Published in final edited form as:

Acta Biomater. 2010 January ; 6(1): 12–20. doi:10.1016/j.actbio.2009.07.023.

Human mesenchymal stem cell differentiation on self-assembled monolayers presenting different surface chemistries

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Abstract

Human mesenchymal stem cells (hMSCs) have tremendous potential as a cell source for regenerative medicine due to their capacity for differentiation into a wide range of connective tissue cell types. Although significant progress has been made in the identification of defined growth factor conditions to induce lineage commitment, the effect of underlying biomaterial properties on functional differentiation is far less understood. Here we conduct a systematic assessment of the role for surface chemistry on cell growth, morphology, gene expression, and function during hMSC commitment along osteogenic, chondrogenic, and adipogenic lineages. Using self-assembled monolayers of ω -functionalized alkanethiols on gold as model substrates, we demonstrate that biomaterial surface chemistry differentially modulates hMSC differentiation in a lineage-dependent manner. These results highlight the importance of initial biomaterial surface chemistry on long term functional differentiation of adult stem cells and suggest that surface properties are a critical parameter that must be considered in the design of biomaterials for stem cell-based regenerative medicine strategies.

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent adult progenitors that maintain the potential to differentiate into numerous cell types found within adult connective tissues [1,2]. A wide range of functionally, morphologically, and transcriptionally distinct phenotypes have been derived from a common MSC precursor [3-9]. This intrinsic multipotent property, coupled with the ability to isolate large quantities of these cells from the bone marrow, highlights the potential of human MSCs as a cell source for regenerative medicine applications [10-13]. Translation of this therapeutic potential into a realizable therapy, however, requires the development of well-controlled procedures for in vitro expansion, differentiation, and maintenance. Strategies for directed MSC commitment to specific lineages typically rely on specific combinations of soluble factors [14]. These cocktails of growth factors, cytokines, and

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other serum proteins have been well documented in the case of at least three mesodermal lineages (e.g. osteoblasts, chondrocytes, and adipocytes) and are commercially available [15].

In addition to soluble inductive factors, properties of the biomaterial substrate, including surface energy, roughness, chemistry, and biomatrix composition, have also been demonstrated to modulate short-term cell function. Specifically, these surface parameters have been linked to cell behaviors such as adhesion, morphology, and/or proliferation across numerous cell lines [16-21]. Several studies also suggest that alterations to one or more of these cellular responses at early time points may ultimately result in downstream effects on phenotype-specific gene expression and functional differentiation [22-26]. Beyond this general observation, however, it has been difficult to dissect the individual contribution of each surface parameter to the numerous phenotypic attributes specific to each lineage. Most reports focus on one particular cell type and examine a specific surface property or phenotypic read-out in isolation. Direct comparison between independent studies have been confounded by vast differences in cell lines, serum components, underlying substrates, culture procedures, and functional assays and time points used by different investigators. Thus, rigorous characterization will be necessary to establish cause/effect correlations between variables if fine control over cell phenotype is to be achieved.

A powerful in vitro model system that has allowed significant control over chemical properties of the underlying substrate is self-assembled monolayers (SAMs) of alkanethiols on gold [27]. A key advantage of this system is the facile creation of well-defined, easily replicated, and well-characterized surfaces presenting a wide range of chemical moieties [28-30]. Using this model system, we have previously demonstrated that surface chemistry modulates the conformation of adsorbed extracellular matrix proteins, such as fibronectin (FN), which in turn direct the binding of specific integrin adhesion receptors [31]. These surface-dependent differences in integrin binding differentially modulate focal adhesion formation and intracellular signaling cascades, ultimately leading to changes in initial adhesion and long-term differentiation of osteoblast- and myoblast-like cells [32-34]. Based on these results, we hypothesized that surface chemistry may also influence phenotypic behaviors of adult human stem cells.

Here we analyzed the influence of biomaterial surface chemistry on human MSC differentiation along three distinct mesenchymal lineages (i.e. osteogenic, adipogenic, chondrogenic). Multiple phenotypic features, including initial adhesion, morphology, long term growth, gene expression, and functional differentiation, were examined for each lineage using substrates presenting four different surface chemistries. Selection of human MSCs as a model system enabled the direct comparison of surface chemistry effects across multiple divergent phenotypes in parallel. These results lay the groundwork for future studies aimed at the identification of surface parameters that are optimal to achieve the fine control over stem cell differentiation required for clinical use of adult stem cells for therapeutic purposes.

