Directing osteogenic and myogenic differentiation of MSCs: interplay of stiffness and adhesive ligand presentation

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Rowlands AS, George PA, Cooper-White JJ. Directing osteogenic and myogenic differentiation of MSCs: interplay of stiffness and adhesive ligand presentation. Am J Physiol Cell Physiol 295: C1037–C1044, 2008. First published August 27, 2008; doi:10.1152/ajpcell.67.2008.—The mechanical properties of the extracellular matrix (ECM) can exert significant influence in determining cell fate. Human mesenchymal stem cells (MSCs) grown on substrates with varying stiffness have been shown to express various cell lineage markers, without the use of toxic DNA demethylation agents or complex cocktails of expensive growth factors. Here we investigated the myogenic and osteogenic potential of various polyacrylamide gel substrates that were coated with covalently bound tissue-specific ECM proteins (collagen I, collagen IV, laminin, or fibronectin). The gel-protein substrates were shown to support the growth and proliferation of MSCs in a stiffness-dependent manner. Higher stiffness substrates encouraged up to a 10-fold increase in cell number over lower stiffness gels. There appears to be a definitive interplay between substrate stiffness and ECM protein with regard to the expression of both osteogenic and myogenic transcription factors by MSCs. Of the 16 gel-protein combinations investigated, osteogenic differentiation was found to occur significantly only on collagen I-coated gels with the highest modulus gel tested (80 kPa). Myogenic differentiation occurred on all gel-protein combinations that had stiffnesses >9 kPa but to varying extents as ascertained by MyoD1 expression. Peak MyoD1 expression was seen on gels with a modulus of 25 kPa coated in fibronectin, with similar levels of expression observed on 80-kPa collagen I-coated gels. The modulation of myogenic and osteogenic transcription factors by various ECM proteins demonstrates that substrate stiffness alone does not direct stem cell lineage specification. This has important implications in the development of tailored biomaterial systems that more closely mimic the microenvironment found in native tissues.

Bone marrow-derived mesenchymal stem cells (MSCs) are an optimal cell source for cellular therapy and tissue engineering applications; they can be autologously derived, expanded ex vivo to clinically relevant cell numbers, and directed to differentiate into multiple cell types. MSCs are capable of significant expansion and can be directed toward adipogenic, chondrogenic, neurogenic, myogenic, and osteogenic lineages (1, 18, 22, 27). In vivo MSCs are known to migrate to sites of inflammation and engraft in a variety of tissues where they appear to play an active role in tissue repair and regeneration through secretion of chemokines as well as differentiation into “mature” cell types (1, 4, 25). This differentiation is likely to be initiated and directed by the MSC sensing changes in its local microenvironment as it engraves in the tissue.

The microenvironment in which the MSCs reside has been shown to be deterministic of their behavior. This complex environment consists of soluble factors, cell-cell interactions, and cell-extracellular matrix (ECM) interactions. The microenvironment provides three major categories of physicochemical signaling: chemical, topographical, and mechanical (29). Cell-ECM interactions are incredibly complex and have been shown to regulate many cellular processes, including proliferation and differentiation (10). Cell-ECM and cell-cell signaling is mediated by transmembrane proteins, predominantly integrins, but also cadherins, cell adhesion molecules, and selectins, that link the cell to the proteins of the ECM and to other cells (10, 20).

Integrins are a family of calcium-dependent heterodimeric transmembrane receptors found in all mammalian cells. Integrins are composed of an α- and β-chain having large extracellular and mostly short cytoplasmic domains. Currently, there are at least 24 known receptor combinations, each of which has some specificity for particular binding sites on ECM proteins (14, 24). Integrin binding to the ECM and the generation of cytoskeletal tension through actin-myosin-driven contraction initiate the formation of a focal adhesion complex, which involves more than 50 proteins that generate a submembrane plaque mediating the interaction between the ECM and cytoskeleton (2, 11).

