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Spatially Coordinated Changes in Intracellular Rheology and Extracellular Force Exertion during Mesenchymal Stem Cell Differentiation

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Abstract

The mechanical properties within the cell are regulated by the organization of the actin cytoskeleton, which is linked to the extracellular environment through focal adhesion proteins that transmit force. Chemical and mechanical stimuli alter the organization of cytoskeletal actin, which results in changes in cell shape, adhesion, and differentiation. By combining particle-tracking microrheology and traction force cytometry, we can monitor the mechanical properties of the actin meshwork and determine how changes in the intracellular network contribute to force generation. In this study, we investigated the effects of chemical (differentiation factors) and mechanical (substrate rigidity) stimuli important in mesenchymal stem cell (MSC) differentiation on the intracellular mechanics and traction stress generation. We found the presence of adipogenic factors resulted in stiffening of the actin meshwork regardless of substrate rigidity. In contrast, these factors increased traction stresses on hard substrates, which was associated with increased expression of contractility genes. Furthermore, MSCs cultured on hard substrates expressed both adipogenic and osteogenic markers indicative of mixed differentiation. On hard substrates, heterogeneity in the local elastic modulus-traction stress correlation was also increased in response to adipogenic factors, indicating that these mechanical properties may be reflective of differences in level of MSC differentiation. These results suggest intracellular rheology and traction stress generation are spatially regulated and contribute insight into how single cell mechanical forces contribute to MSC differentiation.

Keywords

Mesenchymal stem cells; Differentiation; Multiple particle-tracking microrheology; Traction force cytometry; Mechanotransduction

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Introduction

Mesenchymal stem cells (MSCs) are adult bone marrow-derived stem cells with the ability to differentiate into connective tissues, including adipocytes and osteoblasts [1]. MSCs are ideal for tissue engineering applications due to their multipotency and immunosuppressive characteristics [2]; however, an incomplete understanding of how microenvironmental factors collectively direct stem cell fate hinders their therapeutic potential. Current techniques, such as histological staining and transcriptional analysis, measure the overall rate of MSC differentiation, not local differences in differentiating cells. A holistic understanding of how the microenvironment, including soluble factors and mechanical cues, directs stem cell differentiation on a cellular level is critical for clinical translation of MSC-based therapeutics.

Early MSC differentiation studies utilized a cocktail of soluble factors to induce differentiation on tissue culture plastic [1]; however, further studies demonstrated that biophysical factors such as matrix rigidity [3], cell shape [4], and geometric cues [5] also influence differentiation. In addition, mechanical cues, including intracellular and extracellular mechanics, can influence the effects of soluble factor cues from growth factors and cytokines [6]. This led to probing of intracellular mechanical properties [7–9] and traction force generation [10–12] and forecasting of stem cell fate based on actin cytoskeleton organization [13].²

During the differentiation process, MSCs rearrange their cytoskeleton [13,14] which leads to changes in intracellular mechanical properties [9] and traction force generation [12]. Traditional intracellular mechanical characterization techniques, such as atomic force microscopy (AFM), typically do not allow for simultaneous measurement of traction forces; consequently, the connection between intracellular and extracellular forces is not completely understood. Multiple particle-tracking microrheology (MPTM), which involves tracking the motion of hundreds of individual particles embedded in the cell cytoplasm, can be used to rapidly probe the local and bulk rheological properties of the cell cytoplasm to determine mechanical properties in a high-content and spatial manner [15]. Cells ballistically injected with nanoparticles can be cultured on elastic substrates for simultaneous measurement of intracellular rheology and cell traction stress.

In this study, we combined MPTM and traction force microscopy to gain further insight into the mechanical properties and force generation of MSCs undergoing differentiation. Human MSCs were cultured on soft (~2 kPa) and hard (~8 kPa) substrates in the presence of control, adipogenic, osteogenic, and mixed differentiation media. We monitored local intracellular rheological properties and traction stress simultaneously and found MSCs increase the elastic character of their cytoplasm in response to adipogenic factors. On soft substrates, which were conducive for adipogenesis, cells did not alter traction stresses in response to differentiation factors; however, on hard substrates traction stresses increased in cells cultured with adipogenic factors. This increase in traction stresses was associated with

