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Small molecule functional groups for the controlled differentiation of human mesenchymal stem cells encapsulated in poly(ethylene glycol) hydrogels

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Abstract

Cell-matrix interactions play critical roles in regeneration, development, and disease. The work presented here demonstrates that encapsulated human mesenchymal stem cells (hMSCs) can be induced to differentiate down osteogenic and adipogenic pathways by controlling their 3D environment using tethered small molecule chemical functional groups. Hydrogels were formed using sufficiently low concentrations of tether molecules to maintain constant physical characteristics, encapsulation of hMSCs in 3D prevented changes in cell morphology, and hMSCs were shown to differentiate in normal growth media, indicating that the small-molecule functional groups induced differentiation. To our knowledge, this is the first example where synthetic matrices are shown to control induction of multiple hMSC lineages purely through interactions with small molecule chemical functional groups tethered to the hydrogel material. Strategies utilizing simple chemistry to control complex biological processes would be particularly powerful as they could make production of therapeutic materials simpler, cheaper, and more easily controlled.

RESULTS AND DISCUSSION

Interactions of cells and extracellular matrix initiate signaling cascades involved in critical cell functions, such as regeneration^{1–3}. An important goal of tissue engineering, specifically is to mimic critical aspects of the extracellular environment and to control cell function through cell-material interactions⁴. The complexity of the natural ECM and cell-matrix interactions makes design of materials for regenerative medicine applications challenging since a variety of factors will influence cell fate. The choice of the chemical environment used for a specific tissue engineering application is dependent on the desired outcome, and many studies have shown that chemical functionality and hydrophilicity play important roles in cell adhesion and function^{5–10}. Comprehensive studies have also demonstrated that surface function of 2D materials plays a role in differentiation of embryonic stem cells down the hepatocyte lineage based on ECM protein presentation¹¹ and epithelial lineage based on charge, hydrophilicity, and branching⁵. The attachment and growth of hMSCs, and the control of neural stem cell differentiation, and articular chondrocyte function, have also been studied¹². However, conclusions about the specific effects of chemical functionalities using approaches to date^{5, 12}, especially on 2D surfaces, are complicated due to surface effects since factors such as stiffness and/or cell spreading can be influenced by the charge and hydrophilicity of the surface, and these factors have been shown to have a significant influence on stem cell fate^{13, 14}.

The goal of this work was to identify tethered chemical functional groups that could influence differentiation of human mesenchymal stem cells (hMSCs) due to the importance of this cell type in potential regenerative medicine applications^{15–17}. Our strategy was to screen immobilized small molecules for their ability to induce hMSCs to differentiate down pathways important for tissue engineering. The small molecules were chosen to incorporate functionalities found in the extracellular environment of the hMSC target cell types. Initial screening for differentiation markers was performed on 2D arrays, and the results were used to design 3D encapsulation materials for inducing hMSC differentiation. We were able to formulate 3D encapsulation materials that had functional groups with different charge and hydrophilicity, but at concentrations of tether molecules that did not affect materials properties such as stiffness and swelling. Furthermore, by encapsulating hMSCs in these materials, they were trapped in a rounded morphology that was the same for each of the materials tested. Finally, encapsulated hMSCs were cultured in standard hMSC media, without added cytokines or steroids typically used in differentiation media. As a result, we were able to definitively demonstrate that tethered small molecules could indeed have a direct influence on the differentiation fate of hMSCs, with charged phosphate groups leading to osteogenesis and hydrophobic t-butyl groups inducing adipogenesis. This work demonstrates that biomaterials with very simple functionalities may be used to control complicated cellular function such as stem cell differentiation, a result that could have interesting fundamental implications, but more importantly could lead to simple, cheap, highly controllable biomaterials formulations for controlling differentiation of hMSCs for several regenerative medicine applications.