MATERIALS AND METHODS

Cell Culture and Reagents

Human MSCs were obtained and expanded in standard mesenchymal stem cell growth media (MSCGM) (Lonza, Walkersville, MD). These cells were derived from bone marrow isolated from the iliac crest of human volunteers by a patented procedure licensed from Osiris Therapeutics (<http://www.lonza.com>). Unless otherwise stated, all cell culture media and supplements were purchased from Lonza and general reagents were acquired from Sigma Aldrich (St. Louis, MO).

Model Biomaterial Surfaces

Gold-coated substrates were prepared by sequential deposition of titanium (100 Å) and gold (200 Å) films via an electron beam evaporator (Thermionics Laboratories, Hayward, CA (2×10^{-6} Torr, 2 Å/sec)) onto oxygen plasma-etched 35 mm polystyrene tissue culture dishes. Self-assembled monolayers (SAMs) of alkanethiols were then assembled on gold coated substrates. Three alkanethiols were purchased from Aldrich Chemical (Milwaukee, WI) and used as received: 1-dodecanethiol (HS-(CH₂)₁₁-CH₃), 11-mercapto-1-undecanol (HS-(CH₂)₁₁-OH), and 11-mercaptoundecanoic acid (HS-(CH₂)₁₀-COOH). The amine-terminated alkanethiol 12-amino-1-mercaptododecane (SH-(CH₂)₁₂-NH₂) was synthesized and characterized by our group [31]. Surfaces were prepared by immersing gold-coated dishes for 12 hours in alkanethiol solutions (1.0 mM in absolute ethanol). Following overnight assembly, SAMs were rinsed 2x with 95% ethanol, 1x with diH₂O, and equilibrated in Dulbecco's phosphate buffered saline (DPBS) for at least 30 minutes. SAMs were characterized by X-ray photoelectron spectroscopy (XPS) and contact angle goniometry [31]. All surfaces were then coated for 30 min with various concentrations (0, 2, or 20 µg/ml in DPBS) of human plasma fibronectin (FN; Invitrogen, Carlsbad, CA) and subsequently blocked for at least 30 min in 1% heat-denatured bovine serum albumin prior to cell seeding. Adsorbed FN densities at each coating concentration have been previously quantified with ¹²⁵I-labeled FN [31]. Control substrates consisted of gold-coated tissue culture polystyrene (no SAM assembly) or uncoated tissue culture polystyrene; no significant differences in cellular responses were observed between these two conditions. In order to achieve equivalent light exposure across all conditions, surfaces represented by gold-coated tissue culture plastic are shown as controls for all functional and morphological assays.

Osteogenic Differentiation

Early passage (≤ 5) hMSCs were plated on SAMs at a density of 10,000 cells/cm² and cultured for 48 hours in MSCGM growth media. After cells reached 70-80% confluence, osteoblastic differentiation was induced with media consisting of DMEM (Invitrogen), 10% fetal bovine serum (Hyclone), 100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, 50 µg/ml L-ascorbic acid, 2.1 mM sodium βglycerophosphate, and 10 nM dexamethasone. Media was changed every 3 days until end point assay. Expression levels of osteoblastic genes were assessed with qRT-PCR (described below) using the following human-specific primers: Runx2 [5'-TGAGAGTAGGTGTCCCGCCT-3' (forward) and 5'-TGTGGATTAAGGACTTGGTGC-3' (reverse)]; bone sialoprotein [5'-GAGGACGCCACGCCTG-3' (forward) and 5'-TCCCCAGCCTTCTTGGG-3' (reverse)]; and osteocalcin [5'-GCAGGTGCGAAGCCCA-3' (forward) and 5'-TCCTGCTTGGACACAAAGGC-3' (reverse)]. Matrix mineralization was assessed by Alizarin red staining for calcium deposits [35]. Relative calcium content was also quantified by dissolving mineralized regions with 1.0 N acetic acid for 24 hours. A normalized volume from each sample (25 µl) was added to 300 µl of Arsenazo III-containing Calcium Reagent (Diagnostic Services Ltd., Oxford, CT). Absorbance was read at 650 nm and compared to a linear standard curve of CaCl₂ in 1.0 N acetic acid.

