Published in final edited form as:

J Biomech. 2014 June 27; 47(9): 2197–2204. doi:10.1016/j.jbiomech.2013.11.017.

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

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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.

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Conflict of interest statement The authors declare no competing financial interests associated with this work.

Appendix A. Supplementary material Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jbiomech.2013.11.017>.

Keywords

MSC; Atomic force microscopy; Bone sialoprotein; Cell stiffness; Osteoblast differentiation; Osteocalcin

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 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.

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/cm² (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 μ M 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 μ m.

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, $k=10\text{--}60$ 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 (Pillariseti 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 $\mu\text{m/s}$ were used. The 2 nN force trigger resulted in indentations of 250–500 nm for typical cells, corresponding to approximate contact areas of 4.3–8.6 μm^2 . To determine cellular Young's modulus, Igor software (Wavemetrics, Portland, OR) was used to apply the Hertzian contact model to the portion of the extension force-displacement curves from 50% to 95% of the maximum indentation, over which range the Young's modulus was generally independent of indentation. The mean Young's modulus of three measurements was calculated for each cell, using indentation offset as a free variable and assuming cellular Poisson's ratio, $\nu=0.5$.

2.5. Immunofluorescence imaging and image processing

2.5.1. Single-cell—Cells were fixed, permeabilized, and simultaneously stained for cell membrane (HCS CellMask Blue, Invitrogen, Carlsbad, CA), BSP (anti-BSP and fluorescein isothiocyanate-conjugated secondary antibody), and OCN (phycoerythrin-conjugated anti-OCN), as detailed in the Supplementary Information.

Following immunofluorescence staining, cell identity was determined by location within the grid using a Nikon Eclipse Ti microscope under halogen light illumination and confirmed by comparison to images taken during AFM. Cell membranes and antibody-labeled BSP and OCN were visualized using the 4',6-diamidino-2-phenylindole, fluorescein isothiocyanate, and tetramethylrhodamine isothiocyanate excitation/emission filter sets, respectively. Individual cells were imaged using a CoolSNAP HQ² camera (Photometrics, Tuscon, AZ).

The relatively high signal-to-noise ratio of the cell membrane images allowed use of the ImageJ rolling ball algorithm to subtract the background from each image. However, due to substantial background autofluorescence, the BSP and OCN images were enhanced using a different method of background subtraction. ImageJ (National Institutes of Health, Bethesda, MD) was used to generate background images for each day of differentiation by averaging the intensity of raw BSP or OCN images and applying a 25 μm Gaussian blur.

Spread cells have previously been shown to be stiffer than spherical cells (Darling et al., 2008), indicating that morphology may play an important role in apparent cell stiffness; therefore, the morphology of each cell measured by AFM was quantified. CellProfiler™ (Carpenter et al., 2006) was used to identify the boundary of each cell by applying a “background global” threshold to each corrected cell membrane image. ImageJ was used to characterize cell morphology by calculating the minor and major axes, Feret’s diameter, perimeter, area, aspect ratio, circularity, eccentricity, perimeter:area ratio, and roundness of each cell boundary. Quantification of cell membrane images was validated by the expected positive correlations among size descriptors as well as correlations between factors, such as aspect ratio and eccentricity, which are directly mathematically linked (see Supplementary Information). For each BSP and OCN corrected image, protein staining was quantified as the fraction of pixels within the cell boundary that exceeded a threshold value (“percent area”) (Cregger et al., 2006; Gerger et al., 2004). Quantification of single-cell morphology and protein staining is illustrated in Fig. S2.

2.5.2. Cell population—hMSC-OBs and hOBs were plated at 8050 cells/cm² on glass coverslips, stained as described above, and mounted on glass slides prior to imaging. Cell membranes, BSP, and OCN were imaged and background corrected *a priori*. The boundaries of naive hMSCs were identified using a classifier generated by ilastik software (Sommer et al., 2011) and 3-class “Otsu global” thresholding in CellProfiler™, and the identified region was used for quantification. Since all other hMSC-OBs and hOBs were confluent, the entire image was used for quantification. BSP and OCN staining was quantified as described above. Threshold values for each fluorescent channel were identical for single-cell and cell population images.