It is well established that cell-matrix interactions play a crucial role in the development of the differentiated phenotype. In the mouse mammary gland, β1-integrin-mediated interactions with the ECM induce synthesis of the milk protein β-casein. In human mammary epithelial cell lines, α5β1-integrin modulates epithelial differentiation and glandular morphogenesis (16). In basal keratinocytes, α6β4-integrins have been shown to be critical in establishing cell polarity (16). Furthermore, knockout mice studies have shown the explicit role of integrins in establishing and maintaining tissue function (see Ref. 15 for a review). These studies indicate that the characteristics of a cell are largely defined by its expression profile of integrin receptors.

Engler et al. (8) recently showed that during ex vivo culture of MSCs, lineage specification could be directed by the elasticity of the matrix on which the cells are grown. By tuning the elastic modulus of collagen I-coated polyacrylamide to that of brain (0.1–1 kPa), muscle (8–17 kPa), and collagenous bone (>34 kPa), the authors rudimentarily simulated the environ-

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ment that cells “feel” in these tissues. Using this approach, the authors demonstrated that MSCs differentiate into neurogenic, myogenic, and osteogenic precursors when cultured on gels of varying elasticity. The MSCs sense and respond to the matrix stiffness by sampling their pericellular environment through integrin adhesions that link the ECM to the cytoskeleton through focal adhesion complexes, a process known as mechanotransduction.

Integrins are not only responsible for cellular adhesion and mechanosensitivity, but they also have a direct role as cell signaling molecules. Each integrin receptor combination has affinity for a single, or a few specific ligand sequences found in different ECM molecules; e.g., \( \beta_{1}\alpha_{5}/8 \)-integrin combinations bind specifically to the RGD amino acid sequence found in fibronectin.

In the present study we used a polyacrylamide gel system with surface coatings of four prominent, but distinct, ECM molecules: collagen I, collagen IV, laminin, and fibronectin. This system permitted us to simultaneously investigate the interplay of integrin signaling and mechanotransduction on the growth and myogenic and osteogenic differentiation of MSCs. We show that there is significant interplay between the particular matrix molecule presented to MSCs and the underlying substrate elasticity that affects myogenic or osteogenic differentiation. For example, we show that particular proteins, such as collagen IV and laminin I, present at only very low levels in bone, do not result in osteogenic marker expression despite the substrate stiffness matching which has previously been described as being conducive to osteogenic differentiation of MSCs. From these results we suggest that these substrates that incorporate physiologically relevant elastic moduli in combination with relevant ECM molecules better simulate the microenvironments encountered by MSCs in vivo. Analysis of cellular behavior under environments more representative of those encountered in vivo, such as discussed in the present work, will provide new insights into several key cellular processes, including differentiation, mechanotransduction, and wound healing, and will allow for the development of better disease models and new generation biomaterials for directed tissue organization from multipotent stem cells.

**EXPERIMENTAL PROCEDURES**

**Materials**

\( N,N' \)-methylen-bis-acrylamide and \( N,N,N',N' \)-tetramethylethylenedianime (TEMED) were purchased from Bio-Rad (Regents Park, Australia). The extended chain length, photoactivatable cross-linker \( N \)-succinimidy1-6-[4'-azido-2'-nitrophenylamino]hexanoate (SANPAH) was obtained from Pierce (Rockford, IL). Collagen I was extracted from rat tail, human placenta collagen IV was sourced from Sigma-Aldrich (Sydney, Australia), natural mouse laminin type I was supplied by Invitrogen (Melbourne, Australia), and fibronectin was sourced from R&D Systems (Minneapolis, MN). Hoechst and Alexa Fluor phalloidin were obtained from Invitrogen. Antibodies for MyoD1 myogenic marker were obtained from Abcam (Cambridge, UK). Antibodies for the transcription factor Runx2 bone marker were sourced from Alpha Diagnostic International (San Antonio, TX). All other chemicals were used as purchased from Sigma-Aldrich.

