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Mechanical stiffness as an improved single-cell indicator of osteoblastic human mesenchymal stem cell differentiation

Tom Bongiorno [a], Jacob Kazlow [b], Roman Mezencev [c], Sarah Griffiths [d], Rene Olivares-Navarrete [e], John F. McDonald [c], Zvi Schwartz [e], Barbara D. Boyan [e], Todd C. McDevitt [b,d], Todd Sulchek [a,b,d,*]

[a] The G. W. Woodruff School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, GA, USA
[b] The Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA, USA
[c] School of Biology, Georgia Institute of Technology, Atlanta, GA, USA
[d] The Parker H. Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA, USA
[e] School of Engineering, Virginia Commonwealth University, Richmond, VA, USA

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ABSTRACT

Although it has been established that cellular stiffness can change as a stem cell differentiates, the precise relationship between cell mechanics and other phenotypic properties remains unclear. Inherent cell heterogeneity and asynchronous differentiation complicate population analysis; therefore, single-cell analysis was employed to determine how changes in cell stiffness correlate with changes in molecular biomarkers during differentiation. Design of a custom gridded tissue culture dish facilitated single-cell comparisons between cell mechanics and other differentiation biomarkers by enabling sequential measurement of cell mechanics and protein biomarker expression at the single cell level. The Young's modulus of mesenchymal stem cells was shown not only to decrease during chemically-induced osteoblast differentiation, but also to correlate more closely with the day of differentiation than did the relative expression of the traditional osteoblast differentiation markers, bone sialoprotein and osteocalcin. Therefore, cell stiffness, a measurable property of individual cells, may serve as an improved indicator of single-cell osteoblast differentiation compared to traditional biological markers. Revelation of additional osteoblast differentiation indicators, such as cell stiffness, can improve identification and collection of starting cell populations, with applications to mesenchymal stem cell therapies and stem cell-based tissue engineering.

1. Introduction

Human mesenchymal stem cells (hMSCs) hold great potential for autologous therapy, highlighted by the properties of immunosuppression, migration to injured tissues, and tissue repair via soluble factor secretion (Hwang et al., 2009). MSC osteoblast differentiation following bone graft incorporation may facilitate subsequent bone formation (Amini et al., 2012). However, the absence of donor- and anatomical location- independent MSC biomarkers hampers the collection of MSCs from bone marrow or adipose tissue for clinical therapies (Amini et al., 2012), which establishes a need to improve phenotype detection by identifying additional MSC biomarkers.

The lack of reliable cell-surface or intracellular markers of terminal MSC osteoblast differentiation precludes techniques such as fluorescence-activated cell sorting from successful phenotype identification. Common markers of MSC osteoblast differentiation, including alkaline phosphatase, osteopontin, and osteonectin, peak prior to mineralization of the extracellular matrix (Aubin and Triffitt, 2002; Vater et al., 2011), and are therefore not optimal for definitive phenotype identification. Two other MSC osteoblast differentiation markers, bone sialoprotein (BSP) and osteocalcin (OCN), are considered to be late osteogenesis markers, but are produced by other cells that form the mineralized matrix (Aubin and Triffitt, 2002; Vater et al., 2011). Isolation of extracellular matrix constituents, such as BSP, OCN, and other common osteoblastic proteins, requires dissociative, cell-destructive methods. Therefore, locally synthesized proteins are difficult to distinguish from matrix-trapped proteins derived from other sources, such as serum. Altogether, these facts emphasize a need for additional cell-specific osteoblastic markers to identify cell phenotype.

Compared to extracellular protein markers, cellular stiffness is easily attributable to individual cells, and thus may serve as a candidate osteoblastic marker. Cellular stiffness has been proposed

Abbreviations: AFM, atomic force microscopy; bone sialoprotein, BSP; osteocalcin, OCN; hMSC, human mesenchymal stem cell; hMSC-OB, osteoblastic hMSC; hOB, human osteoblast

* Corresponding author at: The G. W. Woodruff School of Mechanical Engineering, Georgia Institute of Technology, 771 Ferst Drive, Atlanta, GA 30332, USA. Tel.: +1 404 385 1887; fax: +1 404 385 8187.
E-mail address: todd.sulchek@me.gatech.edu (T. Sulchek).