As an initial screening tool to identify promising hydrogel formulations, human mesenchymal stem cells (hMSCs) were cultured on arrays of PEG functionalized with various small molecules (Figure 1, left hand column). Immunostaining was used as an initial screen to determine concentrations of small-molecule functionalization that induced elevated expression of proteins important for chondrogenesis, osteogenesis, and adipogenesis due to the ease of the experimental design on 2D surfaces. Promising immunostaining formulations were then further screened using FISH analysis to ultimately identify formulations for 3D encapsulation experiments. Since the goal of this work was to identify 3D encapsulation materials, a complete evaluation of differentiation on 2D surfaces was not performed. Small molecules were chosen to capture chemical aspects of the native extracellular space of relevant tissues to determine if differentiation could be controlled through cell-material interactions. Carboxylic acid functionalities resemble the exposed functional groups of native cartilage, which is rich in glycosaminoglycans¹⁸, phosphates were chosen because they play an important role in mineralized tissue development such as bone formation^{10, 19}, and hydrophobic functional groups were chosen as adipose cells are rich in lipids²⁰ and release fatty acids into their extracellular space²¹.

Figure 1 summarizes the immunostaining results for hMSCs cultured on various concentrations of the different functional moieties (the array design used is shown in Figure S1). Cultured hMSCs were monitored at day 10 using immunostaining of collagen II, osteopontin (OPN), and peroxisome proliferating antigen receptor gamma (PPARG). Collagen II is the major extracellular component of cartilage and an indicator of chondrogenesis, osteopontin (OPN) is an extracellular matrix protein found in bone and was monitored as a measure of osteogenesis, and PPARG is a critical regulator of adipogenesis.

A detailed explanation of 2D immunostaining results can be found in the supplemental material. Briefly, Figure 1a demonstrates that, while there is no change for collagen II production at the lower concentrations, an increase was observed for 50 mM acid-functionalized surfaces relative to the control gel (0 mM). While OPN was unchanged for most treatments, hMSCs cultured on phosphate-functionalized gels showed increased OPN production at 5 and 50 mM concentrations relative to the control gel. The hydrophobic t-butyl- and fluoro-functionalized

hydrogels elicited an elevated expression of PPARG by hMSCs at 5mM and the greatest at the 50 mM concentration relative to the control gel whereas the other gels remained unchanged or decreased expression. In summary, for each of the surfaces, the 50 mM concentration led to the biggest increase in expression of markers for differentiation; acid-functionalized gels increased the chondrogenic marker collagen II, phosphate-functionalized gels increased the osteogenic marker OPN, and hydrophobic surfaces, especially the t-butyl surface, increased the adipogenic marker PPARG.

Figure 2 summarizes the results for FISH analysis of hMSC differentiation based on hydrogel formulations with the highest functional group concentration, 50 mM due to this concentration showing the highest increases in expression via immunostaining. For FISH analysis, we monitored expression of aggrecan, a component of cartilage extracellular matrix and an early indicator of chondrogenic differentiation (Figure 2a), CBFA1 gene expression as a measurement of osteogenic differentiation (Figure 2B), and PPARG as a measure of adipogenic differentiation (Figure 2C). A detailed summary of FISH results can be found in the supplementary material. Briefly, aggrecan was unchanged for all treatments at day 0 but was found to be produced at constant or decreased levels relative to control gels for cells cultured on all surfaces at day 4 (Figure 2a). By day 10, all surfaces exhibited decreased aggrecan expression, except for the acid-functionalized gel, which exhibited an increased expression. At day 0, hMSCs showed no significant CBFA1 expression differences, however, at day 4 cells cultured on phosphate-functionalized hydrogel spots exhibited a 2-fold increase of CBFA1 gene expression over PEGDM alone while the other surfaces lead to the same or decreased expression. At day 10, the CBFA1 gene expression of hMSCs seeded on phosphate-functionalized gels showed a large increase compared to PEGDM, while fluoro-functionalized gels also demonstrated a moderate increase in CBFA1 gene expression and the other gels demonstrated decreased expression. Only hydrophobic t-butyl and fluoro groups exhibited increased PPARG expression at day 4 and day 10. Taken together, protein production and gene expression demonstrate that hMSCs cultured in the presence of 50 mM acid, phosphate, and t-butyl groups promote the expression of markers that can be associated with differentiation pathways of chondrocytes, osteoblasts, and adipocytes, respectively.