**Fabrication of Protein-Coated Gel Substrate**

Polyacrylamide gels (~300 \( \mu \)m thick) of a range of stiffness were prepared from solutions of various ratios of acrylamide:bis-acryl- amide, ammonium persulfate and TEMED in an anesthetic, oxygen-free environment. Four physiologically relevant stiffness values were selected (0.7, 10, 30, and 80 kPa) and subsequently coated with ECM proteins (collagen I, collagen IV, laminin, and fibronectin). After gelation, the surfaces were rinsed with PBS to remove any unpolymerized monomer. A uniform coating of adhesive protein was covalently bound to gel surfaces using SANPAH. Briefly, using aseptic techniques, a thin layer of 1 mg/ml SANPAH solution was placed on top of the gels, which were then exposed to ultraviolet light for 10 min to bind the SANPAH to the surface of the gels. Protein binding to the surface was subsequently carried out for 18 h at 4°C. Following protein binding, gels were rinsed with PBS prior to cell seeding.

**Characterization of Gel Substrates**

The compressive Young’s modulus of the gels was characterized using a Bio-DMA (Enduratec ELF 3200, Bose) under contact load at a strain rate of 0.5 mm/s. Verification of the presence of proteins cross-linked to the surface of the gels and the penetration depth was carried out by immunofluorescent staining and subsequent fluorescent imaging.

**Cell Culture**

Human bone marrow-derived MSCs (isolated from volunteers’ hips using commonly employed techniques) were kindly donated by Dr. Gary Brooke (Mater Medical Research Institute, Brisbane, Australia). MSCs were purified by selecting for adherent cells on tissue culture plastic after two passages and were confirmed to be CD90<sup>-</sup> and CD105<sup>-</sup> by FACS analysis. The MSCs were maintained in an incubator at 37°C and 5% CO2. Cultures used for experiments were passage 4 and were grown in low-glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 50 \( \mu \)g/ml streptomycin, and 50 U/ml penicillin for 4, 7, or 14 days. Medium was changed every 4 days. Cells were seeded at low density (2,500 cells/cm<sup>2</sup>) onto protein-coated gels in serum-free media and were allowed to attach for 4 h before medium supplemented with serum was introduced into the culture. This was to ensure that the cells bound to the surface-presented ECM molecule and not to adsorbed serum proteins. This step allows us to be sure that the responses noted are due to the prescribed ligand and not a cocktail of proteins nonspecifically bound to the surface of the gel.

**MSC Multipotency Controls: Confirming Adipo/Myo/Osteogenicity**

A sample of the donated MSCs was taken and tested to ensure multipotency by culturing in various conditioned media that induced the MSCs to differentiate along either adipogenic, myogenic, or osteogenic lineages.

**Adipogenic culture conditions.** MSCs were cultured to confluence in normal growth medium. The medium was then exchanged with adipocyte differentiation medium consisting of high-glucose DMEM supplemented with 10% FBS, 0.2 mM indomethacin, 1 mM dexamethasone, 0.5 mM IBMX, and 10 \( \mu \)g/ml insulin. This same differentiation medium was exchanged 3 days later. After a week of culture in differentiation medium, the medium was replaced with high-glucose DMEM supplemented with 10% FBS and 10 \( \mu \)g/ml insulin and was exchanged every 3–4 days thereafter. Two weeks after the induction of differentiation, the MSCs were fixed and stained for intracellular lipid inclusions using Oil Red O.

**Myogenic culture conditions.** MSCs were plated at low density in normal growth medium. Twenty-four hours after seeding, the medium was changed to myogenic induction medium consisting of low-glucose DMEM supplemented with 10% FBS, 10 \( \mu \)M 5-azacytidine, and 10 ng/ml basic FGF. After 24 h, the myogenic induction medium was replaced with normal growth medium supplemented with 10 ng/ml basic FGF. This medium was exchanged every 3 days for the remainder of the culture. Two weeks after the induction of myogenic...
differentiation, cells were fixed, stained for skeletal heavy chain myosin, and their morphological characteristics, especially the presence of myotube-like organization, identified.