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as an indicator of multiple cellular processes, including cancer metastasis (Cross et al., 2007; Darling et al., 2007; Suresh, 2007; Xu et al., 2012) and apoptosis (Hu et al., 2009; Lam et al., 2007), as well as stem cell differentiation (Chen et al., 2010; Ofek et al., 2009; Pajerowski et al., 2007; Pillarisetti et al., 2011; Tan et al., 2012) and differentiation potential (González-Cruz et al., 2012; Hammerick et al., 2011).

Previous cell mechanics experiments suggest that hMSC stiffness can change during osteoblast differentiation (Darling et al., 2008; Docheva et al., 2008; Titushkin and Cho, 2007; Yourek et al., 2007; Yu et al., 2010), but the network of factors that influences the observed stiffness changes is poorly understood. Moreover, the factors that affect cellular stiffness are confounded by the mechanical heterogeneity of cell populations and, in the case of stem cell studies, asynchronous differentiation kinetics (Fig. 1A). Thus, inherent heterogeneity and asynchronous differentiation of stem cell populations motivate the need for single-cell forms of analysis (Di Carlo et al., 2012).

In contrast to the population-wide correlations employed by other studies, a recent study elegantly correlated the mechanical properties and differentiation potential of individual stem cell clones (González-Cruz et al., 2012). However, investigations of *single-cell* relationships between mechanical properties and traditional biomarkers are needed to determine how effectively individual parameters indicate the state of differentiation. Consequently, the objective of this study was to evaluate cell stiffness as a single-cell marker of hMSC osteoblast differentiation in comparison to conventional phenotypic markers (BSP and OCN).

The stiffness, morphology, and differentiation state of hMSCs undergoing osteoblast differentiation were assessed via atomic force microscopy (AFM) and imaging of a fluorescent membrane lipid dye and immunofluorescent BSP and OCN stains, respectively. Custom gridded Petri dishes were used to match individual cells measured by AFM to those assayed by subsequent fluorescence imaging. To investigate the utility of cell mechanics in reflecting differentiation state, single-cell correlations between the day of differentiation and either mechanical or molecular markers were compared.

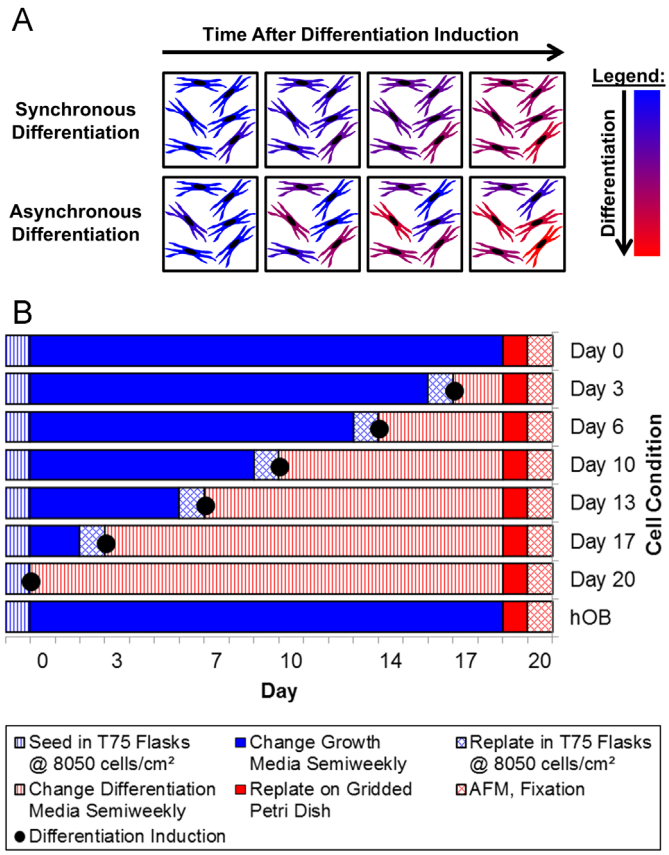

Fig. 1. hMSC Differentiation. (A) Synchronous and asynchronous differentiation modes can result in the same population-average differentiation state. However, the asynchronous differentiation of MSCs necessitates single-cell assays for the most rigorous analysis of differentiation biomarkers. (B) The "staggered" differentiation scheme was employed such that earlier time points were induced to differentiate prior to later time points. Thus, all cells completed osteoblast differentiation simultaneously, regardless of the differentiation time point. The scheme permitted the Young's modulus to be measured for all cells during a single AFM session.

