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Development of a Dynamic Stem Cell Culture Platform for Mesenchymal Stem Cell Adhesion and Evaluation

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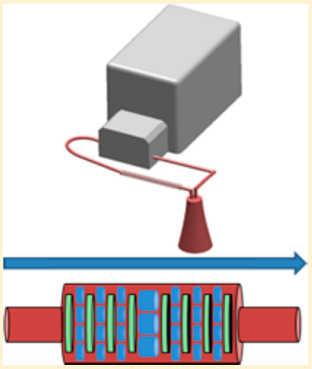
ABSTRACT: The importance of providing a physiologically relevant environment for cell culture is well recognized. The combination of proper environmental cues which are provided *in vivo* by the bloodstream and extracellular matrix must be reproduced to properly examine cell response *in vitro*, and cannot be recapitulated using traditional culture on polystyrene. Here, we have developed a device, the dynamic stem cell culture platform (DSCCP), consisting of a biomimetic scaffold cultured within the dynamic environment of a perfusion bioreactor. By varying scaffold parameters including stiffness and protein inclusion at the material surface, we found that human mesenchymal stem cells (hMSCs) were able to adhere to modified substrates, while still maintaining multipotency. Culture in a perfusion bioreactor showed cell survival and proliferation, particularly on modified substrates. The DSCCP represents a complete platform for cell adhesion and subsequent evaluation, including the response of a cell population to drug treatment.



KEYWORDS: *stem cells, cell adhesion, hydrogel, bioreactor*

INTRODUCTION

Significant contributions to the field of drug discovery research have been achieved using traditional *in vitro* culture of a 2D monolayer of cells seeded onto tissue culture polystyrene (TCPS).[1] However, there are some drawbacks to TCPS in that it lacks the ability to provide a cellular microenvironment that imitates the native environment. A system which can provide environmental cues to a cultured cell population is critical for drug testing as environmental cues are found to have significant impacts on cell phenotype, function, and therefore the response of cells to drugs.[2,3] It has been well documented that the cellular microenvironment can send signals to a cell through cell−cell and cell−matrix interactions, as well as through mechanical forces. Downstream effects of these interactions include alterations in gene expression, cell migration, proliferation, and differentiation.[2,4]

In order to develop an *in vitro* environment suitable for drug discovery applications, we chose to investigate two major components: cell−matrix interactions and mechanical cues provided by a biomaterial scaffold, and a dynamic environment provided by a perfusion bioreactor. The combination of both of these components will result in a simple *in vitro* device, the dynamic stem cell culture platform (DSCCP), that can be translated to many cell types, matrix molecules, and subsequent evaluations, including investigation of cell response to drug treatments.

Biomaterials can be fabricated to control both cell−cell and cell−matrix interactions, optimizing adhesion events and the resulting downstream reactions. Cell adhesion is critical for many cellular functions, including spreading, proliferation, and migration. A key finding in cancer research discovered that integrin interactions played a major role in the resistance of breast cancer cells to paclitaxel,[5] and that cell adhesion protected cancer cells from drug-induced apoptosis. The fabrication of biomaterials to include specific extracellular matrix (ECM) molecule ligands which mimic the *in vivo* integrin interactions that result in drug-resistant tumors can create a more efficient model for drug evaluation.

There have been many recent developments in the field of biomaterials for drug evaluation, including the development of modified 2D substrates as well as 3D environments for drug screening applications. Poly(dimethylsiloxane) (PDMS) 2D substrates modified to include ECM molecules have been shown to successfully increase the adhesion of the human intestinal Caco-2 cell line, and provide a foundation for creating miniaturized biomimetic environments for drug evaluation.[6] The complex 3D nature of tumors has led to the development of 3D models for drug evaluation, specifically in cancer research. Scaffold-free 3D culture of multicellular tumor spheroids (MTS) has shown that formation of 3D spheroids resulted in a significantly different outcome when spheroids are exposed to traditional cancer treatments when compared to their TCPS counterparts.[7] The cell−cell interactions that are

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recapitulated in the MTS system demonstrated decreased levels of cell death following exposure to drugs commonly used for cancer treatment such as doxorubicin and paclitaxel.

Synthetic biomaterials provide a level of control over the structure and composition of the polymer that cannot be achieved when using natural materials. Tumors represent a complex *in vivo* environment, which must be studied by systematically evaluating individual interactions. In order to achieve this, recent studies have used modified poly(ethylene glycol) (PEG) hydrogels that include a specific ECM protein, exhibit specific mechanical properties, or degrade at a specific rate.[8] Studies of this nature allow researchers to break the complex tumor microenvironment into distinct parts and evaluate the effect of each of these parts on the cell response to drug treatment.

The mechanical properties of the cell microenvironment can also influence cell functions similar to those impacted by cell adhesion. Substrate stiffness has also been shown to affect cell migration,[9,10] differentiation,[11,12] and self-renewal.[13] Increasing the stiffness of PEG hydrogels has previously been shown to increase the osteogenic differentiation of mesenchymal stem cells (MSCs),[11] while soft substrates resulted in weaker adhesion and the promotion of MSC chondrogenesis.[14] It is well documented that tissue stiffness is increased in the tumor microenvironment, and several groups have sought to investigate how the substrate stiffness can impact the response of cells to drug therapy in terms of cell attachment, organization, proliferation, and survival.[8,15]

The mechanical environment is also influenced by an important factor of nutrient and drug delivery: the bloodstream. Nutrient exchange *in vivo* occurs continuously through the diffusion of molecules from the bloodstream into the tissues. In addition to nutrient delivery, the blood also provides mechanical stimulation in the form of shear stress, which can influence cell behavior. All intravenously administered drugs reach the targeted tissue via transport in the blood, making it an important aspect of drug evaluation which cannot be ignored when creating *in vitro* drug evaluation models. We have previously developed a tubular perfusion system (TPS) bioreactor in our laboratory, which we have used extensively for the culture of MSCs, to increase nutrient transport and cell survival as well as induce osteogenic differentiation.[16]

In order to develop a platform which provides a more accurate representation of the *in vivo* environment by mimicking the cell−ECM interactions, mechanical properties, and nutrient delivery of native tissue, we investigated the impact of several parameters on MSC adhesion, morphology, and pluripotency. The development of a product which can recapitulate the native cellular microenvironment is imperative for the future of drug development and evaluation. We chose to investigate MSCs because they are found in multiple tissues throughout the body including bone marrow, adipose tissue, the synovial membrane, and trabecular bone and can be differentiated into a number of lineages including chondrocytes, adipocytes, and osteoblasts.[17,18] In addition to the importance of drug evaluation on a cell type capable of downstream differentiation, the epithelial-to-mesenchymal transition (EMT) has been indicated as an important driving force for tumor development, invasion, and metastasis.[19] Recently, it has been reported that EMT may initiate the induction of a cancer stem cell (CSC) population.[20,21] CSCs are thought to be one of the key reasons that tumors can develop drug resistance and contribute to the unpredictable nature of tumor development.[22] Therefore, the investigation of drug treatment on a stem cell population such as MSCs is of high importance.