**Osteogenic culture conditions.** MSCs were cultured to confluence in normal growth medium. The medium was then exchanged with osteogenic differentiation medium consisting of low-glucose DMEM supplemented with 10% FBS, 2 mM t-glutamine, 01 mM dexamethasone, 50 μM ascorbate-2-phosphate, and 10 mM β-glycerophosphate. This medium was exchanged every 3 days for the remainder of the culture. Two weeks after induction of osteogenic differentiation, cells were fixed and stained for mineralization using Alizarin Red.

**Immunocytochemistry: Cell Morphology, Proliferation, and Differentiation**

Primary antibodies for MyoD1 and Runx2 were conjugated to various amide reactive fluorescent Alexa Fluor dyes via commonly employed protocols. To ascertain conjugated antibody efficacy, positive controls for the immunofluorescence staining of Runx2 and MyoD1 were conducted on SaOS2 osteoblasts and C2C12 myoblasts, respectively. After culture, cells were fixed with 4% paraformaldehyde for 20 min at room temperature. Cell membranes were permeabilized using 1% Triton X-100 and were then incubated with the conjugated antibodies in a 3% BSA PBS solution for 4 h and subsequently imaged. Cell morphology was visualized by using Alexa Fluor phalloidin to stain for F-actin. Nuclei were visualized by staining with Hoechst. Proliferation was ascertained through multiple cell counts. The relative expression of various differentiation markers between different stiffness gels was determined using fluorescence intensity measurements.

**RESULTS AND DISCUSSION**

**Gel Properties**

The mechanical properties of polyacrylamide can be easily tuned by altering the density of cross-links in the gel. Increasing the quantity of either the amount of acrylamide monomer or bis-acrylamide cross-linker results in a gel with a higher Young’s modulus in density and also only located on the surface of the gel. The isolated MSCs displayed the potential to differentiate along adipic, myo, and osteogenic lineages after treatment with the respective differentiation protocols described in experimental procedures (Fig. 3). Lipid globules were observed in the adipogenic MSCs at the end of 2 wk. Dense cell packing and calcium deposits were found in osteogenic MSCs. A reduction in size and acquisition of myoblast and myotube-like morphologies was seen in myogenic MSCs. These results demonstrate
that the MSCs used in the present study were indeed multipotent and responsive to differential stimuli.

**Cell-Gel Interactions**

Cell attachment, spread area, morphology, and proliferation. Cells were allowed to attach to the gels in serum-free conditions, as described in **EXPERIMENTAL PROCEDURES**. Over the 14 days, no changes in the proliferation or the morphology were observed between cells that were incubated in serum-free conditions for the 4-h attachment period versus those that were allowed to attach in the presence of serum (data not shown).

The attachment of MSCs to the gels was found to be dependent both on the stiffness and on the particular ECM molecules presented on the surface of the gel. Figure 4A shows the attachment of MSCs to the various substrates investigated after 24 h. In general, stiffer substrates facilitated higher attachment over soft substrates. Furthermore, on higher stiffness substrates, variability in cell attachment between the proteins was greater. At 0.7 kPa, the number of cells attached was approximately the same, regardless of the protein. At 9 kPa, collagen I- and collagen IV-coated gels had slightly higher numbers of attached cells than fibronectin- or laminin-coated gels. At 25 kPa, the trend observed in terms of numbers of cells attached was fibronectin > collagen I > laminin > collagen IV, whereas at 80 kPa, the trend observed was fibronectin > collagen IV > collagen I > laminin.

Further to differences in the attachment of MSCs across the various substrates, variations in cell spread area (Fig. 4B) and morphology were observed. In general, cells on softer substrates tended to be round and occupy a much smaller footprint on the surface of the gel, whereas stiffer substrates encouraged greater cell spreading—an observation that corroborates with other published results (6–8, 12, 30). Interestingly, the stiffness that facilitated the greatest cell attachment for a given protein did not necessarily result in the greatest spread area and vice versa. The development of actin stress filaments can be clearly seen on substrates with stiffnesses >5 kPa (Fig. 5). As with the attachment results, MSC spread area on gels with a stiffness of 0.7 kPa and 9 kPa varied only slightly across the different proteins. At 25 kPa, the trend observed in terms of spread area of MSCs was collagen IV > collagen I > fibronectin > laminin, whereas at 80 kPa, collagen I ≈ fibronectin > collagen IV > laminin. Of the four proteins investigated, laminin-coated substrates demonstrated the least variability in spread area across the range of stiffnesses.

