Effect of Substrate Topography on Human Mesenchymal Stem Cell Behavior and Morphology

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Abstract
Recent research has proven that the physical, extracellular environment of a cell affects cell morphology and, in stem cells, differentiation. (Markert 2009, Engler et al 2006)
This is a preliminary study to further explore the effects of various topographical features on stem cell morphology and differentiation. We hypothesized that a change in the topographical features of a PDMS substrate will result in significant changes, measurable in cell morphology and that hMSCs will differentiate into osteoblasts, given the rigidity of the PDMS substrate.
In this study, human mesenchymal stem cells (hMSCs) were grown on a PDMS substrate of three different topographies: control (no features), posts, and pits. (see Figure 1) After seeding, cell samples were fixed after one, three, seven, and nine days for each of the topographies tested.
Preliminary results show a significant difference between cells grown on the control substrate and on the posts or pits features. Differences in cytoskeleton structure and overall morphology may be attributed to stem cell differentiation or simply the stem cells’ adaptations to the extracellular environment. Further studies will be required to validate our findings and determine specific cell fate.

Introduction

Background
In all multicellular organisms, cells are organized into tissues that work together to perform functions for the organism as a whole. These tissues consist of more than just cells and include, around and between the cells, a nonliving extracellular matrix (ECM). The ECM varies significantly between species, tissue types, tissue health (especially...
cancerous v. noncancerous, (Fleming, et al 2010)), and even tissue age (Bianchi-Frias 2007). Because of this, scientists have sought to understand the effects of the ECM on cell morphology, proliferation, and gene expression (Ranucci and Moghe 1999, Mata et al. 2002, Teixeira et al. 2006, Kidambi et al. 2007, Biggs et al. 2009).

The effects of this extracellular environment on cell behaviors have been studied for years (Curtis and Wilkinson 1997). Recently, there has been an increasing body of research to understand the effects on stem cells and, in particular, ways of manipulating stem cells to alter their proliferation or differentiation (Markert et al. 2009). Currently, the two main approaches of manipulating these cells revolve around altering their environment chemically or physically. The physical environment around a cell can be defined both by its rigidity and its topography.

Changing the rigidity of the substrate on which the cells are seeded and grown has been proven to affect the differentiation of stem cells: soft substrates will cause differentiation toward adipocytes, whereas a rigid substrate will likely cause differentiation toward osteoblasts, etc. (Engler et al 2006).

A study by Britland, et al (1996) showed that much of the cellular response depended on the scale of the substrate cues: when topographical features were less than 500 nm, the cells aligned themselves according to chemical cues; when topographical features were sized greater than 5 μm, 80% of the cells responded to the physical cues, overcoming the chemical. Additional studies have sought more information concerning the specific roles of chemical versus topographical cues (Lim and Donahue 2007).

The extracellular environment of a cell has been shown to affect much more than simple morphology. A study by Manwaring, Walsh, and Tresco (2004) showed that the
topography of a substrate affects the outside of a cell by changing the structure of the ECM excreted by the affected cells. More studies are currently emerging concerning the effects of substrate topography or ECM on the internal functions of a cell.

Many of the studies done have shown that the response to the extracellular environment—chemical or topological—are highly varied according to cell type (Curtis and Wilkinson, 1997). In fact, it seems that each cell type reacts differently to each different substrate and scientists are continuing to look for explanations as to why. In particular, they are studying the internal cell characteristics such as alignment and structure of actin filaments, the placement and makeup of focal adhesions, as well as other mechanics that lead to such cellular responses. If internal and external characteristics of cells could be linked to predictable responses to substrate topography, this knowledge could then be applied to refining innovations in tissue engineering and manufacturing, prosthesis design, and regenerative medicine.

Stem cells and their potential to be accepted into the body and differentiate into a number of differing cell lineages and tissue types make them ideal for many goals in the bioengineering and medical communities. We feel that a deeper understanding of the internal mechanics of a cell and careful observations of cell reactions to substrate topography over time will be a step towards being able to take this knowledge and apply it towards better bioengineering products and more effective medical treatments.