²Abbreviations: MPTM, multiple particle-tracking microrheology; CM, control media; AM, adipogenic media; OM, osteogenic media; MDM, mixed differentiation media

an increase in heterogeneity in the correlation between local intracellular rheology and local traction stress as well as decreased adipogenesis. The percentage of areas in the cell with highly elastic cytoplasm and high local traction stress also increased, suggesting cells spatially regulate cytoplasmic elastic modulus and traction stress in response to these factors. Gene expression analysis revealed myosin light chain kinase and myosin heavy chain 9 were both upregulated, which is potential mechanism by which these cells increase traction stresses. This study establishes that MSCs spatially alter cytoplasmic rheology and traction stresses during differentiation.

Materials and Methods

Cell Culture

Human MSCs isolated from a 22 year old healthy male donor (Donor 7071L) were obtained from Texas A&M Institute for Regenerative Medicine and cultured in α MEM (Corning) with 20% FBS (Atlanta Biologicals), 1% penicillin-streptomycin (Corning), and 1% L-glutamine (Corning). For differentiation assays, control media (CM) consisted of α MEM with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine. Cells were seeded at a density of 25,000 cells/cm² in order to allow for differentiation along the adipogenic lineage [4]. Adipogenic media (AM) was CM supplemented with 10 μ g/mL human insulin, 0.5 μ M dexamethasone, 0.5 μ M isobutylmethylxanthine, and 50 μ M indomethacin [16]. Osteogenic media (OM) was CM with 10 nM dexamethasone, 50 μ M ascorbate-2-phosphate, and 20 mM β -glycerolphosphate (Sigma Aldrich) added [16]. Mixed differentiation media (MDM) was created by mixing AM and OM at a 1:1 ratio. Media was replaced every 3 days for all differentiation assays.

Substrate Synthesis

Polyacrylamide substrates were synthesized for cell analysis as previously described[17]. Two different rigidity substrates were fabricated: soft with 8% acrylamide (Sigma Aldrich) and 0.02% bis-acrylamide (~2 kPa, GE Healthcare Life Sciences) and hard (8% acrylamide and 0.06% bis-acrylamide, ~8 kPa). Briefly, glass coverslips were activated by incubation in 1% 3-aminopropyltriethoxysilane followed by 0.5% glutaraldehyde. Acrylamide solutions were made with 200 nm red particles (Invitrogen) and ammonium persulfate and tetramethylethylenediamine were added to initiate gel polymerization. This solution was then added to glass slides and an activated coverslip added on top. In order to allow for cell adherence, the surface was activated with 0.2 mg/mL sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino)-hexanoate (G-Biosciences) and coated with 0.2 mg/mL rat tail collagen I (Corning), which has previously been shown to play a critical role in MSC differentiation [18], overnight at 4°C with agitation. Substrates were sterilized under UV light and incubated in media for at least an hour before use. Mechanical properties of the substrates were assessed via compression testing using a Bose Endura TEC ELF 3200 Uniaxial Testing System (data not shown). The Young's modulus was calculated from the slope of the linear region of the stress vs. strain curve at less than 10% strain.

Gene Expression Analysis

mRNA was isolated from hMSCs using Ribozol reagent (Amresco) and its quality confirmed using spectrophotometry. cDNA was reverse transcribed from mRNA using iScript cDNA synthesis kit (BioRad). PrimerBLAST was used to design primers for osteogenic and adipogenic differentiation (Table 1). In order to quantify gene expression, SsoAdvanced SYBR Green Supermix (BioRad) was used along with primers to amplify target sequences in a Step One Plus thermocycler (Applied Biosystems). Gene expression was determined using a comparative C_T method [19] with GAPDH as an endogenous control for all experiments. Data is relative to the glass CM condition for analysis of differentiation genes. For contractility gene analysis, data is reported as gene expression relative to CM for the corresponding substrate rigidity.

Differentiation Staining

After 5 weeks in differentiation media, cells were fixed in formalin and stained for either lipid droplets using 1.5% Oil Red O (Sigma Aldrich) or calcium deposits using 1% Alizarin Red (Sigma Aldrich) [20]. Images of fat droplets were taken using a Nikon Eclipse TS100 Inverted Microscope. A segmentation algorithm in MATLAB (Mathworks) was used to determine the area covered by Oil Red O. Alizarin Red was extracted using established protocols [21] and the absorbance measured at 450 nm.