Aside from the aforementioned increased roundness of cells on softer substrates, the morphology of the cells on stiffer gels was largely quantitatively comparable across the proteins, except for cells cultured on laminin. On gels of stiffnesses >0.7 kPa, these cells possessed a more elongated and spiny morphology than cells cultured on the other proteins. The differences observed in morphology, spread area, and attachment demonstrate that both the protein and the stiffness modulate MSC behavior and that attachment is not solely a function of substrate stiffness or the adhesive ligand alone, but rather a combination of the two. Similar to these stiffness-protein combinatorial effects that we observe in MSC behavior, the peak migration rates of smooth muscle cells have been shown to be biphasic, depending on both the stiffness of the substrate and the surface density of fibronectin (21).

The MSC proliferation rate also followed a general trend of increasing proliferation with increasing substrate rigidity; however, on higher stiffness substrates, the proliferation rates were similar (Fig. 6). While cell growth was observed on gels with a stiffness >9 kPa, the cell number on the softest gels stayed virtually the same over the 14 days. The differences in behavior of the cells on different stiffness gels can be attributed to integrin signaling through focal adhesion complexes. On stiff gels, phosphorylation of focal adhesion kinase will be high, resulting in growth factor activation of ERKs to promote proliferation (23).

Cell differentiation. The multilineage potential of the purified MSC population used in the present study was determined using well-characterized and reproducible induction protocols (as described above in **MSC Multipotency Controls: Confirming Adipo/Myo/Osteogenicity**). The MSCs demonstrated the potential to differentiate toward adipocytes, osteoblasts, and myoblasts.

The ability of the various stiffness gels, in combination with different adhesive proteins, to direct MSC lineage specification toward myogenic or osteogenic precursors was determined by immunofluorescence microscopy against lineage-specific transcription factors MyoD1 (myogenic) and Runx2 (osteogenic). The fluorescent images were analyzed using Adobe Photoshop to measure the fluorescence intensity of the positively stained nuclei. All images were taken using the same exposure settings for each transcription factor, and measured intensities were normalized to background fluorescence. Representative fluorescent images of the cells are shown in Fig. 7, and results of the fluorescence intensity analysis for the expression of both transcriptions factors MyoD1 (myogenic) and Runx2 (osteogenic).
transcription factors on the various substrates investigated are presented in Fig. 8.

The results indicate that MSC differentiation is responsive to both the substrate stiffness as well as the adhesive ligand presented on the surface of the gel. Certain specific combinations of substrate stiffness and ECM molecule appear to have provided a strongly directive microenvironment for MSC lineage specification into both osteogenic and myogenic lineages.

Differentiation of MSCs toward osteogenic precursors occurred predominately on the stiffest substrate gel (80 kPa), with the strongest expression of Runx2 being observed on the 80 kPa collagen I-coated gel. At 80 kPa, expression of Runx2 by MSCs cultured on fibronectin-, laminin-, and collagen IV-coated gels was lower than those cultured on collagen I-coated gels by \( \frac{1}{10} \), \( \frac{1}{7} \), and \( \frac{1}{14} \), respectively. The strong osteogenic response of the MSCs to the 80-kPa collagen I-coated gel may be attributed to the fact that this combination best mimics the natural microenvironment of bone. Type I collagen comprises 80% of the total protein present in bone, and the stiffness of trabecular bone is \( \approx 80 \) kPa (8, 28).