**Purpose**

In this study we will observe and measure human mesenchymal stem cells (hMSCs) and their reaction to differing topographical features on a polydimethylsiloxane (PDMS) substrate. In this, we hypothesize that: 1) A change in the topographical features
of a PDMS substrate will result in significant changes, measurable in hMSC morphology, and 2) hMSCs will differentiate into osteoblasts, given the rigidity of the PDMS substrate.

**Methodology**

In order to verify these hypotheses we will be using several stains and microscopy techniques to observe the morphology, proliferation, and differentiation of stem cells to a specific lineage.

The morphology of a cell is determined by many things, but perhaps the most defining characteristic of a cell’s shape is its cytoskeleton. The cytoskeleton is an ever-changing fibrous network that provides definition to the shape of a cell as well as a means of transportation within a cell. The cell is able to modify this at will, and changes visible in the cytoskeleton are indicative of various cellular activities. This network is composed of several different kinds of protein including actin. Actin is found as a globular monomer (g-actin) within the cell, or polymerized into f-actin fibers which are then incorporated into the cytoskeleton. One way that cells are able to manipulate their internal structure is by breaking the f-actin polymers down into g-actin, and re-polymerizing to create f-actin in new sizes and directions. Actin is also known to closely associate with proteins anchored within the cellular membrane and their related functions. (Doherty and McMahon 2008)

To be able to visualize the f-actin fibers within cell samples, we will use the fluorescing dye rhodamine phalloidin. All phallotoxin derivities, such as rhodamine phalloidin, bind to actin molecules with nearly a one-to-one ratio. Important for our studies, rhodamine phalloidin in particular binds only to f-actin, the actin that is already
in use by the cell, and not g-actin, which is in a transitional state during remodeling of the cytoskeleton. This dye is also purported to work best on cells with a compromised membrane, as the dyes are not permeant in most live cells. Accordingly, we will not be able to use live cells and view their cytoskeleton at the same time. Once fixed and permeabilized, the actin in the cells will fluoresce red.

One of the other measures of changes in morphology will be to stain and observe the focal adhesions of cells growing different topographies. Focal adhesions are important because they are cell membrane proteins that provide the link between the actin filaments inside of the cell and the ECM outside of the cell. Being able to visualize the number and location of each of these membrane proteins will help us to understand the way that the cell is reacting on the inside to the world on the outside (Ezzell et al. 1997). In order to do this, we will use an antibody staining process that targets the vinculin, a protein found in each focal adhesion. The primary antibody, anti-vinculin (isolated from mice), attaches to the focal adhesions when incubated with the cell. This antibody acts as a marker and binding site for the secondary antibody, fluor 488 goat-anti-mouse IgG(H+L). This secondary antibody is important because it fluoresces green and is easily visualized using fluorescent microscopy.

The other major defining structure within a cell is the cell’s nucleus. The nucleus of a cell contains the entire genome of a cell, so visualizing the nucleus is advantageous for several reasons. First, an intact nucleus is necessary for a living, healthy cell; thus, being able to visualize the nucleus of a cell is an easy way to test for cell viability. Second, most eukaryotic cells contain a single nucleus; thus, even if cells are clumped or overlapping, a nuclei count should give a fairly accurate cell count. In order to dye the
nucleus, we will be using 4',6-diamidino-2-phenylindole (DAPI). DAPI binds to the DNA of a cell, located within the nucleus, and fluoresces blue.

For overall morphology of living cells, however, a different approach is needed. CellTracker dye is able to permeate the cell membrane, then react with the thiol groups within the cell to fluoresce green. This can be used in living cells to view total morphology, including cytoplasm in places where there may not be any actin fibers. CellTracker is also useful to record cell motility; hMSCs are not known for their motile nature on flat substrates, but it is possible that a change in substrate could change this cell behavior as well.

