Mesenchymal stem cells (MSCs) are adherent multipotent stem cells from bone marrow and potentially numerous other tissues\cite{1} that serve as an attractive model system for evaluating the influence of extracellular cues on stem cell differentiation. MSCs have been shown to commit to several lineages including: bone, cartilage, fat, and smooth muscle, as well as transdifferentiation to skeletal muscle and neural fates\cite{1b,2}. Our research group\cite{3} and others\cite{2a,2b,4} have used MSCs to demonstrate the importance of cytoskeletal tension during lineage specification and commitment. For example, MSCs that were cultured either on stiff substrates\cite{4} or patterned on surfaces that promote cell spreading or cytoskeletal tension\cite{3,4b} all favored an osteogenic program that depended on increased contractility of the actomyosin cytoskeleton. Other reports have demonstrated the use of materials that are modified with cell adhesion ligands to promote MSC osteogenesis;\cite{5} we reasoned that the molecular characteristics of the adhesion ligands—including the affinity and density—would influence the cytoskeleton of the cell and may, therefore, serve to direct the differentiation pathways of adherent MSCs. Herein we report that the biomolecular interactions between cells and their substrates can be tuned to promote osteogenesis, myogenesis, or neurogenesis of cultured MSCs. This work provides an example of the use of molecular engineering to control the influence that materials have in regulating cell function.

We used self-assembled monolayers (SAMs) of alkane-thiylates on gold (anchored through the thiol group) as model substrates, because these surfaces allow excellent control over the ligand–receptor interactions that mediate cell adhesion, in part, because they are structurally well-defined and, in part, because the use of monolayers that are terminated with an oligo(ethylene glycol) group are highly effective at preventing nonspecific adsorption of proteins.\cite{6} Monolayers, to which short peptide-adhesion ligands are immobilized, have been used to study several aspects of cell adhesion, including that of embryonic and mesenchymal stem cells, and are an established model for these applications.\cite{7} We prepared substrates by immobilizing either the linear peptide GRGDSC (linRGD) or the cyclic peptide RGFDIC (cycRGD, where f denotes an F residue having the D configuration) to monolayers presenting a maleimide group at a density of 1% (high density) or 0.1% (low density) against a background of tri(ethylene glycol) groups (Figure 1a).\cite{8} The cyclic peptide has approximately two orders of magnitude higher affinity for the αvβ3 integrin—an important receptor in the adhesion and osteogenesis of MSCs\cite{9}—than does the linear peptide.\cite{10} We used self-assembled monolayer desorption ionization mass spectrometry (SAMDI) mass spectrometry to confirm immobilization of the peptides to the maleimide group (Supporting Information, Figure S1).

We cultured MSCs under standard growth conditions (see Supporting Information) for ten days on substrates having a bare gold film, a fibronectin-coated gold film (Fn), or on monolayers presenting linRGD, cycRGD, or a scrambled form of the linear peptide that we have demonstrated to be inactive (KRDGV).\cite{11} For the monolayers, peptides were present at a density of 1% relative to total alkanethiolate (high density) or 0.1% (low density). We then fixed and stained the cells to observe alkaline phosphatase (AP) expression, which is an early marker for osteogenesis. We detected elevated AP expression for cells on the fibronectin (48% of cells stained for AP) and cycRGD substrates (44% on high density, 30% on low density) compared to a lower level of expression for cells adherent to the bare gold film (25%; Figure 1b). MSCs cultured on monolayers presenting linRGD, at either high or low density, expressed AP at levels that are comparable to cells cultured on the unmodified bare gold. Control experiments, which used monolayers presenting no peptide or the scrambled RDG peptide, showed insignificant levels of cell adhesion and were not included in the analysis. We confirmed these trends by using reverse transcriptase PCR (RT-PCR) to quantitate the amount of AP mRNA transcript. Again, we found higher expression for cells cultured on monolayers presenting cycRGD and the fibronectin-coated substrate relative to those cultured on the linRGD-terminated monolayers (Figure 1c). These results suggest that fibronectin and the monolayers presenting cycRGD promote osteogenesis.