Morphological characteristics of hMSCs cultured on acid-, phosphate-, and t-butyl-functionalized surfaces suggest chondrogenic, osteogenic, and adipogenic phenotypes, as well. Figure 3 demonstrates light micrographs (a–d) and F-actin immunostaining (e–h) for hMSCs cultured on PEGDM (Figure 3a, e) and 50 mM acid- (Figure 3b, f), phosphate- (Figure 3c, g), and t-butyl- (Figure 3d, h) functionalized surfaces. The image in Figure 3a and the F-actin staining shown in Figure 3e demonstrates that hMSCs exhibit a round morphology on the acid-terminated surface, reminiscent of two-dimensionally cultured chondrocytes.^{22,23} hMSCs cultured on OPN hydrogel surfaces exhibit a similarly spread morphology to *in vitro* osteoblasts (Light micrograph, Figure 3c and F-actin staining, Figure 3g), which is consistent with the hypothesis that phosphate-functionalization may promote osteogenesis. In addition to increased adipogenic protein expression, cells seeded on gels containing hydrophobic functional groups also appear morphologically similar to adipocytes, including evidence of the formation of intracellular lipid droplets (grey deposits shown in Figure 3d. Moreover, hMSCs cultured on t-butyl-functionalized gels are significantly less spread (Light micrograph, Figure 3d and F-actin staining, Figure 3h) than hMSCs cultured on PEGDM and have very few projections, similar to adipocytes. Therefore, based on immunostaining, FISH, and morphological characteristics, we were able to identify promising formulations for testing small-molecule functionalized hydrogels for their ability to induce differentiation of encapsulation hMSCs.

Ultimately, we aim to discover materials that promote hMSC differentiation down a specific, tissue-related pathway. We tested the most promising formulations identified using the 2D

arrays to determine if a 3D hydrogel environment could be accurately predicted to promote specific hMSC differentiation. Specifically, we chose to encapsulate hMSCs in t-butyl and phosphate-functionalized hydrogels to test for osteogenic and adipogenic differentiation, respectively, without the use of differentiation media. The 3D environment allows hMSCs to be encapsulated at a fixed density for all gel compositions, and all of the cells are maintained in a similar rounded morphology, allowing the effects of the chemical functionality on hMSC differentiation to be tested in the absence of these variations that often occur on 2D surfaces. Immunoblotting was used to evaluate osteogenic and adipogenic markers, and histology and immunohistochemistry were performed on the constructs after 21 days of encapsulation to examine the distribution and components of elaborated matrix.

Immunoblotting of CBFA1 and PPARG was performed to evaluate osteogenesis and adipogenesis, respectively. Figure 4A–C shows the results and quantification for hMSCs encapsulated in t-butyl, phosphate-functionalized, and control PEGDM hydrogels after 0, 4, 10, and 21 days of culture. Immunoblots (Figure 4B) for hMSCs encapsulated in phosphate-functionalized hydrogels identify detectable upregulation of CBFA1 after 10 days in culture, with further increases observed at day 21, indicating differentiation down an osteogenic pathway. For t-butyl-functionalized hydrogels, PPARG production was upregulated starting at day 10 and remained constant through day 21, suggesting increased hMSC adipogenesis (Figure 4C).

Standard histological staining as well as immunohistochemistry indicative of osteogenesis or adipogenesis was used to evaluate specific matrix production by encapsulated hMSCs. Masson's trichrome, which stains collagen blue, muscle and cytoplasm red, and nuclei black, was used to verify osteogenic differentiation. Figure 4D illustrates that Masson's trichrome staining (left column) shows very little collagen or muscle tissue development by cells encapsulated PEGDM or in t-butyl functionalized hydrogels. In contrast, hMSCs cultured in phosphate-functionalized hydrogels produce a collagen-rich matrix, deposited pericellularly, which is common for non-degradable hydrogels like those utilized here^{24, 25}. Immunohistochemical staining of the bone matrix extracellular protein, osteopontin, revealed very little to no staining for PEGDM or t-butyl-functionalized gels. However, hMSCs cultured in phosphate-functionalized hydrogels elaborated a fairly rich matrix of osteopontin, as shown by the nearly uniform light brown staining (Figure 4D, second column). Bone tissue is composed of a rich collagen matrix and other structural proteins such as osteopontin with hydroxylapatite mineral deposits. The development of a collagen and osteopontin-rich matrix by hMSCs encapsulated in phosphate-functionalized hydrogels supports the assignment of osteogenic differentiation as determined through immunoblotting.