Chondrogenic differentiation

Early passage (≤ 5) hMSCs were plated on SAMs at a density of 30,000 cells/cm² and cultured for 48 hours in basal media. Standard chondrogenic induction media supplemented with 10 ng/mL TGF-β3 was added to confluent cells and changed every 3 days until end-point assay. Detailed differentiation procedures and lot numbers for supplements at proprietary concentrations are provided by Lonza (https://bcprd.lonza.com/group/en/products_services/products/catalog_new.ParSys.0007.File0.tmp?path=eshop/IMS_DOCS/DE/DE3358CF211E88F19E960017A48DB3BC.pdf). Primers used to evaluate human

chondrogenic gene expression with qRT-PCR included: type II collagen [5'-GACAGCATGACGCCGAGG-3' (forward) and 5'-GGCTGCGGATGCTCTCAAT-3' (reverse)]; type X collagen [5'-TCCTTGAAGTTGGTTCATGGAGT-3' (forward) and 5'-GGTGTGGGTAGTGGTCCTT-3' (reverse)]; and aggrecan [5'-GCCTGCGCTCCAATGACT-3' (forward) and 5'-GAACACGATGCCTTTCACCA-3' (reverse)]. Chondrogenic extracellular matrix was visualized with Alcian blue staining for acidic mucosubstances as previously described [35]. Relative Alcian blue staining intensity was also quantified by incubating unfixed cells with 1% Alcian blue dissolved in 3% acetic acid for 10 min followed by washing 3x with 3% acetic acid, 1x with diH₂O, solubilizing Alcian blue in 400 µl of a 1% SDS solution, and reading the absorbance at 605 nm.

Adipogenic differentiation

Early passage (≤ 5) hMSCs were plated on SAMs at a density of 30,000 cells/cm² and cultured for 48 hours in basal media until cells reached confluence. Adipogenic differentiation was induced by exposure to 2.5 cycles of standard induction/maintenance media: 72 hours in adipogenic induction media containing dexamethasone, 3-isobutyl-1-methyl-xanthine (IBMX), recombinant human insulin, and indomethacin, followed by 48 hours maintenance media containing recombinant human insulin. Detailed differentiation procedures and lot numbers for proprietary supplements are provided by Lonza (https://bcprd.lonza.com/group/en/products_services/products/catalog_new.ParSys.0007.File0.tmp?path=eshop/IMS_DOCS/DE/DE3358CF211E88F19E960017A48DB3BC.pdf). Primers used to evaluate human adipogenic gene expression with qRT-PCR included: peroxisome proliferation-activated receptor $\gamma 2$ (PPAR $\gamma 2$) [5'-AGCAAAGAGGTGGCCATCC-3' (forward) and 5'-CCTGCACAGCCTCCACG-3' (reverse)]; lipoprotein lipase (LPL) [5'-TGCCCTAAGGACCCCTGAA-3' (forward) and 5'-CAGGTAGCCACGGACTCTGC-3' (reverse)]; adipon [5'-CTGAGGCTGCAGTGAGTTGTG-3' (forward) and 5'-ACAAGGTTTCACTCTGTTGCC-3' (reverse)]; and glucose transporter 4 (GLUT4) [5'-GGGCGGGTAATTTCATTGAAA-3' (forward) and 5'-CGGATGTGGCAGCTCTAGTG-3' (reverse)]. Lipid levels were qualitatively assessed by a standard Oil Red O staining protocol described elsewhere [36]. Briefly, cells were washed in PBS, fixed with 10% formalin for 30 min, and washed in diH₂O. Immediately before use, 30 mL of a stock solution of Oil Red O (3 mg/ml in 99% isopropanol) was mixed with 20 mL diH₂O, filtered, and applied for 10 min to cells pre-equilibrated with 60% isopropanol.

Quantitative RT-PCR

Total RNA was isolated using the RNeasy RNA isolation kit (Qiagen, Valencia, CA). cDNA synthesis was performed on DNaseI-treated (27 Kunitz units/sample) total RNA (0.5 µg) by oligo(dT) priming using the SuperscriptTM First Strand Synthesis System for RT-PCR (Invitrogen). Gene expression was assessed by quantitative RT-PCR using SYBR Green intercalating dye (Invitrogen) and human lineage-specific primers (see above). Primer specificity was confirmed by ABI Prism 7700 Dissociation Curve Software. Standards for each gene were amplified from cDNA using real-time oligonucleotides, purified using a Qiagen PCR Purification kit, and diluted over a functional range of concentrations. Transcript concentration in template cDNA solutions was quantified from a linear standard curve, normalized to 0.5 µg of total RNA, and expressed as nanomoles of transcripts per µg of total RNA. Detection limits for each gene were determined by reactions without cDNA and fall below the y-axis minimum.

