiffness [8]. MSCs were found to commit to lineages based on the similarity to the committed cells' native matrix; soft polyacrylamide gels (<1 kPa) promote neurogenesis, intermediate stiffness gels (~10 kPa) promote myogenesis and stiff gels (>30 kPa) promote osteogenesis.

In addition to stiffness, the composition and presentation of adhesion ligands on a substrate have been shown to influence MSC differentiation [1–3,13–15]. Cooper-White and co-workers demonstrated that different matrix proteins — collagen, fibronectin and laminin — grafted to hydrogel substrates of different stiffness will significantly influence the expression of myogenic and osteogenic markers. This work suggests that the identity of adhesion ligand and its presentation to the cell can play an important role in promoting competing differentiation outcomes. Kilian and Mrksich recently showed how the density and affinity of surface bound adhesion peptides could modulate the expression of markers associated with neurogenesis, myogenesis and osteogenesis, further confirming the importance of the type and presentation of ligand in guiding stem cell differentiation [3].

Another important physical parameter that has emerged as an important cue in guiding the differentiation of stem cells, and is influenced by stiffness and the presentation of adhesion ligands, is cell shape [4,16–19]. For instance, Chen and colleagues demonstrated that MSCs captured on small islands tended to prefer adipocyte differentiation when exposed to a mixture of osteogenic and adipogenic soluble cues while cells captured on large islands developed a higher degree of cytoskeletal tension and preferred to

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adopt an osteoblast outcome [16]. In a related study, Kilian et al. demonstrated that MSCs patterned in geometries with subcellular concave regions and moderate aspect ratios increase the actomyosin contractility of the cell and promote osteogenesis [17]. In both of these studies, keeping cell shape the same across a population of MSCs was shown to normalize the differentiation outcome when compared to unpatterned cells that take on a host of different geometries.

An important lesson that has emerged from these studies is that there is clearly interplay between matrix mechanics, adhesion ligand presentation and cell geometry during differentiation [4,5,20]. The majority of research efforts have focused on varying one physical cue while exploring the influence on biological activity. However, in vivo cell fate is likely influenced by a combination of geometry, mechanics and ECM composition [21,22]. Thus we reasoned that combining these cues would prove useful in designing materials that more closely emulate the in vivo microenvironment and "fine-tune" a desired differentiation outcome.

In this paper, we use soft lithography to micropattern multiple matrix proteins — alone and in combinations — on hydrogel substrates with the mechanical properties of soft tissue to explore the physical and biochemical cues that guide MSCs towards adipogenesis and neurogenesis outcomes. Immunofluorescence staining and real-time PCR are employed to assess the expression of key markers during differentiation. We explore the translation of our findings to a pseudo-3D hydrogel format that more closely represents the in vivo environment.

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 and produced by Osiris Therapeutics. Mouse anti-ß3 tubulin was purchased from Sigma (T8660), rabbit anti-PPARγ was purchased from Cell Signaling (C26H12), and chicken anti-MAP2 was purchased from abcam (ab5392) Technologies. Tetramethylrhodamine-conjugated anti-rabbit IgG antibody, Alexa Fluor 647-conjugated anti-mouse IgG antibody, Alexa488-phalloidin and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen. Glass coverslips (18-mm circular) for surface preparation were purchased from Fisher Scientific. These cells were derived from bone marrow isolated from the iliac crest of human volunteers. MSCs were tested for purity by Lonza, and were positive for CD105, CD166, CD29, and CD44, negative for CD14, CD34, and CD45 by flow cytometry, and had ability to differentiate into osteogenic, chondrogenic, adipogenic lineages (http://www.lonza.com). The use of human MSCs in this work was reviewed and approved by the University of Illinois at Urbana-Champaign Biological Safety Institutional Review Board.

2.2. Polyacrylamide gel fabrication and protein immobilization

We used the protocol of making 0.48 ± 0.16 kPa gels by using the mixture of 3% of Acrylamide and 0.06% of Bis-acrylamide, and for the polymerization, 0.1% of Ammonium Persulfate (APS) and Tetramethylethylenediamine (TEMED). Hydrazine hydrate 55% (Fisher Scientific) was utilized for 1 h to convert amide groups in polyacrylamide to reactive hydrazide groups. 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, laminin, or type 1 collagen (for combinations of ligands, the final concentration was normalized to 25 μg/mL) 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. Pseudo-3D microwells were fabricated by templating the polyacrylamide gels on an SU-8 photolithography master displaying the inverse features used in fabricating the PDMS stamps. After subjecting the microwells to hydrazine treatment and oxidized protein, adhesive tape was applied to the gel and removed quickly to shear off the top layer of protein-conjugated gel.