Oil red, which stains intracellular lipid deposits red, was used to verify adipogenesis. Oil red staining is negative for PEGDM and phosphate-functionalized hydrogels but positive for hMSCs cultured within t-butyl-functionalized hydrogels (Figure 4D, third column). As adipogenesis proceeds, cells accumulate lipids intracellularly, and therefore the presence of Oil red-positive staining for hMSCs cultured in t-butyl-functionalized PEGDM hydrogels is indicative of adipogenic differentiation. Further, the distribution of PPARG was examined. While PEGDM and phosphate-functionalized hydrogels showed no PPARG staining, t-butyl-functionalized hydrogels showed robust PPARG staining (Figure 4D, right most column), and, as PPARG is a regulator of adipogenic differentiation, this serves as strong evidence that the t-butyl-functionalities are promoting adipogenesis. Therefore, we conclude that, based on immunoblotting, histology, and immunohistochemistry results, 3D encapsulation of hMSCs in phosphate- and t-butyl-functionalized PEGDM hydrogels leads to osteogenic and adipogenic differentiation, respectively.

Signals from the extracellular matrix, such as physical structure and chemical environment, are known to play a critical role in guiding stem cell differentiation^{26, 27}, but the complexity of the native ECM is difficult or impossible to completely recreate synthetically. Therefore, there is a great deal of motivation to identify simplified synthetic mimetics for guiding differentiation down specific phenotypic pathways. Screening materials based on knowledge about the tissue environment in which a stem cell resides and choosing chemical strategies accordingly could therefore have a significant impact on tissue engineering strategies. Phosphates, typically calcium phosphate, are widely used osteoconductive and osteoinductive materials in which sequestering of charged proteins, such as the acidic sialoprotein osteopontin lead to subsequent adhesion and promotion of osteogenesis of hMSCs. Phosphates play an important role in mineralized tissue development^{10, 19}, and when incorporated into biomaterial scaffolds, may promote differentiation, as well as nucleation and mineral deposit formation¹⁰. Cell attachment is characterized by binding of integrin receptors to molecules of the extracellular matrix, and the dynamic interaction of focal adhesions with the underlying substrate is significant for integrin mediated signal transduction and the biological response of the cell.

During adipogenesis, ECM remodeling defines the onset of the differentiation process. Remodeling during adipogenesis is characterized by the conversion of a fibronectin-rich stromal matrix of the pre-adipocyte to the basement membrane of an adipocyte^{28–30}. The expression of ECM components is highly regulated during the process of adipocyte differentiation: types I and III collagen, fibronectin, and β 1-integrins are down-regulated, whereas type IV collagen and entactin are up-regulated³¹. Also important for MSC adipogenesis is ECM-regulated cell morphology. Growth of pre-adipocytes on a matrix that induces a spread cell morphology inhibits adipocyte differentiation, and this effect is overcome by the disruption of actin filaments, which promotes rounding-up of cells³². Thus, both ECM production and cell morphology play roles in MSC adipogenesis.

For 3D studies presented here, cells are entrapped in a rounded morphology, which would be expected to be beneficial for adipogenesis. Our results indicate that phosphate functionalized PEG hydrogels induce osteogenesis while t-butyl functionalized hydrogels induce adipogenesis. Deconvoluting the role of cell morphology from cell-matrix interactions is difficult in 2D studies, but a 3D PEG environment restricts spreading of encapsulated cells, due to the small mesh size. Thus, the role of the cell-matrix interactions can be more directly studied. Since adipogenesis is more efficient when cells adopt a rounded morphology³², encapsulation in a hydrogel may actually further enhance differentiation. Spreading may be less crucial for osteogenesis, as hMSC osteogenic differentiation is observed for rounded cells in 3D. While matrix formation is also likely to play a role in hMSC fate for cells encapsulated in functionalized PEG gels, these cells only see a simplified chemical environment at early time points. Therefore, based on the fact that phosphate groups promote osteogenesis while t-butyl groups promote adipogenesis, it is possible that initial interactions between functionalized PEG gels and hMSCs are sufficient for determining the ultimate fate of the stem cell.