Data Analysis

Experiments were performed at least three times in triplicate. Data are reported as mean $\pm$ standard error of the mean (SEM) and statistical comparisons using SYSTAT 8.0 were based on analysis of variance (ANOVA) and Tukey's test for pairwise comparisons, with a p-value < 0.05 considered significant. In order to make the variance independent of the mean, statistical analysis of real-time PCR data was performed following logarithmic transformation of the raw data.

RESULTS

Self-assembled monolayers (SAMs) of ω -functionalized alkanethiols on gold were used as model substrates to investigate the effects of surface chemistry on human stem cell lineage commitment. Four functional end groups were chosen to represent a wide range of surface properties: CH₃ (hydrophobic), COOH (negatively charged at pH 7.4), NH₂ (positively charged at pH 7.4), or OH (neutral hydrophilic). Prior to cell seeding, SAMs were coated with 0, 2, or 20 $\mu\text{g/mL}$ human plasma fibronectin (FN) and blocked with heat-denatured bovine serum albumin in order to minimize non-specific adsorption of serum-derived proteins. This step provides control over the initial surface properties, a feature that is important because surface chemistry modulates protein adsorption, conformation, and/or bioactive properties, ultimately resulting in complex cellular responses that are difficult to link to one particular serum component. For example, we have demonstrated that surface chemistry modulates FN conformation and, consequently, integrin binding specificity and downstream cellular function in the MC3T3-E1 osteoblast-like cell line [31-33].

Here, we use hMSCs to study the effects of surface chemistry on phenotypic behavior of multipotent adult stem cells during commitment along three distinct (adipogenic, osteogenic, and chondrogenic) lineages. Cells were seeded on all 4 SAMs, which were coated with a range of FN concentrations. Previous analyses by our group have demonstrated that FN adsorption density varies as a function of coating concentration in a surface-dependent manner [31]. Specifically, a saturation density of 40 ng/cm^2 can be achieved on OH SAMs with a coating concentration of 20 $\mu\text{g/mL}$. By contrast, saturation thresholds are significantly higher for CH₃, NH₂, and COOH SAMs, as the 20 $\mu\text{g/mL}$ FN coating concentration achieves a 3- to 4-fold increase in FN density (range 150-250 ng/cm^2). Because these SAMs exhibit different protein adsorption profiles, FN coating concentrations can be adjusted (2 $\mu\text{g/mL}$ for CH₃, COOH, and NH₂ SAMs) to achieve equivalent surface densities (40 ng/cm^2) across all surfaces.

We first examined the effect of surface chemistry and FN coating concentration on initial cell adhesion. Phase contrast micrographs were taken after 24 hours hMSC seeding in MSCGM growth media at densities optimum for differentiation (Figure 1). Specifically, an initial seeding density of 10,000 cells/ cm^2 promotes osteogenesis, while a higher density of 30,000 cells/ cm^2 has been reported favorable for adipogenesis and chondrogenesis [3,15]. hMSCs consistently displayed a spindle-like, fibroblastic morphology across all surfaces. After 24 hours, FN coating appeared to have a negligible effect on initial cell adhesion and spreading on COOH, NH₂, and OH SAMs. By contrast, hydrophobic CH₃ SAMs were refractory to initial cell adhesion, but spreading and proliferation could be induced by precoating with FN at 2 or 20 $\mu\text{g/mL}$. This observation indicated that FN coating (or another adhesive protein) would be necessary to achieve equivalent cell adhesion across all surfaces to facilitate direct side-by-side comparisons within each media condition inducing functional differentiation.