Though all of the previous tests will allow observation and measurement of changes in cell morphology, it does not address the possibility of stem cell differentiation. Isolated from human bone marrow, hMSCs can differentiate into any number of cell lineages. However, the PDMS substrate to be used is rigid enough that stem cells grown on it would likely differentiate into osteoblasts. Identifying osteoblasts is much simpler than other cells, as they are known for creating calcium deposits in the ECM immediately around them. Testing for calcium can be done using alizarin red, a dye that bonds with the calcium to form a heavy red-orange, birefringent precipitate. A positive stain of hMSCs would indicate that the cells had begun to differentiate into the expected lineage of osteoblasts; a negative stain would mean that the cells remained undifferentiated or they differentiated into a new, unexpected lineage.
Methods

Creating Substrate

Substrate was created using polydimethylsiloxane (PDMS). Pure PDMS, still in a liquid state, was mixed with a curing agent in a 10:1 ratio and poured over pre-constructed masks to create three different topographies. The control substrate was created on a glass plate, leaving a smooth surface devoid of as many features as possible. One of the two molded topographies created a substrate with posts, while the second substrate topography featured pits. The mixture was allowed time to rest, eliminating as many air bubbles within the substrate as possible and was placed in an oven for two hours until dry. The PDMS sample was then peeled off of each of the masks and ready to use. Once finished, both features had a pitch of 6 μm; posts had a height of 200 nm and pits had a depth of 200 nm. (Figure 1)

Figure 1. Topography of PDMS substrate. Unobstructed view of control (A), pits (B), and posts (C). DIC image of cells growing on control (D), pits (E), and posts (F).
**Preliminary: Cell Adhesion Test**

The first set of experiments was done to ensure that hMSCs would be able to adequately adhere to the PDMS without further surface modification. Each of the three PDMS substrate topographies was punched into circular samples able to fit in a 4-well plate, each individual well having a surface area of 2 cm$^2$. Three trials of each of the topographies were seeded at a concentration of 6000 cell/cm$^2$ in media consisting of 83% DMEM cell culture medium, 15% fetal bovine serum (FBS), 1% L-glutamine, and 1% antibiotics. Media was changed every two days and cells were observed for attachment and confluence. On Day 7, two of the well-plate samples were fixed with 4% formalin, then observed with Differential Interface Contrast (DIC) Microscopy and representative sample images were recorded. Cell adhesion was determined to be adequate on all three topographies, so we proceeded to the formal experiment. The final samples were placed back in incubation, and media changed as necessary, for further experiments and analyses.

**Seeding and Growing hMSCs**

Twelve samples of each of the three PDMS topographies were punched and placed in four-well plates. Each plate used three wells, one for each topographical sample. All samples were sterilized with ethanol and ultraviolet light. Cells were seeded with media at a density of 6000 cells/cm$^2$ and incubated. On the following day (Day 1), two four-well plate samples were fixed with 4% formalin, stored in phosphate buffer solution (PBS) and refrigerated for later imaging. This process was repeated on Day 3, Day 7, and Day 9. Two additional samples were fixed on Day 7 for later calcium-testing. The remaining two samples were left unfixed for Cell Tracker testing.
Observing Cell Morphology and Behavior

Cell Tracker
Two unfixed Day 7 sample well plates were incubated for 1 hour with Cell Tracker, washed, and then covered with a phosphate buffer solution. These samples were viewed using florescent microscopy and representative images were recorded using Metamorph software.

Calcium Staining
Three sample well-plates were fixed with 4% formalin, one remaining Day 21 sample left from the preliminary adhesion test and two Day 7 samples. They were stained for 15 minutes with Alizarin Red, and then rinsed with a phosphate buffer solution (PBS). All three samples were viewed as DIC and a representative sampling of cells were photographed using Metamorph software.

Focal Adhesions, Actin fibers, and Nuclei
Images of all fixed cell samples (Day 1, Day 3, Day 7, and Day 9) were taken the same day to ensure consistent results.

Fixed cell samples were treated with 3% bovine serum albumin to block any nonspecific binding of dyes. Cell membranes were then permeabilized for 3 minutes in iced acetone, then rinsed with PBS. Samples were treated for one hour in the primary antibody (mouse anti-vinculin), rinsed, then treated concurrently with both the secondary antibody (Fluor 488 goat anti-mouse IgG (H+L)) and rhodamine phalloidin for 30 minutes. Samples were washed, then treated with DAPI (4′,6-diamidino-2-phenylindole) for a total of five minutes.

