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Geometric guidance of integrin mediated traction stress during stem cell differentiation

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ABSTRACT

Cells sense and transduce the chemical and mechanical properties of their microenvironment through cell surface integrin receptors. Traction stress exerted by cells on the extracellular matrix mediates focal adhesion stabilization and regulation of the cytoskeleton for directing biological activity. Understanding how stem cells integrate biomaterials properties through focal adhesions during differentiation is important for the design of soft materials for regenerative medicine. In this paper we use micropatterned hydrogels containing fluorescent beads to explore force transmission through integrins from single mesenchymal stem cells (MSCs) during differentiation. When cultured on polyacrylamide gels, MSCs will express markers associated with osteogenesis and myogenesis in a stiffness dependent manner. The shape of single cells and the composition of tethered matrix protein both influence the magnitude of traction stress applied and the resultant differentiation outcome. We show how geometry guides the spatial positioning of focal adhesions to maximize interaction with the matrix, and uncover a relationship between αvβ3, α5β1 and mechanochemical regulation of osteogenesis.

1. Introduction

Stem cells in their niche are in contact with the extracellular matrix (ECM) which provides multiple structural and biochemical cues to direct their behavior [1–8]. Cells adhere to the ECM through several different cell surface receptors including integrins which are involved in mechanosensing and bi-directional transmission of mechanical force [9]. This interaction allows cells to sense and respond to their microenvironment via contractile forces and to adaptively remodel tissues with dynamic mechanical forces, guiding broad aspects of their functions such as cell migration, growth, differentiation, and survival [10–15]. For this reason, the careful design of the cellular recognition interface on deformable biomaterials is a critical aspect for the regulation of distinct stem cell functions.

Mesenchymal stem cells (MSCs) are multipotent cells which have the ability to differentiate into several cell types including chondrocytes, adipocytes, myoblasts and osteoblasts in vitro, and this process is regulated by biophysical and biochemical dynamics of signal-activated gene regulation [16–25]. Controlling the microenvironment properties such as matrix elasticity [17,26,27], cell and tissue shape [19,28,29], and adhesive proteins [20,30] can regulate lineage specification of MSCs. For example, MSC lineage specification to neurogenesis, myogenesis, or osteogenesis outcomes can be directed by matrix elasticity [17]. Specifically, MSCs cultured on stiff substrates (~34 kPa), which promote cell spreading, are guided to an osteogenesis outcome due to increased contractility of the actomyosin cytoskeleton. Cytoskeletal tension can be modulated not only by matrix elasticity but also by cell shape. For instance, cells cultured in shapes which promote cytoskeletal tension prefer to adopt an osteogenic fate while those in relaxed shapes prefer to undergo adipogenesis [19,31]. In addition, MSC osteogenesis can be tuned on fibronectin coated substrates with variable stiffness (10–40 kPa) by controlling the geometry of single micropatterned cells [29]. Other reports have shown that combining different adhesion ligands (fibronectin, laminin, or collagen) with hydrogels of variable matrix elasticity, influences MSC differentiation between osteogenesis and myogenesis lineages [21].

MSCs interact with extracellular matrix proteins through various integrins including α1-6, αV, α11, αX, β1-4, and β7-8 [10,13]. Combinations of two different chains, integrin α and β subunits, define the surface receptors that recognize ECM proteins such as: fibronectin, vitronectin, collagen, and laminin [32,33]. These integrin

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transmembrane receptors act as mechanosensors and mechanotransducers to connect the actin cytoskeleton to the ECM and enable dynamic interactions with the microenvironment through focal adhesions. For example, MSCs primarily bind to fibronectin through the common integrin heterodimers α5β1 or αVβ3 [34]. A previous report showed that α5 integrin expression in MSCs was elevated during osteogenic differentiation while cells expressed higher level of α6 integrin during adipogenic lineage specification at 7 days [10]. The surface geometry and local biochemical microenvironment of biomaterials have been shown to influence focal adhesions, cytoskeletal tension and differentiation in adherent MSCs [19]. However, the relationship between integrin mediated traction stress and MSC differentiation has not been described.

In this paper we show how control of cell shape can be used to study the relationship between focal adhesion, traction stress, and the differentiation of single mesenchymal stem cells. We use immunofluorescence staining to investigate the protein expression of key markers during osteogenesis and myogenesis. Traction stress measurements are employed to assess the force generated by MSCs with different combinations of these cues. We show through immunofluorescence that the expression of early and late osteogenic markers is dependent on the engagement of α5β1 and αVβ3 integrins.

2. Materials and methods

2.1. Materials

All materials were purchased from Sigma unless otherwise noted. Tissue culture plastic ware and glass coverslips (18-mm circular) were purchased from Fisher Scientific. Cell culture media and reagents were purchased from Gibco. Rabbit anti-Runx2 (ab23981) and anti-Osteopontin (ab8448) were purchased from Abcam. Mouse anti-MyoD (MAB3878) Mouse anti-α5β1 (MAB1969) and αVβ3 (MAB1976Z) were purchased from Millipore. Blebbistatin, Y-27632, FR180204 (ERK inhibitor), SP600125 (JNK inhibitor), and SB202190 (p38 inhibitor) were purchased from Calbiochem. Tetramethylrhodamine-conjugated anti-rabbit IgG antibody, Alexa Fluor 647-conjugated anti-mouse IgG antibody, Alexa Fluor 555-conjugated anti-rabbit IgG antibody, Alexa488-phalloidin and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen.