2.6. Statistics

Due to the unequal sample size, heteroscedasticity, and non-normal distribution of the AFM data, parametric bootstrapping was performed (10,000 iterations) using a MATLAB (MathWorks, Natick, MA) routine. Differences in mean Young’s moduli for the 7 hMSC-OB time points and the hOB sample were compared by bootstrapping one-way ANOVA, yielding $p=0.0002$. Post-hoc analysis was performed using pairwise, heteroscedastic bootstrapping Student’s *t*-tests.

225 total cells from the 7 hMSC-OB time points and the hOB sample were tested for pairwise correlations among phenotypic properties. Using JMP software (SAS Institute, Cary, NC), pairwise, nonparametric Spearman’s correlation coefficients were calculated to test for monotonic trends among the 14 single-cell differentiation, mechanical, protein staining, and morphological variables and among the day of differentiation and population

protein staining variables. For the purposes of correlation calculations, the “hOB” time point was considered to be after Day 20 of hMSC differentiation. Two-tailed p -values ($H_0: \rho=0$) were calculated for each correlation coefficient using Student’s t -test.

All obtained p -values were adjusted using Holm’s procedure for multiple comparisons. Since Holm’s adjusted p -values tend to be conservative, $\alpha=0.10$ was chosen. Original and adjusted p -values are listed in the Supplementary Information.

3. Results

3.1. Cell mechanics

Before investigating the relationships between cellular stiffness and molecular differentiation biomarkers, stiffness trends during differentiation were examined. The stiffness values of passage 4 hMSCs from two donors were not significantly different (Fig. S3, $p=0.368$), so donor 8002 L was used for the remainder of the study. Analysis of force-indentation curves generally yielded higher Young’s moduli for hMSCs than hOBs (Fig. 3A). Although the stiffness data were highly variable (coefficient of variation > 0.5), stiffness generally decreased during osteoblast differentiation (Fig. 3B). Importantly, Day 20 hMSC-OBs were significantly softer than naive hMSCs ($p_{\text{adjusted}}=0.083$), but not hOBs (Fig. 3C, $p_{\text{adjusted}}=1.000$).

3.2. Pairwise correlations among single-cell parameters

Spearman’s correlations were calculated among the differentiation, mechanical, protein staining, and morphological parameters for each cell (Fig. 4A). A significant inverse relationship between Young’s modulus and the day of differentiation was supported by the Spearman’s correlation coefficient ($\rho=-0.214$, $p_{\text{adjusted}}=0.055$) and the partial Spearman’s correlation coefficient ($r=-0.145$, $p=0.033$, Supplementary Excel Sheet 1), substantiating the observed decrease in stiffness during differentiation. Young’s modulus correlated positively and significantly with major axis, Feret’s diameter, perimeter, and area, but negatively and significantly with circularity, indicating that the stiffest cells were generally large and elongated (Fig. 4A).

The strong, positive correlation between single-cell BSP and OCN suggested coordinated protein expression changes, which is expected since BSP and OCN are both osteoblast differentiation markers. However, correlations between the day of differentiation and protein staining for individual cells were weak and not statistically significant (BSP, $\rho=0.198$, $p_{\text{adjusted}}=0.107$; OCN, $\rho=0.181$, $p_{\text{adjusted}}=0.230$). For individual days of differentiation, the correlations between protein staining and Young’s modulus were also weak ($|\rho|<0.45$) and not statistically significant (Fig. 4B and S4; BSP, $p_{\text{adjusted}}=1.000$; OCN, $p_{\text{adjusted}}=0.960$).

The increase in BSP and OCN staining intensity during differentiation was more pronounced for population than single-cell staining (Fig. 5). The weak correlations ($|\rho|<0.2$) between single-cell protein staining and the day of differentiation corroborated the weak, not statistically significant correlations between single-cell protein staining and Young’s modulus.