These samples were viewed using DIC and florescent microscopy and were recorded using Metamorph software. A sample of representative cells was photographed
from each topography, recording the following for each: a DIC image, vinculin, DAPI, and rhodamine phalloidin images. Each viewfield was colorized according the original dye florescence and then overlaid for analysis.

**Data Analysis**

**Cell Tracker**

Each of the cell tracker images was imported into ImageJ software for analysis. Images in which individual cells were visible without significant overlapping were converted to a binary image. An algorithm was applied to divide connected cells, and then cells were analyzed for circularity (Figure 2). The area of each cell was calculated, then the circularity of a cell was determined by comparing the area of the cell to a circle with the same circumference (Figure 3).

![Figure 2. Analysis of Cell Tracker images in ImageJ to calculate cell area and circularity. (Figure created by Hannah Wirtshafter)](image-url)
Calcium Staining

Calcium staining is typically carried out to provide evidence that hMSCs have differentiated into bone-like cells. However, data collected from Alizarin Red staining for calcium in our samples was inconclusive. Staining was nonspecific, with most samples having a light red tint when viewed through the eyepiece. In addition, use of DIC microscopy resulted in black and white images in which red stained cells were entirely indistinguishable from non-stained cells.

Focal Adhesions, Actin fibers, and Nuclei

The staining and recording of actin fibers with rhodamine phalloidin and the nuclei with DAPI were both highly successful, producing clear images. The staining of the focal adhesions with anti-vinculin antibodies was less successful. Nonspecific staining lead to poor images that were not useful for analytical purposes.

Each composite image was analyzed & identified according to several characteristics: number of cells in the field, physical association between cells, morphology, and actin fiber definition. (Table 1 and Figure 9)

For each day, on each of the three topographies, the percent of samples displaying each characteristic was calculated and displayed graphically using Microsoft Excel.
Table 1. Characterizing composite images of random cell samplings. For example, when examining the morphology, a cell sample could be characterized as exhibiting elongation or spreading.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Defined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells per view field</td>
<td>Counted according to number of DAPI stained nuclei</td>
</tr>
<tr>
<td>Morphology</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Elongation</td>
</tr>
<tr>
<td></td>
<td>Cells appear stretched and thin</td>
</tr>
<tr>
<td>Physical association between cells</td>
<td>Alone</td>
</tr>
<tr>
<td></td>
<td>Cells have no physical connection to other cells in the field</td>
</tr>
<tr>
<td>Actin Definition</td>
<td>Defined</td>
</tr>
<tr>
<td></td>
<td>thick, individual fibers are visible throughout the cell in any direction</td>
</tr>
</tbody>
</table>

Results

Cell Tracker

Analysis of individual cell area showed a visible trend, though sample size limited the statistical significance. Cells on the control substrate had the smallest average area, cells on posts averaged slightly larger, and the cells grown on pits were calculated to have the largest average area. Results of circularity calculations proved more significant. Cells grown on the control had the largest average circularity, while cells grown on either the posts or the pits were calculated to be significantly less circular. (Figure 4)

The correlation between a cell’s area and circularity was also graphed and calculated (Figure 5). A strong negative correlation was found between the two characteristics: small cells were more circular and large cells less circular.
Figure 4. (A) Effect of topography on average cell area. This graph shows a trend, but differences are not statistically different. (B) Effect of Topography on Cell Circularity. This graph shows that modified topography decrease cell circularity significantly. The * indicates statistical significance.

Figure 5. Negative correlation between cell circularity and area for all topographies tested. Cells observed were either small and round or large and noncircular.

**Calcium Staining**

No valid results could be obtained from samples stained with Alizarin Red for cell calcium deposits, due to nonspecific staining (Figure 6).
Figure 6. Alizarin Red staining of cells on pits at Day 7 and Day 21 (20x); results inconclusive due to nonspecific staining.