To develop the DSCCP, we investigated three major objectives. First, we investigated the role of stiffness on MSC adhesion and morphology by attaching nonspecific amine groups to PEG hydrogels of varying stiffness. Second, we investigated how adhesion and differentiation may be altered by the inclusion of an MSC specific protein, fibronectin. Lastly, we examined how the inclusion of shear force and dynamic nutrient delivery, achieved by hydrogel incorporation in the TPS bioreactor, impacts MSC adhesion. We hypothesize that we will see increasing MSC adhesion with increasing stiffness as well as with the inclusion of MSC-specific protein. We anticipate that the inclusion of fibronectin will not affect the pluripotency of MSCs, and that the dynamic environment provided by the TPS bioreactor will result in increased cell adhesion and survival. Overall, this will indicate that the DSCCP can serve as a fitting model for drug discovery applications.

MATERIALS AND METHODS

Hydrogel Fabrication. Poly(ethylene glycol) diacrylate (PEGDA) hydrogels (M_n = 700, Sigma-Aldrich, St. Louis, MO) were created using free radical polymerization using a method previously established in our laboratory.[23] Hydrogels were tested at two concentrations: soft (5% w/v PEGDA) or stiff (20% w/v PEGDA). Solutions containing 5.0 or 20.0 mg PEGDA/100 μL water were used to vary stiffness. 15 mM ammonium persulfate (APS) with a 1:2 component:solvent ratio (where the solvent used was water) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) (Sigma-Aldrich) were used in a 1:1 ratio as initiators. The desired volume of PEGDA was mixed with water, and then APS and TEMED were added sequentially, vortexing after each addition. The solution was quickly poured into a custom designed Teflon mold to cross-link into hydrogel disks 1 mm thick and 20 mm in diameter. The hydrogels finished cross-linking in 2 min. For surface modified gels, 25 μL of 12 μmol/mL acryloyl-PEG-NH_2 (Nanocs, Inc., Boston, MA) solution or 0.64 μmol/mL acryloyl-PEG-fibronectin solution was added to the surface of the hydrogel, after 90 s of cross-linking. Hydrogels were cross-linked for an additional 2 min after modification. Acryloyl-PEG-fibronectin was fabricated by the reaction of a 50-fold molar excess of acryloyl-PEG-NHS (Nanocs, Inc.) with fibronectin (FN) (Millipore, Billerica, MA) in sodium biocarbonate buffer (pH = 8.5) for 2 h, followed by dialysis and drying. All hydrogels were washed in phosphate-buffered saline (PBS) for 24 h following fabrication. Hydrogels used in cell adhesion and spreading experiments were prepared in a sterile environment, and all precursors were filtered using a 0.22 μm sterile filter.

Dynamic Mechanical Analysis. The bulk mechanical properties of PEGDA hydrogels fabricated at 5, 10, and 20% w/v PEGDA were calculated using the Q-800 dynamic mechanical analyzer (DMA; TA Instruments, New Castle, DE) and Q Series Explorer software. 4 mm thick samples were cyclically compressed at 1 Hz to a strain of 7%. From the applied strain, the response of the material and the Young's modulus were determined from the linear region of the stress−strain curve. Six samples at each PEGDA concentration were tested.

Human Mesenchymal Stem Cell Culture. hMSCs (Lonza, Walkersville, MD) were expanded prior to the study in media consisting of DMEM (Life Technologies, Frederick, MD) supplemented with 10% fetal bovine serum (Life

Technologies), 1.0% v/v penicillin/streptomycin (Life Technologies), 0.1 mM nonessential amino acids (Life Technologies), and 4 mM L-glutamine (Life Technologies) using protocols set forth by the manufacturer and previously described.[16,23] hMSCs were expanded on tissue culture polystyrene flasks with medium changes every 3 days according to the manufacturer's specifications. hMSCs at passage 4 were used for all experiments. Cell cultures were incubated at 37 °C, 5% CO_2, and 80% humidity.

Cell Adhesion and Spreading. Prior to hydrogel seeding, adherent hMSCs were lifted with trypsin/EDTA (Life Technologies) and counted using trypan blue uptake to determine viability. Sterilized hydrogels were placed into a 12 well plate and seeded with a concentrated cell solution of 100,000 hMSCs/100 μL media for 1 h at 37 °C. Additional medium was added after 1 h, and cells were cultured for 48 h. Cells seeded onto TCPS served as a positive control for cell attachment. After 48 h of incubation, hydrogels were soaked in PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ for 30 min to remove nonadherent cells as well as any remaining medium. To aid in the visualization of cell attachment and spreading, a live–dead assay was performed following standard protocols. Hydrogels were incubated with 2 mM ethidium homodimer and 4 mM calcein AM (Life Technologies) for 30 min. Images were taken using a fluorescent microscope (Axiovert 40 CFL with filter set 23; Zeiss, Thornwood, NY) equipped with a digital camera (Diagnostic Instruments 11.2 Color Mosaic, Sterling Heights, MI). Images were analyzed using the Zeiss software Axiovision 4.8 to quantify relative cell number, aspect ratio, and the average cell area on each hydrogel. Six images were analyzed for each condition. If individual cells in an aggregate were indiscernible due to overlapping or aggregating, the area was counted as one cell. Figure 1 shows the major processing steps within the Axiovision software that were used for all images.