Microrheological Characterization

Multiple particle-tracking microrheology (MPTM) was used to determine the cytoplasmic rheological properties of hMSCs undergoing differentiation as described [20]. Previous studies have demonstrated that traction force [12] and intracellular mechanical properties [7] are altered after 1 week in differentiation media; hence, cells were analyzed after 1 week in differentiation media in order to evaluate cytoskeletal changes before traditional techniques (i.e. qRT-PCR and staining) can detect differentiation. Human MSCs were ballistically injected with 200 nm green fluorescent particles (Invitrogen), allowed to recover, and then seeded on polyacrylamide substrates. After one week in differentiation media, videos of particle motion were taken using a Nikon Eclipse Ti inverted microscope equipped with a Photometrics QuantEM CCD camera. Imaging was performed with a 40x oil immersion lens with 1.5x zoom. The x-y coordinates where each video was taken were saved for traction force analysis. An average of 15 cells with 10–20 particles per cell was analyzed per experiment. Particle traces were created by applying a bandpass filter, determining the centroid of the particle as described [22], and the trajectories linked using a Hungarian linker algorithm. Mean square displacements (MSD) of particles were determined using a previously described custom MATLAB algorithm [20] and the following equation: $\langle \Delta r^2(\tau) \rangle = \langle [x(t+\tau) - x(t)]^2 + [y(t+\tau) - y(t)]^2 \rangle$. Cytoplasmic fluid is locally isotropic [23]; therefore, it is sufficient to track displacements in the x and y directions to estimate the 3D projection. The MSDs were then related to the frequency-dependent complex shear modulus (G^*) using the following equation [15]:

$$G^*(\omega) = \frac{2k_B T}{3\pi a \left\langle \Delta r^2 \left(\frac{1}{\omega} \right) \right\rangle \Gamma[(1+\alpha(\omega))(1+\beta(\omega)/2)]}$$

k_B is Boltzmann's constant, T is temperature (37°C), a is the particle size, Γ is the gamma function, α is the first derivative and β is the second derivative of the MSD curve. The elastic (G') and viscous (G'') moduli are the imaginary and real components of G^* , respectively. Phase angle was calculated using the following equation: $\delta = \arctan(G''/G')$, where $\delta=0^\circ$ is an elastic solid and $\delta=90^\circ$ is a viscous liquid.

Traction Force Microscopy

After particle tracking videos were collected, images of the cells and stressed gels were taken. Cells were then lysed with 0.5% SDS (BioRad) and an image of the unstressed gel was obtained. In addition, an image of the stationary bottom of the gel was taken before and after detachment to correct for drift. An average of 15 cells was analyzed per experiment. A custom-written MATLAB algorithm was then used to determine the cell-generated displacement field and calculate traction forces [24].

Rheology-Traction Force Correlation Analysis

Local rheological properties were correlated with local traction stresses using a custom-written MATLAB algorithm. After MPTM and traction stress analysis, the mean traction stress for a 3-pixel dilation around each particle location was calculated. This calculated mean local traction stress was normalized to the mean traction stress of all conditions of the same substrate rigidity and correlated with the elastic (G') and viscous (G'') modulus of the cytosol surrounding the particle in the cell.

Immunofluorescence Staining and Analysis

MSCs cultured in differentiation media for 1 week were fixed in formalin, permeabilized with Triton X-100 (BioRad), blocked with serum, and stained for F-actin and vinculin using rhodamine phalloidin (Invitrogen) and rabbit anti-vinculin (Invitrogen) followed by goat anti-rabbit Alexa Fluor 488 (Invitrogen). Imaging was performed with a Zeiss LSM 700 confocal microscope at 40x magnification.

Statistical Analysis

Gene expression and staining experiments were performed in triplicate. For MPTM and traction force analysis, 4–6 experiments were performed. A student's t-test was used for all statistical analysis with $p < 0.05$ being statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Data were reported as the mean $\pm$ standard error of the mean unless otherwise noted.