Interestingly, similar levels of expression of Runx2 were observed on both the 80-kPa and 25-kPa fibronectin-coated gels, in stark contrast to the much reduced expression of Runx2 on the 25-kPa collagen I-coated gel. Ignoring for a moment the differences in the underlying substrate stiffness, the peak levels of Runx2 expression were significantly different between proteins, with collagen I having a \( \approx 2 \times \) higher readout than fibronectin, \( \approx 4.5 \times \) higher readout than laminin, and \( \approx 7 \times \) higher expression than collagen IV. Of particular note are the very low values of Runx2 expression on collagen IV- and laminin-coated gels, regardless of gel stiffness. These results suggest that substrate stiffness alone is not sufficient to instigate osteogenic differentiation of MSCs, at least within the range of stiffness investigated, and that the differentiation process appears to be modulated, for a given substrate stiffness,
by the type of ECM molecule present. Cells sense matrix elasticity by anchoring via integrin adhesions to ECM molecules and pulling via cytoskeletal contractions. As shown in Fig. 5, cells on soft substrates spread less, behavior that can likely be attributed to the cells having more mobile, less permanent, anchors (6). Cells on stiff substrates develop permanent anchors through focal adhesions and generate high cytoskeletal tension, which is evidenced by enhanced actin stress fibers and large spread area. Figure 8 thus also suggests that below a compressive modulus of 25 kPa, independent of the adhesive ligand presented, there is not enough cytoskeletal tension to ultimately result in differentiation toward an osteogenic lineage (19). On the basis of these results we postulate that unless a cell develops cytoskeletal tension exceeding a certain threshold stiffness (such as is generated on substrates with moduli of ≥25 kPa), then 1) osteogenic differentiation will not occur and 2) if this requirement is satisfied, then the cell must also find itself in the presence of an osteogenic ligand for Runx2 expression (and, presumably, further differentiation) to take place.

MyoD1 expression, on the other hand, showed less ECM dependence compared with Runx2, being expressed in detectable amounts in cells cultured on all gels with stiffnesses higher than 9 kPa, regardless of protein coating. Maximum expression of MyoD1 occurred on fibronectin-coated 25-kPa gels, which was ~2× higher than either collagen I or IV and 4× higher than on laminin. There was a noticeable shift between peak expression of MyoD1 between proteins for a given stiffness. While collagen I- and laminin-coated gels gave rise to the highest expression at a stiffness of 80 kPa, cells cultured on fibronectin- and collagen IV-coated gels had peak expression at a gel stiffness of 25 kPa. Furthermore, while maximum expression was observed on 25-kPa fibronectin-coated gels, similarly high levels of MyoD1 were expressed in cells cultured on 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels. At 80 kPa, the expression of MyoD1 from MSCs on fibronectin is ~1.7× lower than either the 80-kPa collagen I-coated gels.
sheets are composed of fine collagen fibers that make up the reticular layer of the endomysium that directly links cells together. Fibronectin plays a similarly important role and is present throughout the different ECM layers in muscle, extending to the reticular layer of the basement membrane, connecting the cell surface to the intercellular matrix (13). Given the lower ECM/stiffness-dependent expression of MyoD1, the results seem to suggest that MSCs have a predisposition to myogenic differentiation in comparison to osteogenic lineages when cultured on these substrates.

There is considerable evidence in the literature that the functional activity of cells, including differentiation, is strongly related to cell morphology and spread area (3, 5, 26). Indeed, cell spread area (controlled via the micropatterning of fibronectin islands on a nonadhesive silicon substrate) and the associated changes in cytoskeletal tension have been shown to be a potent modulator of functional cell fate. MSCs permitted to spread out on a large area (10,000 μm²) have been shown to become highly osteogenic compared with those constrained to a small area (1,024 μm²), which instead become highly adipogenic (19). Cells restricted to a small attachment area cannot form geometrically separated adhesions that act as anchors to allow tensioning of the cytoskeleton, whereas cells allowed to spread over a large area can form numerous geometrically opposed focal adhesion complexes and can generate significant cytoskeletal tension. However, on unrestricted (in terms of available area for attachment) elastic substrates, the cytoskeletal tension is determined not by the available adhesive area but by the substrate elasticity. For the same adhesive ligand, the net effect of increasing the stiffness of the substrate is thus to cause a corresponding increase in the cytoskeletal tension, as shown by the development of actin stress fibers in Fig. 4 and the measured spread area in Fig. 6, where increases in gel stiffness from 0.7 to 80 kPa in our study increased MSC spreading area from ∼5,000 to 45,000 μm², respectively. However, we also note that on different ligand-coated substrates, different spread areas were observed on gels of the same stiffness. For example, MSCs bound to an 80-kPa laminin-coated gel had spread areas of approximately half of those bound to an 80-kPa fibronectin-coated gel (P < 0.05), and yet, the level of MyoD1 expression on both was similar (P > 0.05). On gels of 25-kPa stiffness, these two ligands resulted in MSCs displaying similar spread areas (P > 0.05) but vastly different MyoD1 expression (P < 0.05), by a factor of ∼5×. A similarly significant difference in Runx2 expression is noted on the 80-kPa gel coated with collagen I and collagen IV, even though the observed MSC spread area is very similar (P > 0.05). It is clear from these observations that the known effect of cytoskeletal tension on differentiation is significantly modulated by the ligand to which the MSC is bound, and not simply the elasticity of the underlying substrate alone.