To further investigate the influence of the monolayers on differentiation, we performed immunofluorescence staining of several lineage-specific markers. For example, cells that differentiate into osteoblasts express the runt-related transcription factor 2 (Runx2). MSCs cultured on fibronectin and cycRGD surfaces show a higher level of nuclear Runx2...
cycRGD (at both high and low densities of ligand) or monolayers presenting the linRGD peptide at low density showed elevated expression of MyoD compared to cells cultured on bare gold. In addition to neural specific markers, we note that the expression of myogenic markers is elevated on the low-density linRGD surface, which suggests that a fraction of cells on this peptide surface are specifying myogenic programs. While the cells continued to proliferate, we noted a decrease in total cell area and nuclear area after one week in culture for cells adherent to the linRGD surfaces (Figure S2b). Changes in nuclear area have previously been shown to influence gene expression and cell differentiation.

The influence of ligand affinity and density on differentiation is consistent with a model wherein substrates presenting ligands of higher affinity and at higher density lead to more spreading of cells and greater tension in the cytoskeleton, which favors an osteogenic outcome. Previous studies have demonstrated the importance of cytoskeletal tension and focal-adhesion assembly in directing the differ-
We immunostained MSCs for filamentous actin and the focal-adhesion protein vinculin. Cells that were adherent to monolayers presenting cycRGD displayed a higher degree of spreading, more stress fibers, and more focal adhesion structures as compared to cells on monolayers presenting the linRGD peptide (Figure 3). We also immunostained MSCs for non-muscle myosin IIa and IIb to evaluate differences in contractility in cells on the different substrates. After normalizing the fluorescence data, a greater fraction of cells expressed high levels of myosin IIb when cultured on monolayers presenting cycRGD (36% on high density, 40% on low density) as compared to cells cultured on monolayers presenting linRGD (13% on high density, 25% on low density; Figure 4a). For myosin IIa, we observed a comparable level of total fluorescence in cells cultured on the various monolayers (Supporting Information, Figure S3). This result agrees with a report that showed expression of this isoform to be relatively insensitive to variations in substrate elasticity.[2b] Since cells cultured on the linRGD surfaces displayed the highest expression of myogenic markers, we immunostained the cells for the muscle-specific myosin heavy chain (MYH). The number of cells expressing high levels of MYH increased significantly as the affinity and density of the cell-adhesion peptide decreased, consistent with a previous report that demonstrated increased myogenesis with a decrease in cell contractility using substrates with variable mechanical properties.[2b]

To confirm the important role that cell contractility plays in differentiation, we cultured MSCs in a medium that was supplemented with blebbistatin, an inhibitor of non-muscle myosins that has little effect on muscle-specific isoforms.[2b] Cells were allowed to fully adhere and then the medium was exchanged with medium containing blebbistatin at a concentration that does not significantly perturb cell shape. We found that treated cells showed a decrease in osteogenesis, as determined by alkaline-phosphatase staining and an increase in expression of MyoD and β3-tubulin (Figure 4b and Figure S4). This result supports our hypothesis that the monolayers having a high-affinity ligand promote a more contractile cytoskeleton in adherent cells, which is

**Figure 2.** The density and affinity of an adhesion ligand influence the differentiation of mesenchymal stem cells. Cells were cultured on monolayers presenting either the cyclic or linear RGD peptides at a high or low density and differentiation was analyzed using a, b) immunofluorescence imaging of markers for osteogenesis (Runx2), myogenesis (MyoD1), and neurogenesis (β3-tubulin) and c) expression analysis by RT-PCR of lineage-specific transcripts. d) Table summarizing the preferred differentiation outcome for cells cultured on the four monolayer surfaces. Error bars represent standard deviations from three replicates. Statistical significance compared to bare gold: *p value < 0.02, **p value < 0.002, *p value < 0.05 as determined using Student’s t-test. Scale bar is 20 μm.

**Figure 3.** The affinity and density of a cell-adhesion peptide influence focal adhesion and stress-fiber formation. Immunofluorescence images of mesenchymal stem cells stained for filamentous actin (green), vinculin (red), and nuclei (blue). Surfaces presenting 1% (a) and 0.1% (b) cyclic RGD peptide and surfaces presenting 1% (c) and 0.1% (d) linear RGD peptide.
The differentiation of mesenchymal stem cells on peptide surfaces is influenced by non-muscle myosin. a) Immunofluorescence images and quantitation of cells stained for non-muscle myosin IIb and muscle-specific myosin heavy chain (MYH). *statistical significance p value < 0.005. b) Lineage marker expression as determined by immunofluorescence for MSCs exposed to the non-muscle myosin inhibitor blebbistatin (+B) and control cultures that were not exposed (−B): markers for osteogenesis (AP), myogenesis (MYO-D), and neurogenesis (β3-tubulin). Error bars represent standard deviations of at least three replicates.


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