Chemically, t-butyl groups and phosphate groups have very different properties, although at the concentrations used, the overall physical properties of the PEG gels were unchanged. Our results indicate that hMSCs differentiate down adipogenic or osteogenic pathways for t-butyl or phosphate functionalization, leading to cell-specific matrix production (Figure 4). One possible explanation for our observations may be that tethered chemical groups lead to direct cell-matrix interactions that induce differentiation down pathways that lead to production of tissue specific matrix molecules. Alternatively, the chemical environment may nucleate the sequestering of only particular cell-secreted molecules based on specific chemical interactions, and the subsequent matrix formation may then direct differentiation. In either case, the matrix

produced by the hMSCs would then interact with the cells in a more complex fashion that supports differentiation down a specific pathway. These results could have profound implications for tissue engineering strategies utilizing synthetic materials, since they suggest that it may only be necessary to control initial elements of cell expression in order to guide more complex overall processes.

Understanding the role of the ECM environment and recreating the important aspects of the natural cellular niche are critical for materials-based, tissue engineering approaches to be successful. The approach reported here provides a method for initially screening chemical or biological factors important to recreating the ECM environment, since the PEG-based chemistry allows for the incorporation of a broad range of biologically relevant molecules such as proteins, peptides, and glycosaminoglycans^{24,33–41}. To date, much of the high-throughput research performed on stem cells has focused on soluble signals and their subsequent effects on cellular functions²⁷. Our approach presents a methodology for studying the context in which the cells are screened by controlling the local microenvironment, thereby allowing for an improved understanding of the effects of extracellular interactions on cell activities in the presence and absence of soluble signals.

Collectively, we demonstrate that rational design can be used to identify synthetic extracellular environments that induce differentiation of hMSCs down specific target pathways utilizing small molecule chemical moieties even when mechanistic details are unknown. Of particular importance is the fact that cell-material interactions can be identified as playing a crucial role in cell fate since the differentiation pathways were induced using control hMSC culture media, in the absence of soluble signals. Previously, differentiation of embryonic stem cells was induced by biological and physical cues⁵, and material compliance was shown to induce hMSC differentiation into adipogenic, chondrogenic, neuronal, or osteogenic lineages²⁶. Due to the choice of PEG as an encapsulation material and the low concentration of functional groups relative to the PEG backbone, we were able to maintain a constant material compliance for this work and focus exclusively on changes in cell behavior due to chemical functionality. Our results indicate that differentiation potential can be controlled through simple interactions with chemical functional groups rather than more complex soluble or tethered biomolecules, which would make production of therapeutic materials simpler, cheaper, and more easily controlled. To our knowledge, this is the first example in which confounding factors such as materials properties and differences in cell morphology are eliminated and a synthetic matrix can therefore be shown to control induction of multiple hMSC lineages purely through interactions with small molecule chemical functional groups.

METHODS

Unless otherwise noted, all materials were obtained from Sigma-Aldrich and used without further purification.

Preparation of substrate and arrays of chemical moieties

Standard glass slides were cleaned using ‘piranha’ solution, dip-coated with 6 wt% poly(2-hydroxyethylmethacrylate) (polyHEMA) in methanol to render the glass surfaces non-cell adhesive²⁹, and dried in a vacuum oven at 60 °C for at least 2 hr.

Arrays of polymer spots were prepared from solutions containing poly(ethylene glycol) dimethacrylate (MW~550) and 0.5, 5, or 50 mM of the monomethacrylated monomers 2-aminoethyl methacrylate (amino, formulation b), tert-butyl methacrylate (t-butyl, formulation c), ethylene glycol methacrylate phosphate (phosphate, formulation d), 2,2,3,3-tetrafluoropropyl methacrylate (fluoro, formulation e), and methacrylic acid (acid, formulation f). The structures of these molecules are shown in Figure 1. I651 (Ciba-Geigy), an ultraviolet

initiator, was added to these solutions at a concentration of 0.5 wt%. These solutions were spotted onto the polyHEMA-coated slides using the Bio-Rad VersArray ChipWriter Pro System to create either 16×6 arrays with different concentrations (0.5, 5, and 50 mM) of each moiety for immunostaining screening or 6×6 arrays of the 50 mM concentrations for FISH analyses. The monomer spots were polymerized with ~4 mW/cm² ultraviolet light to create polymer gels for subsequent cellular analyses.

Mesenchymal stem cell culture

hMSCs were purchased from Cambrex and cultured in low-glucose Dulbecco's modified eagle medium (Gibco) supplemented with 10% FBS (Invitrogen), 1% penicillin/streptomycin (Gibco), 0.25% gentamicin (Gibco), and 0.25% fungizone (Gibco). hMSCs at passage 3 were used in this study.