2.3. Cell culture

Human mesenchymal stem cells (MSCs) were thawed from cryopreservation (10% DMSO) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) low glucose (1 g/mL) media 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. Immunofluorescence and histology

After incubation for 10 days, surfaces were fixed with 4% formaldehyde (Ted Pella, Inc.) for 20 min, and cells were permeabilized 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-PPARγ (Cell Signaling Tech., 1:200 dilution) or MAP2 (Santa Cruz, 1:200 dilution) and mouse anti-β3 Tubulin (abcam, 1:200 dilution). Secondary antibody labeling was performed using the same procedure with tetramethylrhodamine-conjugated anti-rabbit IgG antibody and Alexa Fluor 647-conjugated anti-mouse IgG antibody (1:200 dilution) along with Alexa Fluor 488-phalloidin (1:200 dilution) and 4′,6-diamidino-2-phenylindole (DAPI, 1:5000 dilution) for 30 min in a humid chamber (37 °C). Immunofluorescence microscopy was conducted using a Zeiss Axiovert 200M inverted research-grade microscope (Carl Zeiss, Inc.), and immunofluorescence images were analyzed using ImageJ to measure the fluorescence intensity. For Oil Red O staining, after fixing cells, each sample stained with a lipid staining solution for adipogenesis (Oil Red O, Sigma) per manufacturer's instructions. Briefly, cells were incubated in 60% isopropanol for 5 min followed by immersion in Oil Red O working solution (3:2; 300 mg/mL Oil Red O in isopropanol:DI water) for 10 min to 1 h.

2.5. RNA isolation and RT-PCR

Adherent cells were lysed directly in TRIZOL reagent (Invitrogen) and total RNA was isolated by chloroform extraction and ethanol precipitation. Total RNA in DEPC water was amplified using TargetAmp™ 1-Round aRNA Amplification Kit 103 (Epicentre) according to vendor protocols. Total RNA was reverse transcribed using Superscript III® First Strand Synthesis System for RT-PCR (Invitrogen). RT-PCR was performed linearly by cycle number for each primer set using SYBR® Green Real-Time PCR Master Mix (Invitrogen) on an Eppendorf Realplex 4S Real-time PCR system. Primer sequences were as follows: C/EBPα — GCAAACTCACCGCTCCAATG and TTAGGTTCCAAGCCCCAAGT, PPARG2 — AGAGCCTTCCAACTCCCTCA and CAAG GCATTTCTGAAACCGA, LPL — CATCCCATTCACTCTGCCT and AGTTCTCCAATATCTA CCTCTGTG, β3Tubulin — CCATTTCTCGACTTTCCAAACTG and CTGCGAACTTGCCT GTGGA, MAP2 — GGAGACAGAGATGAGAATTCCT and GAATTGGCTCTGACCTGGT, GAPDH — CTCTGCTCCTCCTGTTCGAC and GTTTCTCTCCGCCCGTCTTC. All reactions were performed linearly by cycle number for each set of primers.

3. Results

3.1. Hydrogel fabrication and single cell patterning

Previous reports of patterning on hydrogels used substrates of relatively high modulus (>2.5 kPa) [23]. In order to study the combinatorial effects of cell shape, substrate stiffness and matrix composition in directing neurogenesis and adipogenesis on soft hydrogels (<1 kPa), we developed a protocol based on soft lithography and chemically modified polyacrylamide (PAAm). Patterning ECM proteins on soft hydrogels via direct contact with an elastomeric stamp is challenging due to the substrate compliance and the presence of surface water, and few studies of microcontact printing on hydrogels have been reported [24]. Here we systematically varied curing, drying and contact times to identify an operating regime in which precise patterning of complex features on PAAm was possible (Fig. 1a). Polydimethylsiloxane (PDMS) stamps were prepared using photolithography to present geometric features in relief or flat surfaces without structure (unpatterned). Polyacrylamide (PAAm) hydrogels were prepared according to established literature methods [25], and we confirmed their stiffness (~0.6 kPa) via atomic force microscopy (AFM) (Fig. S1). The PAAm gels were treated with hydrazine hydrate and the stamps were inked with an oxidized glycoprotein solution to promote covalent immobilization after microcontact printing [26]. After seeding cells