A technical hurdle in this analysis was achieving the balance between long term cell adhesion and functional differentiation because cells tended to peel away from the substrate before sufficient time for phenotype induction had elapsed. For this reason, we initially evaluated both low and high FN coating concentrations on all 4 surface chemistries under all three lineage-

specific culture conditions. Phase contrast micrographs were taken after 12 days exposure to osteogenic media (Supplementary Figure 1), 12 days exposure to chondrogenic media (Supplementary Figure 2), and 13 days exposure to adipogenic media (Supplementary Figure 3). During the osteogenic induction period (Supplementary Figure 1), cells grew to confluence and displayed a cobblestone phenotype typical of osteoblasts on COOH, OH, and NH₂ surfaces without FN, but did not exhibit mineralized nodule formation. No evidence for cell growth, proliferation, and/or differentiation was observed on CH₃ SAMs without FN. A FN coating concentration of 2 µg/mL increased adhesion to the CH₃ surface, but was generally insufficient to maintain confluent cell growth, as peeling and cellular ‘de-adhesion’ were observed across all surfaces. At 20 µg/mL FN, mineralized nodules were observed on NH₂ SAMs and cell numbers appeared to be generally constant across all surfaces. Similarly, for both chondrogenic (Supplementary Figure 2) and adipogenic (Supplementary Figure 3) media conditions, only the 20 µg/mL FN concentration resulted in equivalent cell numbers and confluence across surfaces throughout the induction period. Importantly, FN-dependent differences in morphology were not evident in adipogenic or chondrogenic cultures. Taken together, these data indicated that surfaces coated with 20 µg/mL FN would be necessary to enable comparison across all surface chemistries and all lineages despite potential differences in coating density with the OH surface.

Next, we investigated the effects of surface chemistry on hMSC differentiation along the osteoblastic lineage. Cell morphology was generally similar across COOH, OH, and NH₂ surfaces independent of surface chemistry after 12 days exposure to osteogenic media (Supplementary Figure 1). Mineralized nodule formation occurred only on NH₂ SAMs at the high 20 µg/mL FN concentration, suggesting that mineral deposition displays FN-dependence on specific surfaces. This observation, coupled with the need to maintain equivalent cell numbers across all chemistries throughout the induction period, provide support for our rationale to conduct all functional analyses at a saturating FN concentration.

Several lines of evidence indicate that NH₂-functionalized SAMs were most favorable for osteogenic differentiation. Mineralized nodules were primarily observed only on this surface (Figure 2a) and Alizarin red staining for calcium deposition displayed markedly enhanced intensity on NH₂ compared to all other SAMs (Figure 2b). This result was confirmed by an independent assay in which calcium content was significantly greater on NH₂ compared to OH and FN-coated control surfaces (Figure 2c). Trends for expression of osteogenic genes as assessed by qRT-PCR analysis are in good agreement with the functional assays (Figure 3). Runx2 and osteocalcin (OCN) were significantly upregulated on NH₂ vs. the control surface, while bone sialoprotein (BSP) showed increases on NH₂ vs. COOH.

In addition to the NH₂ SAM, it is important to note that the other three SAMs also showed some evidence for functional osteogenic differentiation. For example, COOH and CH₃ displayed quantitatively higher calcium content compared to control surface (Figure 2c). However, this trend was not completely captured by Alizarin red staining intensity, which was enhanced on CH₃ and OH, but not on COOH, relative to control surfaces (Figure 2b). Discrepancies between assays for the OH surface are likely caused by ‘patchy’ cell adhesion, resulting in lower cell numbers and, consequently, quantitatively lower calcium content despite macroscopically equivalent staining intensity. Although NH₂ SAMs showed the most consistent upregulation of all 3 osteogenic markers, specific instances of surface-dependent changes in expression of osteogenic genes were also observed (Figure 3). Runx2 levels were elevated on OH and COOH SAMs relative to the control substrate, while BSP was significantly enhanced on OH relative to COOH SAMs. These data indicate that surface effects may change the overall magnitude of one or two individual phenotypic markers, but it is the expression of the full osteogenic differentiation program (as in the case of the NH₂ SAMs) that predicts the most profound effect of a single surface variable on lineage commitment.

Next, we investigated the effects of surface chemistry on hMSC differentiation along the chondrocyte lineage after 12 days exposure to chondrogenic induction media. Macroscopic differences in cell morphology between low and high FN concentrations were not observed (Supplementary Figure 2). These observations coupled with generally equivalent cell numbers across surface chemistries only at 20 $\mu\text{g/mL}$ FN provide strong support for comparison of surfaces at the high coating concentration despite potential differences in coating density with the OH surface (Figure 4a).