Assessing differentiation of hMSCs on chemical moiety arrays

Immunostaining for proteins associated with differentiation of hMSCs was performed. 16×6 arrays were sterilized via exposure to UV light, copiously washed with PBS to remove any unreacted monomers, and seeded with hMSCs at 20,000 cells/cm². After 10 days of culture, cells were rinsed in PBS and fixed in 4% paraformaldehyde. Antigen retrieval (PPARG only) was performed by incubating sections in boiling citric acid (10 mM) for 10 min. The samples were blocked in 10% normal goat serum and 0.5% BSA for 30 min, incubated separately in primary antibodies [mouse anti-human collagen II (Abcam), osteopontin (Abcam), or PPARG (Abcam)] at 1:1000 for 4 h, then incubated in secondary antibody [goat anti-mouse Alexa Fluor 532 (Molecular Probes)] at 1:100 for 2 h. Samples were mounted with ProLong® Gold antifade reagent with propidium iodide (Molecular Probes) and allowed to cure overnight. Fluorescent images were taken with a Bio-Rad VersArray ChipReader and evaluated for the intensity of staining, normalized to the number of cells as indicated by propidium iodide staining, with a threshold for analyses as 2x background.

Differentiation of hMSCs was analyzed via fluorescent *in situ* hybridization (FISH). Arrays were sterilized via exposure to UV light, copiously washed with PBS to remove any unreacted monomers, and seeded with hMSCs at 20,000 cells/cm². After 4 and 10 days of culture, cells were rinsed in PBS and permeabilized in cytoskeletal buffer (CB) (100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 10 mM PIPES, pH 6.8), CB plus 0.5% Triton X-100 and CB for 30 s each step. Cells were then fixed in 4% paraformaldehyde for 10 min and stored at -80 °C for up to 2 weeks. For FISH, samples were hybridized overnight in a humidity chamber at 65 °C. Probe production details and sequences are listed in supplementary data.

Probes were prepared by hybridizing one target probe (Cy5-labeled) with β-actin, (Cy3-labeled) with each array. After hybridization, slides were washed in 10% formamide, 2× saline-sodium citrate buffer (20x SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7), at 39 °C for 3 × 5 min and 2× SSC for 3 × 5 min, and at room temperature using 1× SSC for 10 min and 4× SSC for 5 min with agitation. Fluorescent images were taken with a Bio-Rad VersArray ChipReader and evaluated for the intensity of staining, normalized to the intensity of β-actin, with a threshold for analyses as 2× background.

hMSC attachment and spreading on different materials was monitored after 10 days in culture by staining the F-actin filaments with phalloidin. Samples were rinsed with PBS and fixed in 4% paraformaldehyde in PBS for 10 min. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. TRITC phalloidin (Chemicon) was diluted 1:100 each in PBS and samples were incubated for 1 h at room temperature, and mounted with ProLong® Gold antifade reagent with DAPI (Molecular Probes) and allowed to cure overnight.

Chemical moiety affects on encapsulated hMSC differentiation

hMSCs were photoencapsulated in a 10 wt% monomer solution in PBS; the solution contained PEG4600DM and 0.05 wt% photoinitiator I2959, a cytocompatible photoencapsulation strategy³⁰. In addition, t-butyl methacrylate and EGMP were added separately at 50 mM to assess moiety effects on hMSC adipogenic and osteogenic differentiation in 3D culture. Cell/monomer solution (40 μ l) containing 25×10^6 cells/ml was polymerized in molds upon ultraviolet light exposure (~ 5 mW/cm², 10 minutes, 365 nm). After polymerization, the constructs were placed in hMSC media and cultured at 37 °C and 5% CO₂, replacing the media every 3–4 days. Directly after polymerization and after 4, 10, and 21 days, constructs were removed from culture and analyzed for CBFA1 and PPARG protein production via immunoblotting using β -actin for normalization (anti-CBFA1 and anti-PPARG, Abcam, anti- β -actin, Sigma).