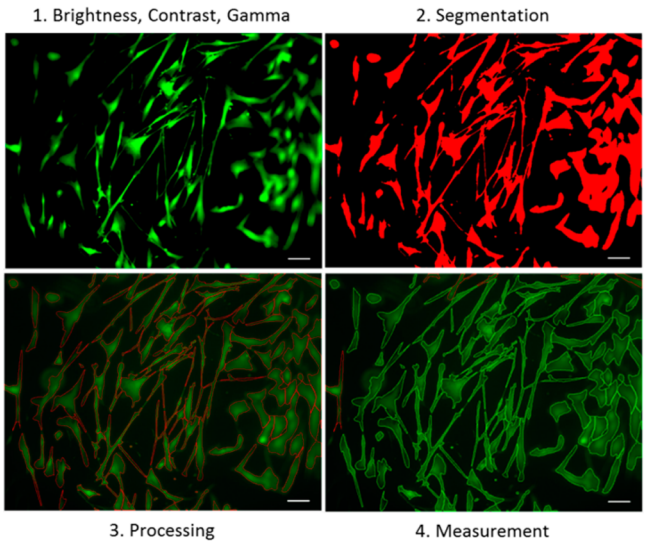

Figure 1. Major processing steps used to analyze all cell adhesion images. Using Axiovision software, four major steps were used to analyze images including the adjustment of brightness, contrast, and gamma; segmentation to select cells; processing to separate individual cells; and measurement of cell number and average cell area. Identical parameters were set for all images analyzed.

Centrifugation Assay. To quantify relative adhesion of hMSCs seeded to modified and unmodified hydrogels, a centrifugation cell adhesion assay was performed as described previously.[24] Hydrogels were fabricated in the wells of a 96 well TCPS-treated plate (Costar, St. Louis, MO). Approximately 5000 hMSCs in growth medium were premixed with Hoechst 33342 fluorescence stain (10% of volume), added to each well, and incubated for 4 h at 37 °C and 5% CO_2. Wells without hydrogels served as a positive control (TCPS). A schematic of the centrifugation cell adhesion assay, which was modified from Kaplan et al.,[24] is shown in Figure 2. Using an inverted

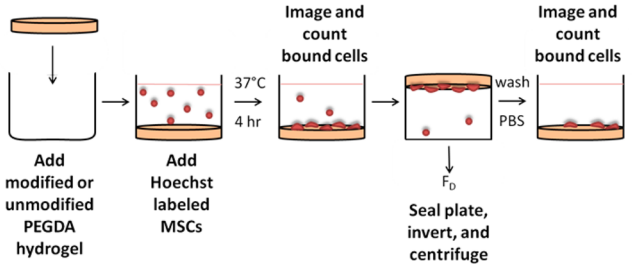

Figure 2. Schematic of the centrifugation assay. Unmodified, amine modified, and FN modified groups were tested. TCPS served as a control.

microscope (Zeiss AxioVert, equipped with a AxioCam MRm digital camera, Zeiss, Inc., Thornwood, NY) and AxioVision Software (Version 8.2, Zeiss, Inc.), 2 × 2 tiled (total tiled image area = 0.542 mm^2) images were captured on wells prior to centrifugation (prespin). The wells were filled with PBS, purged of air bubbles, and covered with acetate sealing tape (Fisher Scientific, Pittsburgh, PA). The plates were centrifuged (upside down) at a relative centrifugal force (RCF) of 50g for 30 s at 22 °C. The force of detachment (FD) was approximately 18 pN and was calculated by the following formula (Reyes and Garcia, 2003[25]):

$$FD = V \cdot d \cdot RCF$$

where V = cell volume = 500 μm^2, d = (cell density − medium density) = 0.07 g/cm^2, and RCF = 50.

The supernatant was removed from the wells, and the wells were washed once with PBS. Excess PBS was removed from the well, and 100 μL of PBS was added to each well. Tiled imaging was repeated (postspin). The cell numbers pre- and postspin were determined by stitching of tiled images and subsequently using an automated cell counting (AxioVision) routine to count the number of fluorescently labeled nuclei. The percent adhesion was determined by subtracting the postspin cell count from the prespin cell count, dividing by the prespin count, and multiplying by 100%.

Induction of Osteogenic, Chondrogenic, and Adipogenic Differentiation. To show that the environment produced by surface modified PEGDA hydrogels neither inhibits nor induces differentiation down the three main mesenchymal lineages (osteogenesis, chondrogenesis, and adipogenesis), a differentiation study was completed to show that, when induced, cells seeded on the gels underwent differentiation and cells that were not introduced to differentiation cues were not induced based on material properties alone. Cells were seeded onto modified and unmodified hydrogels as described above. After incubation for 24 h in growth medium, cells were cultured for 21 days in osteogenic

(DMEM with 10% FBS, 1 mM sodium pyruvate (Life Technologies), 100 U/100 μg penicillin−streptomycin, 10^{-7} M dexamethasone (Sigma-Aldrich), 50 μg/mL ascorbic acid (Sigma-Aldrich), and 10 mM β-glycerophosphate), chondrogenic (high glucose DMEM supplemented with ITS (BD, Franklin Lakes, NJ), 4 mM L-proline (Sigma-Aldrich), 50 μg/mL ascorbic acid, 1% sodium pyruvate, 10^{-7} M dexamethasone, and 100 U/100 μg penicillin−streptomycin, and 10 ng/μL TGF-β3 (R&D Systems, Minneapolis, MN)), or adipogenic (DMEM with 10% FBS, 1 mM sodium pyruvate, 100 U/100 μg penicillin−streptomycin, 10^{-6} M dexamethasone, 10 μg/mL insulin (Sigma-Aldrich), 0.5 mM IBMX (Life Technologies), and 200 μM indomethacin (Life Technologies)) media as described by Yang et al.[26] Growth media as well as groups cultured on TCPS were used as controls. All media were changed every 3 days. After 21 days in culture, cells were lifted from the surface of the hydrogels and seeded onto chamber slides (Fisher Scientific) for 24 h. At this time cells were fixed with 10% formalin for 10 min, and stored at 4 °C in PBS. Histological staining was completed to show calcification, glycosaminoglycan production, and the presence of lipid droplets. Mineralization was visualized using Von Kossa staining with a Nuclear Fast Red (Poly Scientific, Bay Shore, NY) counterstain following standard protocols. Glycosaminoglycan production was visualized by staining with 0.5% Alcian Blue solution (Poly Scientific) followed by a nucleic counterstain with Nuclear Fast Red. For the visualization of lipid vacuoles in adipogenic samples, a working solution of Oil Red O (Poly Scientific) was prepared and applied to fixed monolayers, followed by counterstaining with hematoxylin.