4. Discussion

During chemically-induced hMSC osteoblast differentiation, decreases in cellular stiffness, size, and circularity were observed. Whereas Young's modulus indicated differentiation of single cells, staining for BSP and OCN indicated differentiation more robustly at the population than the single-cell level.

The cellular softening observed during hMSC osteoblast differentiation may have resulted from concurrent changes in the underlying cellular structure. Recent studies predicted stem cell differentiation fate based on the combination of several actin filament morphology descriptors (Treiser et al., 2010). F-actin staining parameters, including mean intensity, total intensity, and the number of F-actin branches, changed during hMSC osteoblast differentiation (Treiser et al., 2010). Furthermore, the cellular softening observed during osteoblast differentiation has been attributed to a simultaneous transition from many thicker actin fibers to a fewer number of thinner actin fibers in hMSCs (Titushkin and Cho, 2007; Yourek et al., 2007) and amniotic fluid-derived stem cells (Chen et al., 2010). In hMSCs, actin stress fibers were long, thin, parallel, and oriented along the major axis of the cell; cytoskeletal rearrangement during osteoblast differentiation resulted in hMSC-OBs with thicker, disordered actin filaments (Rodríguez et al., 2004; Titushkin and Cho, 2007; Yourek et al., 2007). Interestingly, the actin organization of the spindle-shaped "rapidly self-renewing" hMSC subset matched the expected hMSC phenotype, but the "flat cell" hMSC subset matched the osteoblast phenotype (Docheva et al., 2008). The presence of hMSC morphology subsets indicates the large degree of hMSC heterogeneity, which may partially explain the large degree of stiffness variation observed in naive hMSCs (Fig. 3B).

To further investigate the notion of cytoskeletal rearrangement during osteoblast differentiation, raw gene expression data for Day 0, 1, 7, and 10–14 hMSC-OBs and hOBs (Granchi et al., 2010) were downloaded from the gene expression omnibus dataset GSE12267 (<http://www.ncbi.nlm.nih.gov/geo/>) and analyzed by gene set enrichment analysis (GSEA) and differential expression analysis (DEA), as described in the Supplementary Information. GSEA indicated that the chromosome, chromatin, and cytoskeleton cellular component ontologies were relevant to the phenotypic differences between hMSCs and hOBs. Furthermore, genes identified by DEA as differentially expressed between hMSCs and hOBs were found to be significantly enriched in cytoskeleton remodeling-related maps (Figs. S5 and S6). Several genes related to actin binding and regulation of the actin cytoskeleton were decreased (ACTN1 [α -actinin-1], ACTG2, ACTR2, ANLN, FBLIM1) or increased (TWF1 [twincillin], ADD3, and GSN [gelsolin]) in hOBs relative to hMSCs (Supplementary Excel Sheet 2). Time course analysis also identified 222 genes with consistent expression increase during osteoblast differentiation (Supplementary Excel Sheet 3).

Gene expression analysis suggested that cytoskeletal remodeling during osteoblast differentiation may result from the combined effects of reduced G-actin polymerization, reduced F-actin cross-linking, and enhanced severing of actin filaments. Reduced G-actin polymerization in hOBs was indicated by decreased expression of actin monomers (e.g. ACTG2), twincillin-mediated sequestration of G-actin (Ojala et al., 2002), and decreased

activity of the Arp2/3 complex. Reduced F-actin cross-linking in hOBs was indicated by decreased expression of α -actinin-1, which is critical in leading edge focal adhesion maturation during cell spreading (Kovac et al., 2013). Decreased expression of α -actinin-1 in hOBs may therefore partially explain the positive correlation between cell size and stiffness (Fig. 4A). Increased expression of twinfilin and gelsolin suggested enhanced severing of actin filaments in hOBs. Gene expression analysis therefore supported actin cytoskeletal rearrangement during osteoblast differentiation.