After 21 days of culture, cell-hydrogel constructs were fixed overnight in 4% paraformaldehyde in PBS overnight, transferred to 22wt% sucrose for 72 hours, frozen in Cryo-gel (Instrumedics, Inc.), and cryosectioned (10 μ m sections). The sections were stained using Masson's trichrome, which stains collagen blue, and with Oil red, which stains intracellular lipid deposits red. All histological chemicals were obtained from Sigma.

Immunohistochemistry was utilized to visualize osteopontin and PPARG. Antigen retrieval (PPARG only) was performed by incubating sections boiling citric acid buffer (10 mM) for 10 min and endogenous peroxidase activity was quenched by incubating in 3% H₂O₂ for 5 min. The slides were blocked in 10% normal goat serum and 0.5% BSA for 30 min, incubated separately in primary antibodies [mouse anti-human osteopontin (Abcam) or PPARG (Abcam)] at 1:100 for 4 h, then incubated in secondary antibody [goat anti-mouse HRP (Chemicon)] at 1:750 for 2 h. The sections were developed using Vector NovaRED Substrate Kit (Vector Labs). The sections were mounted with ProLong® Gold antifade reagent (Molecular Probes) and allowed to cure overnight and imaged using conventional fluorescence microscopy (Nikon Eclipse TE300 and associated SPOT software).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Small molecule incorporation alters hMSC protein expression on PEG hydrogels. Chemical structures of functional moieties incorporated (left column) and protein expression of hMSCs (right column, as measured by immunostaining) quantitatively analyzed for collagen II (A), OPN (B), and PPARG (C) after 10 days of culture in control media on unmodified PEG and 0.5, 5, and 50 mM of amino (black bars), t-Butyl (black (top) to white (bottom) gradient bars), phosphate (grey bars), fluoro (white (top) to black (bottom) gradient bars), and acid (white bars). Values are reported as the fluorescent intensity average of 6 samples per composition, relative to the number of cells, as analyzed by propidium iodide counterstaining, and normalized to expression by cells cultured on PEGDM surfaces. Error bars represent one

standard deviation. An asterisk (*) denotes statistical significance compared with PEGDM ($p < 0.05$).

Figure 2.

Small molecule incorporation alters hMSC gene expression on PEG hydrogels. Gene expression of hMSCs (as measured by *in situ* hybridization) quantitatively analyzed for AggreCAN (A), CBFA1 (B), and PPARG (C) at days 0 (black bars), 4 (white bars), and 10 days (grey) of culture on (a) unmodified PEG and 50 mM of (b) amino, (c) t-Butyl, (d) phosphate, (e) fluoro, and (f) acid. Values are reported as the fluorescent intensity average of 6 samples per composition per timepoint, relative to β -actin expression, and normalized to expression by cells cultured on PEGDM surfaces. Error bars represent one standard deviation. An asterisk (*) denotes statistical significance compared with PEGDM ($p < 0.05$).

Figure 3. hMSC morphology is altered in response to small molecule incorporation into PEG hydrogels. Representative light and fluorescent micrographs of TRITC-phalloidin (red) and DAPI (blue)-stained of hMSCs depicting morphology cultured on PEGDM (a and e), acid (b and f), phosphate (c and g), and t-butyl (d and h)-functionalized surfaces (bar = 200 μ m for light micrographs, bar = 50 μ m for fluorescent micrographs).

Figure 4.

Encapsulation of hMSCs in phosphate and t-Butyl functionalized PEG hydrogels alters CBFA1 and PPARG expression. CBFA1, PPARG, and β -actin expression of hMSCs encapsulated in control, t-butyl, and phosphate-functionalized PEG hydrogels and cultured for 0, 4, 10, and 21 days in control media. Immunoblots (A) were quantified with ImageJ software and CBFA1 (B) and PPARG (C) expression levels over the 21-day culture period were normalized to β -actin expression (PEGDM: X; t-butyl functionalized: closed diamonds; phosphate-functionalized: open circles), error bars represent one standard deviation. Differentiation was further verified using histological and immunohistochemistry staining (D) of matrix evolution by encapsulated hMSCs. Masson's trichrome stains collagen blue (left column) and Oil Red

stains intracellular lipid deposits red (third column). OPN and PPARG staining was performed using protein-specific primary antibodies and HRP-labeled secondaries, and visualized with Vector NovaRed Substrate Kit (Vector Labs) (bar = 100 μm).