Dynamic TPS Bioreactor Culture. hMSCs were seeded onto 20% w/v PEGDA hydrogels that were unmodified, amine modified, or FN modified following the procedures previously outlined. After 24 h, a subset of each group was examined using a live−dead assay as described above. The second subset was loaded into the bioreactor. The bioreactor was set up as described previously by our laboratory.[16] Briefly, a tubing circuit composed primarily of platinum-cured silicone tubing (Cole Parmer, Vernon Hills, IL) connected a growth chamber to a medium reservoir (Figure 3). Prior to use, the entire tubing circuit was sterilized by autoclave. The growth chamber was made up of platinum-cured silicone tubing (i.d. of 1 in.) and contained the different hydrogel groups. Hydrogels were separated inside of the growth chamber by pieces of quarter-inch silicone tubing to prevent aggregation. hMSC growth medium was pumped through the recirculating system using a peristaltic pump (Cole Parmer) at 1.0 mL/min. This flow rate was chosen based on previous studies[16,27] to prevent aggregation while still exposing cells to direct shear force. The entire system was placed in an incubator at 37 °C for the duration of the study. After 48 h in the bioreactor, hydrogels were washed in PBS and imaged using a live−dead assay and the same techniques as before. Following culture within the bioreactor, it was observed that hMSCs on modified hydrogels formed distinct clusters (see Figure 9). A minimum of six samples from each group were analyzed for cell number, and images of modified hydrogels without clustering were not included in analysis as they were not representative of the modified environment. As a result, the modified hydrogel groups from the bioreactor have an $n = 9$ while all other groups have an $n = 6$.

Statistics. All data was analyzed using one-way analysis of variance and Tukey's multiple-comparison test to determine statistical differences between hydrogels. A confidence interval of 95% ($\alpha = 0.05$) was used for all analyses, and means and SDs are shown in each figure.

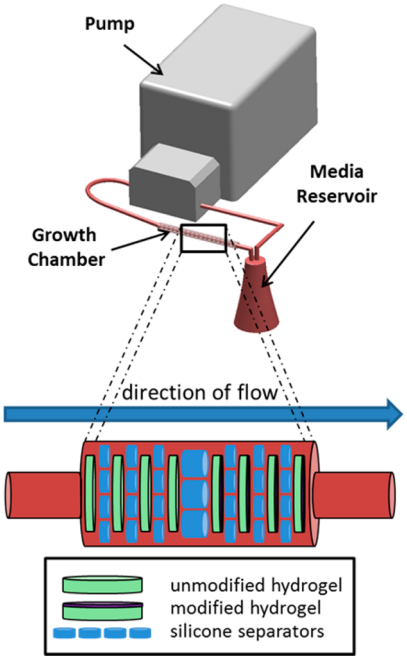

Figure 3. Schematic of the overall design of the DSCCP consisting of the TPS bioreactor (top) with detailed depiction of the growth chamber (bottom). Here, it is shown that unmodified (left) and modified (right) hydrogels can be cultured together within the same growth chamber to ensure evaluation under the same dynamic environment. Individual hydrogels as well as hydrogel groups were separated by pieces of autoclaved silicone tubing (blue) to prevent aggregation and maintain separation between experimental groups.

RESULTS

DSCCP Design. Shown in Figure 3, our complete device consists of a biomimetic scaffold within the TPS bioreactor. The TPS bioreactor is composed of a peristaltic pump and medium reservoir that continually perfuses medium through a growth chamber. The growth chamber is shown in detail below the pump. Depicted in the figure, unmodified (left) and modified (right) hydrogel groups can be cultured together within the same chamber at different axial positions. Hydrogel groups were separated by larger pieces of silicon tubing to distinguish groups, and individual hydrogels were separated by thin pieces of the same tubing to prevent hydrogel aggregation without inhibiting the flow of media. Our laboratory has previously completed detailed modeling of the nutrient profiles and shear forces within the growth chamber at varying flow rates, and we have found that, at the currently used flow rate (1.0 mL/min), there are no significant differences in the nutrient profile or shear forces over a growth chamber of our size (approximately 7 cm).[16,27] The DSCCP can be customized on many levels, as parameters such as perfusion flow rate, scaffold properties, cell type, and the perfusion medium can be modified to investigate a desired cell function.

Dynamic Mechanical Analysis. The resulting Young's modulus of each hydrogel formulation is shown in Figure 4. Hydrogels formulated at 20% w/v PEGDA were found to have a statistically greater Young's modulus than both the 5 and 10% w/v gels. There were no statistical differences between the

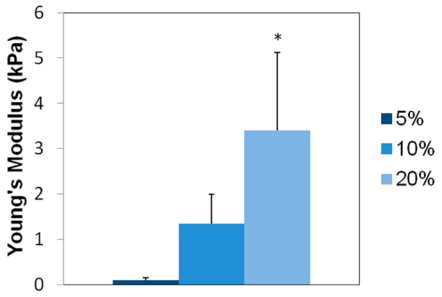

Figure 4. The Young's modulus of PEGDA hydrogels fabricated at 5, 10, and 20% w/v. Six samples at each PEGDA concentration were tested. Results show that hydrogels formulated with 20% w/v PEGDA were found to have a statistically greater Young's modulus than all other hydrogel formulations (*, $p < 0.01$).

softer gels. The Young's modulus increased with increasing PEGDA concentration, from 0.1 to 1.3 to 3.4 kPa for 5, 10, and 20% gels respectively.