Previous work has directly linked actin reorganization to cell stiffness changes. Cells characterized by ordered actin filament geometry tended to be stiffer than cells with disordered actin fibers (Ketene et al., 2012; Xu et al., 2012). Furthermore, α -actinin-1, which was increased in hMSCs relative to hOBs, crosslinks actin filaments and increases the stiffness of the actin filament network (Esue et al., 2009; Xu et al., 1998). Therefore, based solely on actin fiber organization, hMSCs would be expected to soften during osteoblast differentiation, as shown previously (Chen et al., 2010; Titushkin and Cho, 2007) and in the present study. Furthermore, the connections between cellular stiffness and reported changes in cytoskeletal gene expression lend credence to the observed softening during osteoblast differentiation.

The mechanics data of spread morphology hMSCs during osteoblast differentiation is characterized by a large degree of variation (Darling et al., 2008; Docheva et al., 2008; Titushkin and Cho, 2007; Yourek et al., 2007). Thus, measures were taken to control substrate-induced stiffening and cell density, which could otherwise confound stiffness data since naive MSC stiffness increases with the duration of growth in vitro (Maloney et al., 2010). Passage 4 cells were used throughout the study to keep the total amount of time in vitro constant and reduce confounding substrate-induced stiffness changes. Furthermore, a “staggered” differentiation scheme was employed, allowing all cells to grow on tissue culture polystyrene for an equal amount of time before AFM analysis. Cells were also trypsinized and replated approximately 1 day before AFM measurements. Adhesion of the replated cells may have resulted in some differences in differentiation from traditional induction assays; however, replating at low cell density limited morphology changes due to uncontrolled cell-cell contact, which can influence cell mechanics measurements (Efremov et al., 2013). Factors that could otherwise confound stiffness comparisons between differentiation time points were controlled, which strengthens the claim that cell stiffness decreases during osteoblast differentiation.

BSP and OCN are commonly used to indicate osteoblast differentiation, but correlations between the day of differentiation and single-cell protein staining were not significant (Fig. 4A). The trend between protein staining and the day of differentiation was stronger for population staining than single-cell staining (Fig. 5C), which may reflect the extracellular localization of BSP and OCN as well as the concept of asynchronous osteoblast differentiation. Unlike single-cell staining, population averaged protein staining cannot detect subsets of cells that express BSP or OCN at any one time, as previously observed in the formation of nodules prior to hMSC osteoblast differentiation (Aubin and Triffitt, 2002). Young's modulus may therefore provide additional and improved information on the osteoblastic state of single cells.

As a label-free property of individual cells, Young's modulus holds great potential in phenotype identification. Furthermore, Young's modulus is attributable to individual suspended cells, and thus potentially lends itself to phenotypic cell sorting applications. Recent developments in cell separation by adhesion (Singh et al., 2013) and stiffness (Hur et al., 2012; Wang et al., 2013) indicate future label-free cell sorting capabilities for cases in which molecular biomarkers are not fully established. Continued investigation of various differentiation lineages, besides the osteoblast differentiation considered in this study, is required to further understand mechanical differentiation indicators. Additional characterization of stiffness and other cell-intrinsic physical properties promises to result in the development of novel, label-free cell identification and sorting capabilities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge Alice Cheng for scanning electron microscopy and Priya Baraniak for helpful discussions. hMSCs were provided by the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine at Scott & White through a Grant from NCRR of the NIH, Grant # P40RR017447. Funding was provided by the National Science Foundation, Grant # CBET-0932510, as well as the NSF Stem Cell Biomaterials IGERT (TB) and the President's Undergraduate Research Award (JK) at Georgia Tech. The study sponsors were not involved in the study design, the collection, analysis, and interpretation of data, the writing of the manuscript, or the decision to submit the manuscript for publication.

Abbreviations

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

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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.

Fig. 2.