No differences in Alcian blue staining intensity were detected between SAMs, indicating that surface chemistry does not have a marked effect on functional hMSC differentiation into chondrocytes (Figure 4b,c). Although pellet culture and 3D aggregates have been shown to be more favorable for cartilage-specific phenotype induction [9], it was necessary to restrict the present analysis to monolayer cultures in order to facilitate comparisons across all conditions. Interestingly, we note that the 'no FN' coating condition on CH_3 SAMs, which is refractory to cell adhesion, promotes cell aggregation into 3D clumps resembling pellets formed in suspension culture. Enhanced Alcian blue staining was observed on this surface, supporting the importance of 3D culture for chondrocyte differentiation (Figure 4d). We also highlight this particular condition as a valuable internal control demonstrating the capacity for current supplementation procedures to induce positive Alcian blue staining with hMSCs. Although qRT-PCR analysis revealed that a specific chondrogenic gene may display small changes on particular surface conditions, no consistent trends between surfaces and no clear correlation between gene expression and cell function emerged (Figure 5). For example, type II collagen was upregulated on NH_2 and CH_3 compared to OH SAMs, while aggrecan was upregulated on OH versus CH_3 and NH_2 SAMs. Type X collagen did not show a statistically significant change in expression across all surfaces.

Adipogenic differentiation was induced by two cyclic supplementation stages: 72 hours induction by treatment with 1-methyl-3-isobutylxanthine, dexamethasone, insulin, and indomethacin, followed by 48 hours exposure to media supplemented with insulin alone. Macroscopic differences in the presence of lipid vacuoles were not observed between 2 and 20 $\mu\text{g/mL}$ FN coating concentrations on COOH, NH_2 , and control surfaces (Supplementary Figure 3), indicating that lipid vacuole coalescence is generally not dependent on initial FN density. Because equivalent cell numbers and confluency among surfaces through the 13 day culture period required 20 $\mu\text{g/mL}$ FN, all functional assays were conducted on SAMs coated with the high FN concentration.

Lipid vacuoles were observed on all surfaces after 13 days, or 2.5 induction/maintenance cycles, with significant enrichment in Oil Red O staining intensity on NH_2 SAMs compared to all other surfaces (Figure 6a-c). Notably, OH SAMs showed markedly reduced Oil red O-positive lipids compared to NH_2 , as well as all other surfaces, suggesting that neutral, hydrophilic surface properties may not be favorable for adipogenic differentiation. Consistent with this observation, expression of the adipogenic marker peroxisome proliferation-activated receptor $\gamma 2$ (PPAR $\gamma 2$) was significantly inhibited on OH compared to control and NH_2 surfaces (Figure 7). Similarly, glucose transporter 4 (GLUT4) expression was decreased on OH versus COOH SAMs. We note that the surface-dependent enrichment in lipid vacuoles observed in this study was not mirrored by increases in expression of adipogenic genes on the NH_2 surface. This result likely reflects the single timepoint (day 10) conducted in this analysis, which may not capture spatiotemporal expression patterns unique to each marker during the developmental program. Therefore, although both adipin and lipoprotein lipase did not show statistically significant differences across surfaces, future analyses across multiple time points may uncover temporally-regulated trends in expression.

DISCUSSION

hMSCs represent a promising cell source for regenerative medicine applications. The use of these cells for tissue engineering strategies would be greatly facilitated by advances in our understanding of interactions between cells and the underlying biomaterial substrate. Furthermore, identification of culture substrate conditions that promote controlled lineage specification would provide a robust method for enriching specific phenotypes within a population of cells. In the present study, we use model biomaterial surfaces presenting well defined surface chemistries in order to explore the role for initial biomaterial surface properties during multiple phenotypic aspects of hMSC commitment along three distinct mesodermal lineages. Strikingly, we observed that the chemical properties of the underlying surface present during initial cell seeding can have marked effects on long-term functional differentiation. The well-characterized differences in adsorbed FN conformation(s) on these surfaces (discussed below) provide a likely explanation for these effects. Notably, surface chemistry differentially modulates hMSC differentiation in a lineage-specific manner. For example, FN-coated NH₂ SAMs promote osteogenic and adipogenic differentiation relative to all other surfaces. Moreover, while FN-coated OH SAMs appear to be permissive for osteogenic differentiation, this chemistry inhibits the adipogenic differentiation program. By contrast, in the presence of chondrogenic induction media, all surface chemistries examined have negligible effects on the expression of chondrocyte-specific markers. Interestingly, we also observed that FN adsorption is required to promote cell growth on surfaces refractory to initial hMSC adhesion (i.e. CH₃ SAMs), suggesting an indirect role for FN in promoting lineage commitment by enabling cell adhesion throughout the induction period. These results represent an important step toward the identification of surface parameters that are optimal for controlled stem cell differentiation along specific lineages.