Cell Adhesion and Spreading. The impact of stiffness on hMSC adhesion and spreading was first investigated by comparing unmodified PEGDA hydrogel disks at 5 and 20% w/v with hydrogels that were modified at the surface by covalent tethering of the nonspecific binding group acryloyl-PEG-NH_2. The amine group would create a charge at the surface of the hydrogel, mimicking the action of TCPS. Figure 5A shows representative images of hMSCs on the four hydrogel groups tested: unmodified 5% w/v PEGDA, unmodified 20% w/v PEGDA, amine modified 5% w/v PEGDA, and amine modified 20% w/v PEGDA. All images were taken at 10× magnification, and scale bars represent 100 μm. Six images of each group were analyzed using a script written with Axiovision 4.8.2 (Zeiss) to determine the average cell count per mm^2 as well as the average cell area (μm^2). Results showed that, on both soft and stiff substrates, the cell number was statistically greater on modified gels when compared to unmodified (see Figure 5B, top). In addition to cell number, we saw that amine modified 20% w/v PEGDA hydrogels exhibited a statistically greater average cell area, indicating increased cell spreading on the surface (see Figure 5B bottom) which is confirmed by microscopy images in Figure 5A.

Based on the initial results, we proceeded with all further studies using the 20% w/v PEGDA formulation. We next examined how the inclusion of specific binding sites on the surface of the hydrogel would impact hMSC adhesion and morphology. Acryloyl-PEG-fibronectin was fabricated through the reaction of FN and acryloyl-PEG-NHS, which was confirmed using FT-IR. Figure 6A shows representative images of hMSCs attached to the surface of unmodified, amine modified, and FN modified 20% w/v PEGDA hydrogels, as well as a TCPS control. Results of image analysis indicated that unmodified hydrogels exhibited a statistically lower cell number (see Figure 6B, top). The addition of nonspecific and specific adhesion modalities increased cell number when compared to unmodified hydrogels with FN modified hydrogels demonstrating no statistical difference in cell number when compared to TCPS. Figure 6B, bottom, shows the average cell area (μm^2) for each group. As with cell number, unmodified hydrogels had the smallest average cell area, and histological images indicate rounded cell morphology. On amine and FN modified hydrogels, there was an increase in cell area, indicating cell spreading. Again, there were no statistical differences in average cell area between modified hydrogels and TCPS. Images also revealed cell clustering on FN modified hydrogels, suggesting that the surface modification is heterogeneous and cells cluster around areas of modification. The assay also revealed that there was little or no cell death for all groups.

Centrifugation Assay. To compare the relative strength of adhesion to the various hydrogels, we utilized a centrifugation cell adhesion assay to apply a detachment force to hMSCs incubated on hydrogel surfaces. We investigated four groups: unmodified, amine modified, and FN modified 20% w/v

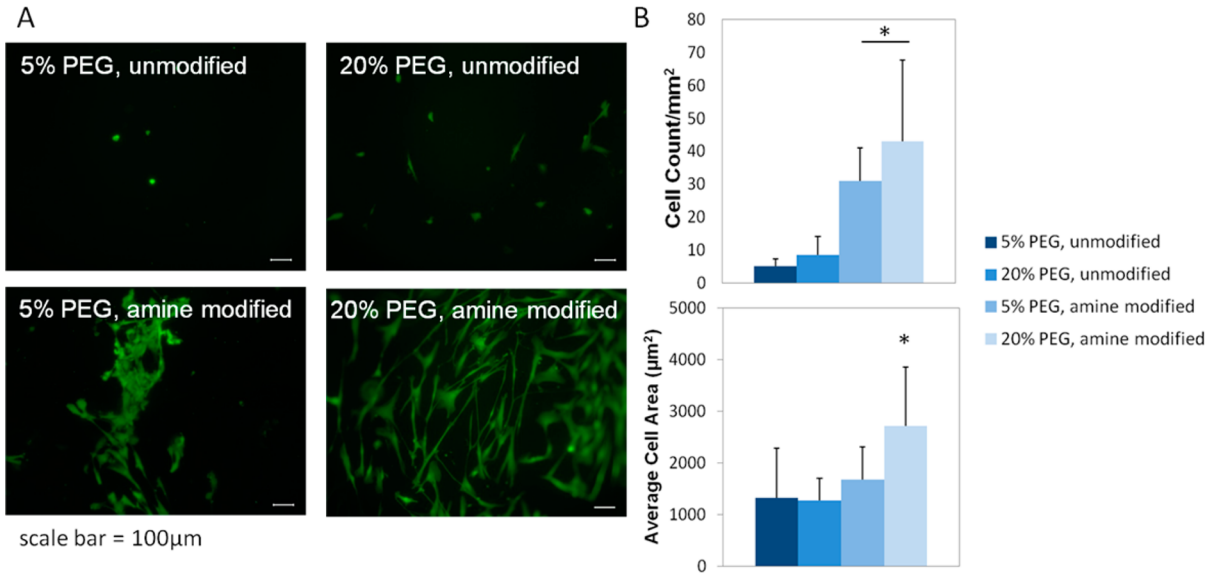

Figure 5. Panel A shows representative images (scale bars = 100 μm) of each group showing increased adhesion and spreading on modified gels, with the greatest degree of spreading on stiff, modified hydrogels. Panel B shows the results of image analysis of hMSCs seeded onto amine modified and unmodified 5 and 20% w/v PEGDA hydrogels. On both soft and stiff substrates, the cell number (top) was statistically greater on modified gels vs unmodified gels (*, $p < 0.01$). The average cell area (bottom) on amine modified 20% w/v hydrogels was statistically greater than that of all other groups, indicating cell spreading on the surface of the hydrogel (*, $p < 0.05$).

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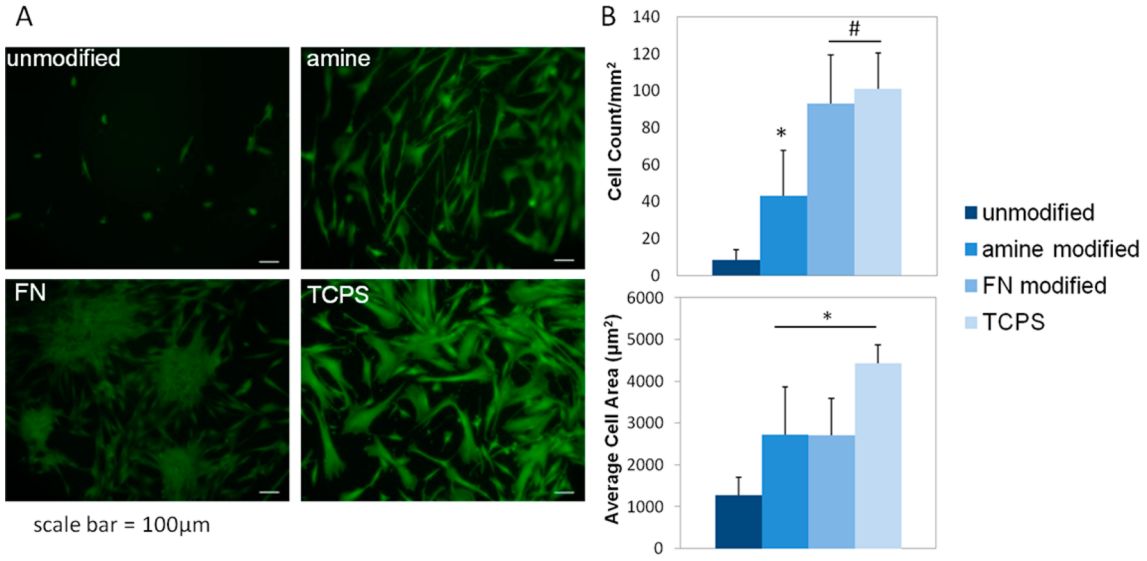