Gridded Petri dish manufacture. (A) The gridded Petri dish design allowed sequential measurement of live-cell stiffness and fluorescent protein biomarker expression at the single cell level, enabling a cell-by-cell analysis of the relationships among differentiation, mechanical, protein staining, and morphological factors. Force-indentation data were used to evaluate the Young's modulus of each cell. (B) Following engraving, the gridded Petri dish was covered with a glass coverslip, which prevented the grid from influencing morphology during cell attachment. (C) Magnified region of interest of Panel B, indicated in blue. The design of the grid was chosen to facilitate pinpointing of individual cells during AFM and fluorescence microscopy; scale bar, 750 μm . (D) Magnified region of interest of Panel C, indicated in red. The locations of individual cells within the grid were recorded during AFM. Dashed black lines indicate grid; scale bar, 25 μm . (E) AFM stiffness measurements were taken using a beaded cantilever to increase cell-probe surface area, thereby allowing measurement of bulk cellular Young's modulus. Scanning electron micrograph; scale bar, 10 μm . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Atomic force microscopy. (A) Three force-indentation curves per cell were fit to the Hertzian contact model to calculate the mean Young's modulus. Representative force-indentation curves yielded mean Young's moduli of approximately 4.3 kPa for the naive hMSC (blue), and 1.9 kPa for the hOB (red). (B) Young's modulus generally decreased as osteoblast differentiation progressed, although a large degree of variation was observed (coefficient of variation > 0.5). Lines indicate mean $\pm$ standard error. Day 0, $n=26$; Day 6, $n=28$; others, $n=30$. (C) Statistical significance of mean Young's modulus differences among the 8 cell conditions is displayed. Importantly, Day 20 hMSC-OBs were significantly softer than naive hMSCs, but not hOBs. Holm's adjusted p -values range from 0 (bright red) to 1 (bright blue). Crosshatch pattern indicates that the difference between mean Young's moduli is not statistically significant ($\alpha=0.10$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Single-cell correlations. (A) For 225 individual cells, values of the 14 differentiation, mechanical, protein staining, and morphological parameters were determined from AFM data and fluorescence image processing. Staining for BSP and OCN was quantified as the fraction of pixels within the cell boundary that exceeded a threshold value ("percent area"). Pairwise Spearman's correlation coefficients among the 14 variables are displayed. Spearman's rank correlation coefficients range from -1 (bright blue) to $+1$ (bright red). Crosshatch pattern indicates a correlation that was not statistically significant based on Holm's adjusted p -values ($\alpha=0.10$). Day 0, $n=26$; Day 3, $n=29$; Day 6, $n=24$; Day 20, $n=27$; hOBs, $n=29$; others, $n=30$. A significant inverse relationship between Young's modulus and the day of differentiation was observed. The strong, positive correlation between bone sialoprotein (BSP) and osteocalcin (OCN) staining appears to suggest that BSP and OCN staining both reflect osteoblast differentiation as expected. However, correlations between the day of differentiation and protein staining were weak and not statistically significant. (B) For each cell condition, the correlation between BSP (top, blue) or OCN staining (bottom, red) and Young's modulus was weak ($|\rho| < 0.45$) and not statistically significant (for other time points, see Fig. S4). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Biomarker changes during hMSC differentiation. (A) The difference in bone sialoprotein (BSP) and osteocalcin (OCN) staining between the representative naive hMSC and hOB images was visually more apparent for population than single-cell images, indicating that BSP and OCN staining may more rigorously indicate osteoblast differentiation for cell populations than single cells. Scale bars, 50 μ m. (B) BSP and OCN staining did not appear to trend strongly with the day of differentiation for single-cell staining, but population staining revealed more abundant protein staining for the later days of differentiation (mean + standard error). (C) Correlations between the day of differentiation and protein staining were weaker for single-cell than population staining. Spearman's rank correlation coefficients range from -1 (bright blue) to $+1$ (bright red). Checkerboard pattern indicates a correlation that was not statistically significant based on Holm's adjusted p -values ($\alpha=0.10$). Single-cell data are repeated from Fig. 4A, but Holm's adjustment was reapplied using $N=3$ for purposes of comparison. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)