Several recent studies have investigated the effect of nanoscale topography, chemical moieties, and substrate-tethered peptides on MSC differentiation in the presence of various induction media [5,26,37-39]. Two features unique to the present study include the numerous assays used to assess cell function (i.e. initial cell adhesion, growth, gene expression, and functional differentiation) and the comparison in parallel of three unique mesenchymal lineages using the same surface conditions. In addition to these features, perhaps the most important difference from previous reports is the use of biomaterial substrates with well-controlled chemistries. Specifically, in many studies a diverse milieu of serum proteins are non-specifically adsorbed to surfaces in a poorly controlled manner, whereas here we created well controlled substrates by adsorption of a single adhesive ligand (in this case fibronectin (FN)) to SAMs prior to cell seeding. We have previously extensively characterized FN adsorption, conformation, density, and bioactivity on the model SAM surface chemistries used in the present work (e.g. COOH, NH₂, OH, and CH₃) [31,33,34]. These analyses revealed that adsorbed FN adopts markedly different conformations in a surface chemistry-dependent manner. Due to these advantages, the experimental design in the present work enabled a more controlled examination of the potential link between a single substrate parameter (namely surface chemistry-dependent FN adsorption) and the resultant phenotype-specific cellular functions. Nevertheless, we also note the limitations of this system, namely that alkanethiol SAMs may not be ideal for long-term cell culture because SAMs lack stability under biological culture conditions [40]. Moreover, cells in contact with these surfaces deposit and remodel extracellular matrices that can obscure biomaterial-dependent effects [41].

We observed that NH₂ SAMs promoted the most robust induction of the osteoblastic differentiation program. At first glance, it is surprising that a parameter such as surface chemistry can affect long-term cell function (10-14 days later) given that most cells quickly remodel their underlying adherent surface [42,43]. However, our results are consistent with previous studies using hMSCs on silanes [37] and an osteoblast-like cell line on SAMs [31].

A body of evidence reported by our group provides mechanistic details that lend support to this observed phenomenon [31,33]. Keselowsky et al. demonstrated that surface chemistry-induced changes in FN conformation lead to alterations in binding of specific integrin adhesion receptors. In particular, $\alpha_5\beta_1$ exhibits binding specificity to the conformation of FN created by adsorption on OH and NH_2 SAMs. By contrast, both $\alpha_5\beta_1$ and $\alpha_v\beta_3$ bind to COOH, while neither display specificity for FN conformations formed on CH_3 . These differential integrin binding profiles modulate short-term changes in focal adhesion formation and activation of intracellular signaling pathways that subsequently lead to alterations in cell adhesion [31,33]. Integrin-specific interactions with FN are essential for osteogenic differentiation, as incubation with β_1 -blocking antibodies inhibited differentiation by the MC3T3-E1 osteoblast-like cell line on NH_2 and OH surfaces [32]. Importantly, integrin binding specificity has also been shown to play a role in MSC-like cells derived from the bone marrow [44-46]. For example, Gronthos et al. demonstrated that β_1 integrins are critical for osteogenic differentiation of hMSCs by using β_1 -blocking antibodies to mitigate marrow stromal cell mineralization [47]. Taken together, these data support a model in which surface chemistry alters adsorbed FN conformation, which in turn directs specific integrin-FN interactions in hMSCs, ultimately inducing signaling pathways that push the population down a long-term osteogenic pathway. Antibody blocking studies, although beyond the scope of the current study, will be essential toward the validation of this hypothesis in adult stem cells.

We posited that surface chemistry-dependent changes in FN conformation may also affect the phenotype of hMSCs in the presence of adipogenic and/or chondrogenic induction media. Although OH SAMs are permissive for osteogenesis, this surface chemistry does not support long-term adipogenic differentiation compared to the other surfaces. Given that expression levels of multiple integrins change during hMSC differentiation along the adipogenic lineage [48-50], it is plausible that integrin-specific signaling may be linked to this inhibitory response. Future studies analyzing integrin expression profiles should provide more information on the direct role for specific integrin-FN interactions during surface chemistry-directed differences in hMSC differentiation into adipocytes.