Figure 6. Panel A shows representative images (scale bar = 100 μm) of each group showing increased adhesion and spreading on modified gels, with similar morphology to TCPS. Panel B shows the results of image analysis of hMSCs seeded onto all groups. The inclusion of amine moieties significantly increased cell number (top) over the unmodified gels (*, $p < 0.01$). Additionally, FN modified hydrogels and the TCPS control also showed significant increases in cell number, but demonstrated no statistical differences between them (#, $p < 0.01$). Modified hydrogels showed statistically greater average cell area (bottom) compared to unmodified gels, indicating a higher degree of spreading. Additionally, modified hydrogels demonstrated no statistical differences in average cell area when compared to TCPS (*, $p < 0.01$).

PEGDA hydrogels, as well as a TCPS control. Results indicate that the unmodified and amine modified hydrogels exhibited relatively low adhesion (9 and 26% respectively), while FN modified hydrogels and TCPS demonstrated much higher levels of adhesion (95 and 87% respectively) (Figure 7). Both

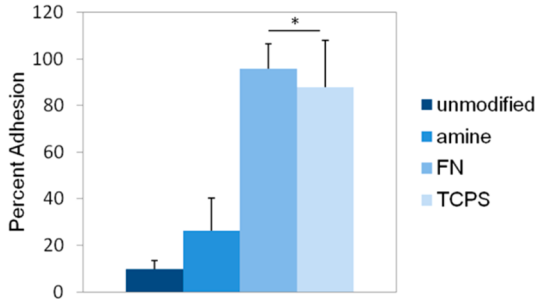

Figure 7. Percent adhesion resulting from the centrifugation assay. Results show that FN modified and TCPS groups had statistically greater percent adhesion compared to unmodified or amine modified hydrogels (*, $p < 0.01$). There were no statistical differences in percent adhesion between FN modified and TCPS groups.

unmodified and amine modified gels had statistically lower adhesion than FN modified and TCPS groups, and there were no statistical differences between unmodified and amine modified or between FN modified and TCPS.

Induction of Osteogenic, Chondrogenic, and Adipogenic Differentiation. hMSCs are a valuable cell type for therapeutic applications due to their ability to readily differentiate down osteogenic, chondrogenic, and adipogenic lineages. In order to demonstrate that the inclusion of amine and FN groups on the surface of PEGDA hydrogels neither inhibits nor induces differentiation, hMSCs were exposed to growth, osteogenic, chondrogenic, or adipogenic media for 21 days. Histological images in Figure 8 demonstrate that hMSCs exhibit the morphology and ECM production of the expected cell type when exposed to proper differentiation signals, indicating that hMSCs seeded onto amine and FN modified surfaces are still capable of undergoing differentiation. It was similarly important to show that hMSCs that were exposed to growth medium maintained the morphology of hMSCs, and remained negative for calcium deposition, glycosaminoglycan production, or the presence of lipid vacuoles, demonstrating that the hydrogel properties alone did not induce undesired differentiation. It is important to note that because cells were lifted with trypsin/EDTA prior to histological staining, there is some loss of matrix.

Dynamic TPS Bioreactor Culture. Image analysis (see Figure 9) revealed that modified hydrogel groups cultured in the bioreactor showed distinct cell clustering, further confirming that the hydrogel surface is heterogeneous. All images with cell clusters were analyzed. Analysis showed that, after the initial static culture, FN modified hydrogels exhibited a significantly higher cell number, while no differences were detected between unmodified and amine modified groups. Following bioreactor culture, modified hydrogels retain a significantly higher cell number, with no differences observed between amine and FN modified groups.

DISCUSSION

The development of the DSCCP which incorporated both cell−ECM interactions through the use of a biomimetic material and mechanical cues provided by the TPS bioreactor was achieved through the investigation of three objectives. The first two objectives involved the development of a biomimetic material that is suitable to facilitate hMSC adhesion and can be used for subsequent evaluations. We first investigated the role of substrate stiffness on hMSC adhesion and morphology through the attachment of nonspecific amine groups on the surface of PEGDA hydrogels of varying stiffness. The stiffness of the surrounding matrix is an important characteristic of disease states and is still typically the method used to initially identify a potential tumor.[15] Based on the results of DMA

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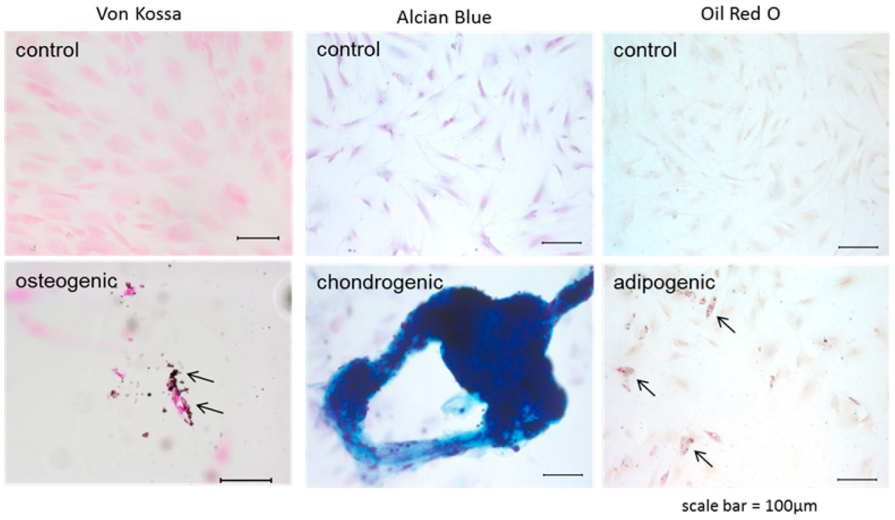