Results

Differentiation Capacity of MSCs in Response to Mechanical and Chemical Factors

In order to analyze the ability of MSCs to differentiate along the adipogenic and osteogenic lineage, qRT-PCR and histological staining were performed after 3 and 5 weeks of culture, respectively. As previously observed [12], cells cultured in mixed differentiation media (MDM) displayed higher expression of adipisin on soft substrates (Fig. 1A) and higher expression of osteocalcin on hard substrates (Fig. 1B) though adipisin and osteocalcin expression in MDM were approximately equal to the negative control on glass and soft substrates, respectively. Though osteocalcin expression was higher for MSCs cultured in MDM on the hard substrate (8 kPa), this intermediate rigidity resulted in mixed differentiation into osteoblasts and adipocytes. These results were confirmed by histology with enhanced Oil Red O coverage on soft substrates (Fig. 1C) and increased Alizarin Red on hard substrates (Fig. 1D). These results suggest that in the presence of mixed chemical cues, the rigidity of the environment directs differentiation.

Adipogenic Factors Increase Elastic Character of MSCs

Multiple particle-tracking microrheology (MPTM) was used to determine the local and bulk rheological properties of the cytoplasm for cells cultured in control or differentiation media on substrates early in the differentiation process (1 week). The use of MPTM allowed for the simultaneous measurement of intracellular rheological properties and traction stresses (Fig. 2A). Substrate rigidity had no effect on the bulk rheological properties determined from the ensemble average particle mean square displacements (Fig. 2B) however, the presence of adipogenic factors in either adipogenic media (AM) or MDM increased the elastic modulus (G') on both soft and hard substrates (Fig. S1A). Differentiation and mechanical factors had less of an effect on the viscous modulus (G'' , Fig. S1B). To further characterize the viscous and elastic properties of the cytoplasm, the phase angle δ was calculated with $\delta = 90^\circ$ for a liquid and $\delta = 0^\circ$ for a solid. The MSC cytoplasm was primarily viscous for all conditions, as indicated by phase angles much larger than 45° . The phase angle was not altered by OM, indicating that osteogenic factors had no effect on the bulk mechanical properties within the range of substrate rigidities used in this study. AM and MDM significantly ($p < 0.01$) decreased the phase angle in both soft and hard environments, suggesting that adipogenic factors were responsible for the intracellular stiffening response in MDM (Fig. 2E).

Stiffer Environments Lead to Altered Force Profiles in Response to Adipogenic Factors

Traction force cytometry was used to determine the traction stress profiles of differentiating MSCs. Traction stresses are affected by two main factors: substrate elasticity, and matrix displacement. Traction stresses were independent of media composition on soft substrates where the mean traction stress was ~ 500 Pa for all conditions (Fig. 3A). In agreement with previous studies [25], the traction stress on hard substrates was increased approximately 2-fold in control conditions and up to 5-fold when adipogenic factors were present. Analysis of the displacements on each substrate revealed that cells in AM and MDM maintained the same matrix displacements on soft and hard substrates, whereas displacements decreased for CM and OM (Fig. 3B). Cells in CM and OM had similar traction stress profiles and

displacements, suggesting increases in forces in MDM are due to adipogenic factors, not osteogenic factors.

Adipogenic and Mixed Differentiation Media Lead to Shifts in Rheology-Traction Stress Spatial Regulation in Hard Environments

In order to determine how local rheological properties are related to local traction stresses, the spatial relationship between these two properties was evaluated. For these calculations, the rheological properties of the cytosol surrounding a single particle were calculated and related to the traction stress exerted on the substrate below that point (Fig. 4). Cells in AM and MDM on both rigidity substrates displayed an increased number of particles with high elastic modulus (G'). In addition, this number appeared to be further increased on hard substrates with AM and MDM. Particles associated with high G' also appeared to be colocalized with areas of high traction stress; therefore, spatial analysis of these two properties was performed to quantify this observation. On soft substrates, spatial regulation of traction stress and elastic modulus was relatively unaffected by differentiation media. The local elastic and viscous (G'') moduli and local traction stress of cells in CM or OM on hard substrates clustered around a low normalized traction force value and similar elastic modulus; however, the presence of adipogenic factors in AM and MDM introduced a large degree of heterogeneity in this relationship (Fig. 5). In addition, the percentage of particles with a corresponding high traction stress increased for cells in AM and MDM on hard, with approximately a 5-fold increase of particles with high G' and high traction stress and a 2.5-fold increase in particles with low G' and high traction stress. A similar trend was found between traction stress and G'' .