2. Methods

2.1. Cell culture

Passage 1 bone marrow-derived hMSCs were obtained from Texas A&M (Donor 8002L). Immunophenotyping after expansion to passage 4 confirmed hMSC phenotype (Fig. S1). hMSC growth medium (16% fetal bovine serum [FBS, Atlanta Biologicals, Flowery Branch, GA], 2 mM L-glutamine, and 1% penicillin/streptomycin [P/S] in alpha minimum essential medium) was changed semiweekly. Normal human osteoblasts (hOBs) were obtained from Lonza, and hOB growth medium (10% FBS, 1% P/S in Dulbecco's modified Eagle's medium) was changed every 48 h. Upon reaching ~85% confluency, cells were washed with phosphate buffered saline (PBS), detached using 0.25% trypsin/EDTA, and subpassaged at 60 cells/cm^2 (hMSCs) or 1:2 (hOBs) until passage 4.

2.2. Osteoblast differentiation

hMSC osteoblast differentiation was induced by semiweekly media changes of hMSC growth medium supplemented with 10 nM dexamethasone, 20 mM β-glycerol phosphate, and 50 μM L-ascorbic acid 2-phosphate (Platt et al., 2009). To improve the consistency of the AFM results, a "staggered" osteoblast differentiation scheme was employed, in which earlier time points were induced to differentiate prior to later time points. Thus, hMSCs undergoing 0, 3, 6, 10, 13, 17, and 20 days of osteoblast differentiation (hMSC-OBs) reached the specified differentiation time points simultaneously (Fig. 1B).

2.3. Gridded Petri dishes

Gridded Petri dish manufacture is illustrated in Fig. 2A. Petri dishes were engraved with a grid pattern chosen to facilitate matching of AFM cell mechanics data to immunofluorescence images (Fig. 2B–D). The grid was engraved using a VLS3.50 laser cutter (Universal Laser Systems, Scottsdale, AZ) with parameters optimized for grid visibility, while minimizing the line width to approximately 75 μm.

To prevent cell attachment to the sites of engraving, each grid was covered with a glass coverslip. Engraved dishes and glass coverslips were soaked in 70% ethanol, sterilized by UV light exposure, and attached using two-part epoxy. After curing for 24 h, the sterile technique was used to apply petroleum jelly to the Petri dish surface, but not the coverslip surface, thereby decreasing the effective dish surface area and limiting the required volumes of cells and immunofluorescence reagents. The fully assembled dishes were sterilized by UV light exposure before cell plating. Gridded Petri dishes yielded similar hMSC morphology compared to glass and tissue culture polystyrene surfaces.

2.4. Atomic force microscopy

Prior to AFM measurements, a 5.5 μm polystyrene bead (Bangs Labs, Fishers, IN) was attached to a tipless silicon nitride cantilever (MLCT-O10, Bruker, Camarillo, CA, Cantilever D, k=10–60 pN/nm) using two-part epoxy with 24 h curing time (Fig. 2E). Compared to pyramidal probe geometry, the spherical probe increased the probe-cell contact area and improved the accuracy of global cell stiffness measurements (Pillarisetti et al., 2011; Titushkin and Cho, 2007).

Approximately 2500 hMSC-OBs or hOBs were plated onto gridded Petri dishes in their respective growth medium. Cells were adhered for 20–32 h and washed with PBS before mechanical characterization using an atomic force microscope (Asylum Research, Santa Barbara, CA) on a vibration isolation table (Herzan, Laguna Hills, CA). Phase contrast microscopy (Eclipse Ti, Nikon, Melville, NY) was used to locate the cells and position the beaded probe over the center of each cell. Thermal calibration (Hutter and Bechhoefer, 1993) yielded the cantilever spring constant, k=19.80 pN/nm. A measurement rate of 0.39 Hz and a probe velocity of 2.34 μm/s were used. The 2 nN force trigger resulted in indentations of

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