Figure 8. Histological images showing the results of differentiation on modified hydrogels. Results show that FN modified gels do not induce differentiation without medium induction (top). Images on the bottom panel show the development of calcium deposits which are stained black in color as a result of Von Kossa, the production of a cartilaginous matrix which has stained darkly for the presence of glycosaminoglycans (blue) using Alcian Blue, or the presence of lipid vacuoles which are stained red in color with Oil Red O. All images were taken at 20×, and scale bars are equal to 100 μm.

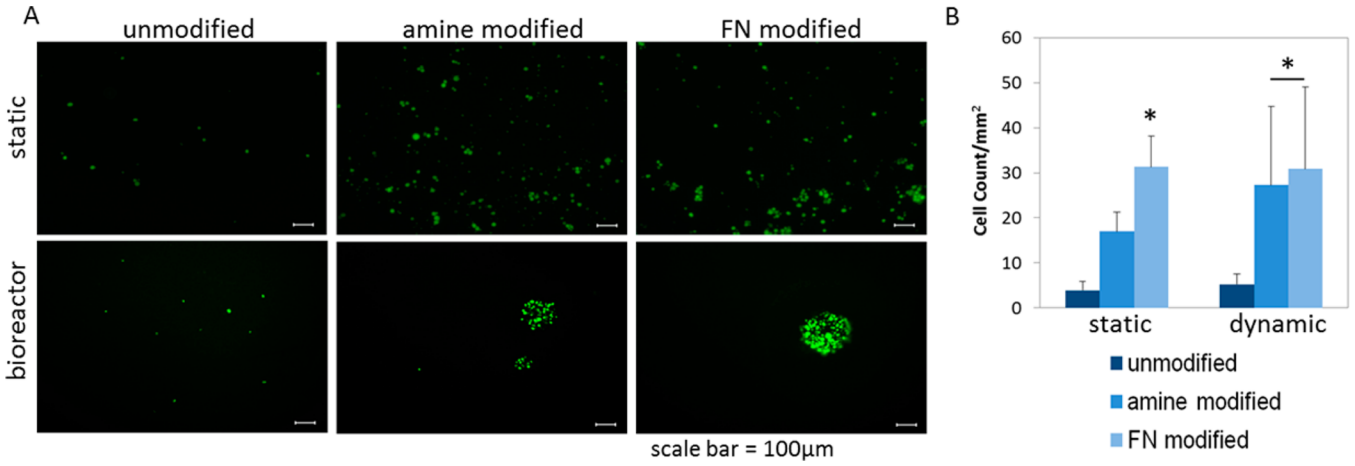

Figure 9. Panel A shows representative images from each group. There is distinct clustering of hMSCs on modified hydrogels following bioreactor culture. All images with clustering were analyzed (n = 9 for amine and FN modified bioreactor groups). Results of image analysis (panel B) showed that, after the initial static culture, FN modified hydrogels exhibited a significantly higher cell number (*, $p < 0.05$), while no differences were detected between unmodified and amine modified groups. After culture within the TPS bioreactor, hMSCs cluster on modified hydrogels and retain a significantly higher cell number (*, $p < 0.05$), with no differences between amine and FN modified groups. These results indicate that surface modified hydrogels can be used in conjunction with shear flow as a dynamic drug evaluation model, but that heterogeneity in surface modification results in cell adhesion and proliferation around points of modification.

analysis, we chose to examine 5% (E = 0.10 kPa) and 20% (E = 3.40 kPa) w/v PEGDA gels to represent soft and stiff substrates respectively. These moduli are also comparable to the mechanical properties of normal mammary tissue (E = 0.17 kPa) and the average tumor (E = 4.05 kPa).[15] Amine groups were chosen as the modifier to mimic the charge presented on TCPS, which is commonly used in 2D *in vitro* models for drug evaluation.

The results demonstrate that adhesion increased significantly on modified gels when compared to unmodified gels regardless of stiffness, indicating that cell−substrate interactions are necessary to facilitate cell adhesion. In addition to increased cell number, we also showed that amine modified 20% w/v PEGDA hydrogels exhibited a statistically greater average cell area, demonstrating increased cell spreading on the surface when compared to all other groups in the study. The presence of amine groups on the material surface allowed us to investigate hMSC adhesion to nonspecific adhesive moieties, which is similar to the function of TCPS which facilitates adhesion based on nonspecific bioactive groups such as hydroxyl and carboxyl groups.[28] The results here agree with numerous studies showing that the inclusion of functional groups increases cell adhesion, proliferation, and subsequent cell responses.[29−31]

Although there was increased adhesion on modified gels over unmodified for both soft and stiff substrates, there were no statistical differences in cell number between modified soft and modified stiff substrates. This indicates that the presence of charge was enough to overcome the limitations of cell binding on soft substrates, or that perhaps a larger difference in stiffness

should be investigated in future studies. The Young's modulus of polystyrene is more than 6-fold higher than that of the 20% w/v PEGDA gels investigated here,[15] and is more in line with trabecular bone, which has a modulus of approximately 50−100 MPa.[32] As a platform for *in vitro* drug evaluation, the mechanical properties of our material are much more in line with the native properties of tissues at both normal and disease state when compared to TCPS. To further develop our platform, we next investigated the inclusion of a specific ECM protein and investigated its effects on adhesion, morphology, and differentiation.

Cell−ECM interactions have been shown to affect cell adhesion, proliferation, and function, including the ability to resist some cancer therapies.[5] In order to develop a platform that accurately mimics the *in vivo* environment, we chose to include a protein from the hMSC ECM, fibronectin. Image analysis results indicated that unmodified hydrogels demonstrated the lowest level of cell adhesion, and that the addition of nonspecific and specific adhesion modalities increased cell number when compared to unmodified hydrogels. Cell adhesion experiments also revealed the heterogeneity of the modified surface, which can be confirmed visually by hMSC clustering, which is particularly evident on FN modified surfaces. The inclusion of FN on modified hydrogels exhibited no statistical difference in cell number or average cell area when compared to TCPS.