While the presence and modulation of both transcription factors under various conditions are certainly encouraging, care must be exercised when attempting to cross-compare the myogenic or osteogenic induction capacity of the various substrates. The presence of a particular transcription factor within the nucleus does not necessarily mean a cell is committed to a particular lineage. Furthermore, the relative abundance of each transcription factor required to trigger further gene transcription and differentiation cascades is not known. While we can certainly infer outcomes based on the results discussed above, tissue physiology, and previously published data, the immunofluorescence analysis performed does not permit the conclusive establishment of which conditions are “more myogenic” over those that are “more osteogenic.” Rather, they give independent insight for each of the transcription factors and together demonstrate the significant interplay between substrate stiffness and adhesive ligand. Thus, while collagen I-coated 80-kPa gels gave high readouts for both Runx2 and MyoD1 expression, it would be incorrect to conclude that this stiffness-protein combination is equally myogenic as it is osteogenic without first investigating other transcription factors or cofactors that are crucial to the myogenic differentiation process, e.g., Myf5, myogenin, and Myf6.

The expression results from both transcription factors indicate that there is significant interplay between mechanotrans-
duction and classical integrin signaling. Furthermore, we note that mechanotransduction is obviously modulated by specific integrin adhesions and thus is responsive to adhesive ligand presentation. The elucidation of how cells respond to substrate stiffness via mechanotransduction is still a much-debated topic.

Critical to this debate is the determination of the exact interplay between the type of integrin receptor that is able to engage with the microenvironment and how that affects/interacts with the mechanotransduction cascade. Although the two-dimensional mechanical environment in which a stem cell finds itself has been shown to play a significant initial role in determining its fate, it would appear to be a primary or developmental step in the MSC differentiation process. Whether these phenotypes are maintained once confluence is achieved or when such micro- environments are presented in three dimensions should be a topic of future investigations.

Conclusion

Current protocols use either toxic DNA demethylation agents or complex cocktails of expensive growth factors to direct stem cell differentiation. The use of demethylation agents could potentially result in genetic abnormalities thereby precluding their use in clinical applications. In the present study, we demonstrate that MSCs can be directed to differentiate along a more osteogenic or myogenic path depending on discrete combinations of substrate stiffness and the ECM protein presented to the cell. The ability to direct stem cell fate via a tissue microenvironment mimic alleviates the necessity of using these mutagenic reagents; the gel-protein system presented here, while perhaps not being suitable for large-scale use itself, demonstrates the potential of such tissue-mimic systems.

The results presented furthermore highlight the role that integrins play in determining cell fate through both mechanosensing and ligand-binding phenomenon. They also highlight the fact that designing a representative artificial in vitro environment may be key to providing insights into how stem cells behave in vivo. Everyday tissue culture is practiced on stiff plastic or glass substrates, environments never found in vivo and that perhaps compromise the analysis of cell behavior in these contexts. Generating artificial environments that more closely mimic those found in natural tissues will provide insights into fundamental cellular processes like differentiation, proliferation, wound healing, and disease modeling.

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