Increased Forces in Response to Adipogenic Factors are Associated with Increased Contractility Gene but not Adhesion Protein Expression

Cells exert forces through sites of adhesion; consequently, increased adhesion protein expression may lead to increased traction stresses. In order to determine if enhanced traction stresses exerted by cells was a result of increased adhesion protein expression, cells were stained for F-actin and vinculin and imaged using confocal microscopy. F-actin and vinculin distribution were similar across substrate rigidity and media conditions (Fig. 6A). Quantification of the ratio of vinculin to F-actin [26] revealed vinculin expression was not upregulated in AM and MDM on stiff substrates (Fig. 6B), suggesting the increase in traction force is not a result of increased adhesion.

Focal adhesion proteins connect the extracellular environment to the actin cytoskeleton, which mediates force generation through actin myosin contraction. Changes in contractility genes may lead to increased traction stress; therefore, we evaluated gene expression of myosin light chain kinase (MLCK), myosin heavy chain 9 (MYH9), and myosin light chain 9 (MYL9). On soft substrates, contractility genes were either downregulated or unchanged in MDM compared to CM; however, on hard substrates both MLCK and MYH9 were significantly upregulated (Fig. 6C).

Discussion

In this study, we simultaneously characterized changes in intracellular rheology and traction force generation during MSC differentiation on soft (~2 kPa) and hard substrates (~8 kPa). High rigidity environments are typically more conducive for osteogenesis, whereas low rigidity environments favor adipogenesis; however, high rigidity environments inhibit adipogenesis to a greater degree than low rigidity environments inhibit osteogenesis [12]. As a result, lower rigidity substrates were selected to allow for differentiation along both lineages. Initially, the cytoplasm was primarily viscous; however, the elastic character increased with exposure to adipogenic factors. MPTM has previously been utilized to show that undifferentiated embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) [27], and MSCs [20,26] have cytoplasm that are primarily viscous. In ESCs and iPSCs intracellular stiffening was also associated with the degree of stem cell differentiation [27,28]. A similar trend is present when comparing the cytoplasm of *C. elegans* embryos to that of more differentiated cells [20,26,27]. The cytoplasm of MSCs in AM and MDM had increased elastic character, suggesting this intracellular stiffening response during stem cell differentiation may be conserved in adipogenesis and may be critical for adipogenesis to occur. In addition, unspread cells are more likely to become adipocytes, whereas more spread cells are more likely to differentiate along the osteogenic lineage [4]. The actin cytoskeleton is a regulator of cell shape; thus, its ability to rearrange and alter its rheological properties may be critical for cell shape changes necessary for differentiation. The elastic character could also be a potential marker for MSC differentiation. Compared to traditional methods, MPTM can be performed without fixing or lysing cells which would allow for screening of differentiation in live cells. In addition, changes in the cytoplasm were detected at 1 week, whereas traditional differentiation analysis techniques such as PCR and staining cannot detect differences until much later in the differentiation process (approximately 3–5 weeks).

We recognize that this intracellular stiffening response seems to contradict previous findings with atomic force microscopy (AFM), which showed that adipocytes have lower elasticities than MSCs and osteoblasts [8]. However, this discrepancy may be a result of inherent differences in mechanical characterization techniques because AFM typically probes large stress fibers [29], whereas MPTM probes much smaller actin filaments on the order of nanometers [30] that are not accessible via AFM. Thus, particles embedded in cells for MPTM interact with smaller actin meshworks in the cell body, whereas AFM tips interact with significantly stiffer stress fibers in the cell cortex [31]. Since fully differentiated adipocytes lose their larger stress fibers [32], mechanical properties probed by MPTM may be more relevant for these more differentiated cells. In addition, decreased stress fiber formation has been associated with increased elastic moduli in cancer cells [33] and in MSCs treated with tumor-conditioned media [26]. Thus, the adipogenic process may lead to loss of stress fibers in favor of a more confined actin meshwork that has previously not been detected using other mechanical characterization techniques.