In order to compare quantitatively the strength of hMSC adhesive interactions with amine groups, FN groups, and TCPS, a centrifugation assay was used to apply a uniform detachment force. Centrifugation assays have been previously investigated for a variety of cell types and substrates and offer a means to obtain a relative quantification of adhesion strength for a cell population.[24,25,33,34] Results show relatively low levels of adhesion for unmodified and amine modified hydrogels, with percent adhesion at 9 and 26% respectively. FN modified hydrogels along with TCPS demonstrated much higher levels of adhesion, with FN mediated adhesion achieving slightly higher levels of adhesion at 95% compared to 87% on TCPS.

The observed increased adhesion when hMSCs are seeded onto FN substrates may be the result of adhesion strengthening. Strengthening can occur due to increased contact area between the cell surface integrins and the ligand of interest due to cell spreading, clustering of integrins on the cell surface to increase attachment to available binding sites, and the assembly of focal adhesions.[35] The strength of integrin binding to FN has been estimated to be 200 nN, as determined through the use of a spinning disk device.[35,36] The binding of cells to FN was also found to plateau at 4 h incubation, indicating saturation of all available integrins for FN binding.[35] In this study, a detachment force of 18 pN was applied to all samples following a saturated incubation time of 4 h. This detachment force is markedly lower than the estimated strength of integrin−FN adhesion, which is confirmed in the result of a 95% attachment rate of hMSCs on FN modified substrates. Nonspecific binding on amine groups was still very low (26%), even at such low detachment forces. Future studies should be completed to investigate how increasing detachment force to levels closer to the estimated integrin binding strength impacts hMSC adhesion on FN modified surfaces.

Integrin binding has been found to impact a multitude of cell functions including differentiation and cell survival.[37] Recently, binding has also been shown to increase cell survival despite treatment with paclitaxel.[5] To examine the function of hMSCs on our *in vitro* platform, we investigated the differentiation potential of hMSCs cultured on our modified 2D surfaces. The maintenance of hMSC multipotency is an important factor when considering drug discovery applications, as a multipotent population can be used to test multiple phenotypes resulting from differentiation, as well as the ability to test how a drug impacts a population of cells during differentiation.[38] The EMT has also been implicated as an important driving force for tumor development, invasion, and metastasis.[19]

To investigate if the presence of an hMSC-specific protein on the biomaterial surface initiated or inhibited hMSC differentiation, cells were seeded onto modified substrates and induced down osteogenic, chondrogenic, and adipogenic lineages. Results demonstrate that hMSCs exhibit the morphology and ECM production of osteoblasts, chondrocytes, or adipocytes when exposed to proper differentiation signals, indicating that hMSCs cultured on FN modified surfaces are still capable of undergoing differentiation. Of equal importance, hMSCs exposed to growth medium alone maintained the morphology of hMSCs, demonstrating that integrin−FN interactions responsible for cell adhesion to the material surface are not enough to induce undesired differentiation.

After developing a substrate with relevant mechanical properties and modified to include functional groups representative of the native cellular microenvironment, our final objective was to examine how the inclusion of shear force and dynamic nutrient delivery impacts hMSC adhesion and survival. Together with the material properties we have developed, the inclusion of dynamic culture is the last piece in recapitulating the cell microenvironment. After a 48 h dynamic culture period, results showed that modified hydrogels retained significantly more cells/mm^2 than unmodified gels. Distinct cell clustering was again observed on modified hydrogels, reflecting the heterogeneity of the surface modification. It appears that hMSCs that were weakly bound to the hydrogel surface were removed as a result of the direct application of shear flow, but hMSCs that were firmly adhered at areas of modification remained and proliferated into a cluster formation. These results indicate that cells seeded onto a modified 2D substrate are able to survive and proliferate in an environment with applied shear force, as a direct result of adhesion at a point of modification.

The DSCCP we have developed here consisting of a biomimetic material cultured within the TPS bioreactor is a fitting model for drug discovery applications. Our platform allows us to evaluate a cell population which is cultured in an environment that encourages cell−ECM interactions and delivers nutrients, growth factors, and drugs as they are delivered *in vivo*, through the perfusion flow of the bloodstream. Using the DSCCP, the response of a cultured stem cell population to growth factors that induce or inhibit differentiation could be evaluated, as well as how the incorporation of a drug into the perfusion medium inhibits or enhances differentiation. The DSCCP allows for real time monitoring of drug distribution throughout the system, and can be used as a way to collect soluble molecules such as growth factors, enzymes, or proteins of interest that have been produced by the cultured cell population. The DSCCP can also be applied to any adherent cell type, ECM protein combination, or flow parameters. This versatility lends itself to several drug discovery applications such as the evaluation of a cancer cell line to treatment with doxorubicin or paclitaxel by injection into the

system, while also investigating the impact of integrin binding or substrate stiffness on cell survival.

CONCLUSION

The use of a relevant microenvironment for *in vitro* drug discovery investigations is critical to the success of such endeavors as the interaction between cells and the surrounding environment is responsible for a multitude of downstream cell functions. In particular, the use of *in vitro* models that are capable of testing a pluripotent stem cell population is of great importance as pluripotent populations are difficult to test through the use of animal models or cadaveric tissue. Here, we successfully developed an evaluation platform, the DSCCP, consisting of two parts: a biomimetic material in which mesenchymal stem cell adhesion was controlled by the interaction of integrins on the cell surface with hMSC specific ligands on the material surface, and a dynamic culture environment that mimics the mechanical and transport properties of the bloodstream. The microenvironment fabricated here was capable of supporting hMSC adhesion, spreading, and pluripotency. The combination of native ECM interactions, the mechanical properties achieved through manipulation of substrate stiffness, and the dynamic delivery of nutrients or desired drug treatments creates a successful platform for *in vitro* drug evaluation. The DSCCP developed here could be translated into a simple, highly controlled, and inexpensive model for *in vitro* drug discovery which could be used for numerous cell types, matrix molecules or growth factors, disease models, and drug treatments.

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Notes

The authors declare no competing financial interest.

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