2.2. Surface preparation

Polyacrylamide substrates were prepared as previously described [29]. Briefly, 10–40 kPa stiffness gels were made by using mixtures of acrylamide/bis-acrylamide according to the desired stiffness [35]. For the polymerization, 0.1% ammonium persulfate (APS) and 0.1% of tetramethylethylenediamine (TEMED) were mixed in the gel solutions and 20 μL of the mixture was pipetted onto hydrophobically treated glass slides. After polymerization, the gels on the coverslips were detached and treated with hydrazine hydrate 55% for 2 h with rocking [36]. 5% Glacial acetic acid for 1 h and then distilled water for 1 h were used to rinse hydrazine and glacial acetic acid, respectively. Polydimethylsiloxane (PDMS, Polysciences, Inc.) stamps were produced by conventional polymerization methods. Sodium periodate (~3.5 mg/mL) was used to generate free aldehydes on matrix proteins and added to 25 μg/mL of fibronectin in PBS for at least 45 min. The protein solution was pipetted onto patterned stamps for 30 min and dried with air. Free aldehydes in proteins were chemically conjugated with reactive hydrazide groups on the gels, resulting in transferring the protein residue on the stamps to the gel surfaces [36].

2.3. Cell source and culture

Human MSCs were purchased from Lonza. The MSCs were harvested and cultured from normal bone marrow. Cells were positive for CD105, CD166, CD29, and CD44 and negative for CD14, CD34 and CD45 by flow cytometry (http://www.lonza.com). Purchased MSCs from bone marrow were cultured and then expanded cells were frozen in cryopreservation (10% DMSO) with passage 2. Cells were thawed 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 3 or 4 days. Passage 4–8 MSCs were seeded on patterned surfaces at a cell density of ~5000 cells/cm^2. MSCs were cultured for 10 days before analysis.

2.4. Immunocytochemistry and histology

Cells on surfaces were fixed with 4% paraformaldehyde (Alfa Aesar) for 20 min. To permeabilize cells, 0.1% Triton X-100 in PBS was employed for 30 min. Cells were blocked with 1% bovine serum albumin (BSA) for 15 min and labeled with primary antibody in 1% BSA in PBS for 2 h at room temperature (20 °C) with mouse anti-MyoD, α5β1, or αVβ3 and rabbit anti-Runx2 or Osteopontin (1:500 dilution). Secondary antibody labeling was performed by the same procedure with Tetramethylrhodamine-conjugated anti-rabbit IgG antibody, Alexa Fluor 488-phalloidin (1:200 dilution), Alexa647-conjugated anti-mouse IgG antibody, and 4,6-diamidino-2-phenylindole (DAPI, 1:5000 dilution) for 20 min in a humid chamber (37 °C). Immunofluorescence microscopy was conducted using a Zeiss Axiovert 200 M inverted research-grade microscope (Carl Zeiss, Inc.). Immunofluorescent images were analyzed using ImageJ; the fluorescence intensity of single cells (over 20 cells) for each condition was measured to compare different levels of marker expression. To stain for alkaline phosphatase, surfaces were rinsed with distilled water and incubated for 30 min in BCIP/NBT solution, rinsed well in PBS and imaged in bright field using a Motic trinocular inverted microscope. All experiments were repeated at least three times. Only single cells that were captured in patterns were used in the analysis. The relative intensity of the fluorescence was determined by comparing each intensity value to the average intensity of one condition. For Figs. 2, 3 and 6, average marker intensities of circular cells in 5000 μm^2 patterned stiff (10 kPa) substrates were selected. For Fig. 5, average mRNA expressions of cells in 5000 μm^2 circle patterned (10 kPa) substrates were selected. The intensity value for single cells was obtained from nuclei (Runx2 and MyoD) or cytoplasmic (Osteopontin) staining intensity minus backgrounds.

2.5. Traction stress measurement

Polyacrylamide gels with desired stiffness (10 and 30 kPa) were fabricated on a glass cover slip (18 mm) as described above [29]. To obtain fluorescent bead-infused gels, the polyacrylamide solution was mixed with a 1 μm-bead suspension (Invitrogen, F-8821) at 1:250 and a small amount (1~2 μl) was applied to gel solutions. Upon the placement of the gel surface faced down, beads in a single layer at the same focal plane were imaged using a fluorescent microscope. Matrix proteins were patterned as described above. An Olympus IX81 fluorescent microscope and 20× objective was used to obtain the live cell images [37]. Throughout the experiment, temperature and carbon dioxide levels were maintained at 37 °C and 5% respectively. Live cell images on gels embedded with fluorescent beads were captured. Bright field images were firstly taken of the cells to visualize cell shape and location, and then fluorescent images of beads were taken. In order to assess the displacement of beads under the null-force condition, cells were removed from the surface using sodium dodecyl sulfate (SDS, Fisher Inc.), resulting in the gel returning to its relaxed initial state without cells. To