The rheological properties of cells were relatively unaffected by the presence of OM (Fig. 2). MSCs and osteoblasts have previously been shown in one study to have similar mechanical properties as measured by AFM [8]; however, there is not a clear consensus as

to whether MSCs differentiating along the osteogenic lineage are stiffer or softer than their undifferentiated counterparts [7,8,34]. Osteogenic differentiation is typically characterized by loss of thin parallel filaments and gain of a few actin filament bundles at the periphery of the cell [14]. MPTM cannot probe thick actin filaments [31] so these changes in cytoplasmic rheology may not be detected, but our results do demonstrate that the actin microstructure is unchanged during osteogenesis. Contrary to previous studies performed on microposts conducive for osteogenesis [12], OM also did not alter the traction stresses exerted by MSCs (Fig. 3). Osteogenesis is typically favored in stiff environments [3,11]; consequently, MSCs may alter their rheological and traction profiles outside of the rigidity range studied here.

A relatively modest increase in substrate rigidity (~2 kPa to 8 kPa) led to dramatic traction stress exertion differences. Though traction stresses were increased in cells cultured in AM and MDM on hard substrates, on soft substrates, which were more conducive for adipogenesis (Fig. 1), traction stress was independent of media composition (Fig. 3A). Previous studies have demonstrated that MSCs undergoing differentiation on micropatterned substrates conducive for adipogenesis do not significantly alter their traction stresses [12]. Inhibition of cell contractility, which can regulate traction stresses, in MSCs cultured with mixed differentiation factors in significantly stiffer glass environments induces adipogenesis [4]. Moreover, in stiffer environments, which are more conducive for osteogenesis, traction stresses are increased. Consequently, inhibiting the high traction stresses generated in less favorable (stiffer) environments in the presence of adipogenic factors may enhance differentiation along the adipogenic lineage by reducing the energy barrier. Previous studies have demonstrated that enhanced mitochondrial respiration is required for adipogenesis [35]; therefore, ATP required for cell contractility purposes would not be available for mitochondrial respiration. Taken together, these results could imply that alteration of traction forces in non-conductive mechanical environments may prevent MSC differentiation along the adipogenic lineage, offering further insight into how matrix stiffness directs cell lineage.

Deformation of the substrate decreased with increasing substrate rigidity for cells in CM, in agreement with studies with fibroblasts [36]. The presence of adipogenic factors on hard substrates was associated with increased levels of substrate deformation similar to substrate deformations caused by cells on soft substrates (Fig. 3B). In addition, these factors were associated with an increase in actomyosin contractility gene expression on hard substrates (Fig. 6). Previous studies have shown cell stiffness measured by magnetic twisting cytometry increases in order to balance tensile stress [37]; however, how local cytoplasmic stiffness changes with local traction stresses has not been elucidated. In agreement with average rheological properties measured (Fig. 2), cells cultured in AM and MDM displayed increased numbers of areas with high cytoplasmic elastic modulus (G' , Fig. 4). This response was amplified on hard substrates and was associated with high traction stresses (Fig. 4), suggesting high local G' may be associated with high traction stress. Spatial analysis revealed adipogenic factors increase the percentage of areas with high G' with corresponding high local traction stresses (Fig. 5). Moreover, AM and MDM induced approximately a 5-fold increase the percentage of particles with surrounding high elasticity and viscosity cytoplasm and high local traction stress. This suggests that the cell increases the number of areas with spatially regulated cytoplasmic rheological properties and traction

stress in response to adipogenic factors. The elastic and viscous character of the cytoplasm is simultaneously spatially altered with traction force in response to AM and MDM. The elasticity and viscosity of the cytoplasm concurrently changing has been documented previously in MSCs treated with tumor conditioned media [20,26], endothelial cells treated with VEGF [38], and in model actin solutions [39,40]. Greater increases in average G' than G'' occurred in response to AM and MDM compared to CM and OM (Fig. S1), phase angle was calculated (Fig. 2C) and decreased in these conditions, suggesting changes in the elastic modulus are dominating this response. In addition, the percentage of particles with low elastic and viscous moduli and low traction stress decreased in AM and MDM compared to CM and OM for both substrate rigidities. This may reflect a mechanical signature of MSCs undergoing adipogenesis and could potentially be used as a subcellular marker for differentiation. Increases in cell stiffness in response to increasing tensile stress has been attributed to myosin-based contractile machinery activation [37]. Expression of MLCK and MYH9 were upregulated in cells cultured on hard substrates in MDM, suggesting that myosin may also play a critical role in mechanotransduction in MSCs differentiating on hard substrates.