By contrast to osteo- or adipogenic lineages, deposition of chondrocyte-like matrix was minimal on all surfaces coated with FN, suggesting that surface chemistry does not affect long-term chondrogenic differentiation in hMSC monolayer cultures. Consistent with our results, previous studies demonstrated that continuous exposure to the ubiquitous RGD-binding domain found within FN may induce relatively low level upregulation of initial chondrogenic marker expression, but ultimately has minimal effects on long-term differentiation [51,52]. Notably, CH_3 SAMs without FN coating (typically refractory for cell adhesion) resulted in formation of 3-D cell aggregates that displayed more intense staining for chondrogenic matrix deposition. These results can be interpreted to suggest that the dominant variable for chondrogenic differentiation of hMSCs is the cell-cell and cell-matrix contacts created within the 3-D architecture of spherical aggregates [9]. Although not examined here, the effect of surface chemistry and conformation of matrix proteins on integrin-mediated cellular behavior (s) within these 3D spheres cannot be ruled out [53-55].

In conclusion, analyses conducted in the present work demonstrate that biomaterial surface chemistry differentially modulates hMSC differentiation in a lineage-dependent manner. These results highlight the importance of initial biomaterial surface chemistry on long term functional differentiation of adult stem cells and suggest that surface properties are a critical parameter that must be considered in the expansion of stem cells and the design of biomaterials constructs for cell-based therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by the NIH (R01-EB004496). J.E.P. was supported by a National Science Foundation Graduate Research Fellowship.

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Figure 1. Multipotent human mesenchymal stem cell morphology

Phase contrast micrographs 24 hours after seeding human mesenchymal stem cells in growth media at low (10,000 cells/cm²) and high (30,000 cells/cm²) densities favorable for osteogenic or chondro/adipogenic differentiation, respectively. SAMs were coated with 0, 2, and 20 µg/mL human plasma fibronectin. Scale bar, 100 µm.

Figure 2. Osteoblastic differentiation

(A) Phase contrast micrographs depicting morphology of unstained cells after 12 days culture in osteogenic media. Scale bar, 100 μ m. (B) Matrix mineralization was assessed by Alizarin Red staining for calcium deposits. Representative images from multiple independent experiments are shown. Scale bar, 50 μ m. (C) Calcium content was quantified after 10 days culture in osteogenic media (mean $\pm$ SEM, n=8; ANOVA: $p < 0.05$; * vs. control ($p < 0.05$), ** vs. control and OH ($p < 0.05$)).

Figure 3. Osteogenic gene expression

mRNA levels were investigated by quantitative RT-PCR after 7 days culture in osteogenic media (Mean $\pm$ SEM, n=4; ANOVA: $p < 0.05$; * vs. control, ** vs. control and CH₃, □ vs. COOH ($p < 0.05$)).

Figure 4. Chondrogenic differentiation

(A) Phase contrast micrographs after 10 days culture in chondrogenic media. Scale bar, 100 μ m. (B) Sulfated proteoglycan accumulation was visualized by Alcian Blue staining and (C) quantified (mean $\pm$ SEM, $n \geq 10$). (D) Comparison of cell morphology (bottom panel) and Alcian blue staining (top panel) on CH₃ surfaces coated with 0 and 20 μ g/mL human fibronectin. Scale bars, upper panel, 80 μ m; lower panel, 40 μ m. Representative images from multiple independent experiments are shown for all panels.

Figure 5. Chondrogenic gene expression

mRNA levels were investigated by quantitative RT-PCR after 7 days culture in chondrogenic media (Mean $\pm$ SEM, $n \geq 3$; ANOVA: $p < 0.05$; * vs. OH, ** vs. CH₃ and NH₂ ($p < 0.05$)).

Figure 6. Adipogenic differentiation

(A) Phase contrast micrographs after 15 days culture in adipogenic media. Scale bar, 80 μm . (B, C) Lipid vacuoles were visualized with Oil Red O staining at (B) low and (C) high magnification. Scale bars, 100 and 50 μm , respectively. Representative images from multiple independent experiments are shown for all panels.

Figure 7. Adipogenic gene expression

mRNA levels were investigated by quantitative RT-PCR after 10 days culture in adipogenic media (Mean $\pm$ SEM, n=4; ANOVA: p<0.05; * vs. COOH, \$ vs. control and NH₂ (p< 0.05)).