Conclusions

In conclusion, we simultaneously measured the local intracellular rheological properties and traction stress generation of MSCs undergoing differentiation on soft and hard substrates. The cytoplasm of MSCs cultured with adipogenic factors was more elastic independent of substrate rigidity. However, these factors only induced increases in traction stresses on hard substrates, which was associated with increased expression of contractility-related genes and decreased differentiation, suggesting force generation is critical for directing differentiation. Increased heterogeneity was present in the correlation between rheological properties and traction stress for MSCs cultured on hard substrates with adipogenic factors. In addition, there was an increase in percentage of areas in the cell with highly elastic cytoplasm and high local traction stress, suggesting local cytoplasmic rheology and traction stress are spatially regulated during differentiation. These results may have important implications for detecting single cell differentiation important in regenerative medicine applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Differentiation capacity of MSCs cultured in different rigidity environments. mRNA expression analysis of adipogenic (A) and osteogenic (B) markers. GAPDH was used as an endogenous control and all data was normalized to the glass CM condition. Quantification of Oil Red O coverage (C) and Alizarin Red intensity (D). * denotes $p < 0.05$.

Figure 2. Microrheological characterization of MSCs undergoing differentiation on soft and hard substrates. Rheological properties and traction stresses of MSCs on substrates were simultaneously measured (A). Frequency-dependent elastic (G' , open circles) and viscous (G'' , open squares) moduli of differentiating MSCs (B). Viscoelastic character was determined by calculating the phase angle at $1 s^{-1}$ (C). ** denotes $p < 0.01$.

Figure 3.

Traction stresses exerted by differentiating MSCs on soft and hard substrates. Quantification of log mean traction stress (A) and bead displacement (B) of cells cultured on different rigidity substrates in the presence of CM, AM, OM, or MDM. ** and *** denote $p < 0.01$ and $p < 0.001$, respectively.

Figure 4.

Spatial analysis of elastic modulus (G') of the cytoplasm and traction stress (TS). Contour maps of traction stress overlaid with local elastic modulus surrounding particles embedded in the cytoplasm (scale bar=10 μm). Particles have been enlarged for visualization purposes.

Figure 5.

Correlation of traction stress with microrheological properties. Correlation of local elastic (G') and viscous (G'') modulus with local normalized traction force (B, n is number of particles analyzed. Soft CM: n=2389, Soft AM: n=3537, Soft OM: n=2031, Soft MDM: n=1889, Hard CM: n=3923, Hard AM: n=3830, Hard OM: n=4125, Hard MDM: n=4466). Heat bar is number of counts (intracellular particles analyzed).

Figure 6.

Analysis of mechanisms for traction stress generation. Maximum intensity projection immunofluorescent images of hMSCs stained for F-actin (red), vinculin (green), and nuclei (blue) (A). Images were used to calculate the vinculin to actin ratio and normalized to Soft CM condition (B). Gene expression compared to CM of myosin light chain kinase (MLCK), myosin heavy chain 9 (MYH9) and myosin light chain 9 (MYL9) (C). * denotes $p < 0.05$.

Table 1

Primers used for qRT-PCR.

Gene		Primer Sequence	Accession Number
GAPDH	Forward 5'	GAAGGTGAAGGTCGGAGTC	NM 002046
	Reverse 5'	GAAGATGGTGATGGGATTTTC	
Osteocalcin	Forward 5'	GGCAGCGAGGTAGTGAAGAG	NM 001199662
	Reverse 5'	CTGGAGAGGAGCAGAACTGG	
Adipsin	Forward 5'	GGTCACCCAAGCAACAAAGT	NM 001928
	Reverse 5'	CTACAAGCACCCACCTCCAT	
MLCK	Forward 5'	CGGAGGGAGTGGAGTACATC	NM 053025
	Reverse 5'	CACAAATTCTGGGGTGCCAA	
MYH9	Forward 5'	AGGACCAGAACTGCAAGCTG	NM 002473
	Reverse 5'	GCGCTCTTCCAAGTCAGTGA	
MYL9	Forward 5'	AAAGGCGTTGCGAATCACAT	NM 006097
	Reverse 5'	ACCCACAGACGAATACCTG	