**Actin fibers and Nuclei**

All three topographies tested proved to be a viable substrate for hMSC growth. Healthy cell shapes were observed on all samples, as well as an increase in stem cell proliferation. (Figure 9)

**Number of Cells per View Field**

Analysis of the number of cells per view field showed that all topographies began Day 1 with 50% or more of the samples taken having five cells or less per view field. Cells grown on the control substrate continued to proliferate throughout the trial. By Day 9, 60% of the samples had more than twenty cells in a single view; the remaining 40% had less than five cells per view. Cells grown on pits showed a decrease in proliferation on Day 3 and Day 7 then a significant increase on Day 9: on Day 7, 100% of the sample views had five or less cells; on Day 9, only 57% had five or less cells while 45% had twenty or more per view field. Cells grown on posts also showed a decrease in proliferation between Day 1 and Day 3. The number of cells viewed increased by Day 7 and remained steady on Day 9. (Figure 10)
**Physical Association between Cells**

Physical association with other cells proved to be a closely related measurement. Views with five cells or less often showed isolated or linked cells. Samples counted to have twenty or more cells were always clumped. By Day 9, both Control and Posts showed 60% of samples to be clumped, while the remaining 40% was split between linked or alone. One occurrence to note is the large number of linked cells for Day 7 of Pits. These cells seemed to have a slightly unique appearance compared to cells on other topographies or even the same topography but on different days. See Pits, Day 7 in Figure 9 for an example. Pits Day 9, however, showed less linked or clumped cells than either of the other two topographies and more than twice the number of alone samples. (Figure 11)

**Changes in Morphology**

All three topographies showed an overall trend towards spreading: samples on day one were more likely to show elongation than samples seen on Day 9. No other significant trends were seen in changes to cells on Control topography. Both Posts and Pits showed a decrease in spreading between Day 1 and Day 3. Posts showed a large increase between Day 3 and 7 that then leveled off between Day 7 and Day 9. Cells on pits showed a steady increase between Day 3 and 7 and between Day 7 and 9. (Figure 7)

**Changes in Actin Fibers**

The percent of cell samples on Control with defined actin fibers rose from 75% on Day 1 to 100% for the rest of the trial. Cells on both pits and posts had a drop in actin fiber definition between Day 1 and Day 3, followed by a sharp increase between Day 3 and 7. (Figure 8)
Figure 7. Changes in Cell Morphology: Trends in cell spreading over time.

Figure 8. Changes in Actin Fibers: Changes in the presence of defined actin fibers over time.
Figure 9. Representative samples of created overlays of actin and nuclei staining. Examples of characterizing samples: (A) Number of cells per view field: Control Day 7 has five nuclei stained with DAPI, so cell count is five. (B) Physical Association between cells: Pits Day 3 is alone, Control Day 3 is linked, and Posts Day 1 and 9 is clumped. (C) Morphology: Posts Day 3 is elongated, Pits day 3 & 9 are spread (D) Actin Definition: Posts Day 7 and 9 is defined, Posts Day 1 & 3 is random.
Figure 10. Number of Cells per View Field: The bar shown for each day totals 100%; each color stacked within it reveals the characteristics of the samples for that day. Example: Control Day 1 – 50% of the samples had 1-5 cells, 25% had 6-10 cells, and 25% had 11-15 cells.

Figure 11. Association with other Cells: The bar shown for each day totals 100%; each color stacked within it reveals the characteristics of the samples for that day. Example: Control Day 1 – 50% of the samples were linked cells and 50% of the samples were clumped with other cells.

**Discussion**

Though only preliminary in nature, we believe that this study shows that there is a significant difference between the cells grown on the control substrate with no intentional topographical features and the cells grown on either of the modified topographies: the posts or the pits.
First, the measurements using Cell Tracker Data to compute the circularity and area of each cell sample shows that the cells found on the modified topographies are larger and less circular (Figure 4). This is important because healthy, confluent hMSCs should be large, flat, and have a highly irregular shape. The control cells were calculated to be smaller and have significantly greater circularity. Small, circular hMSCs have two possible explanations: either the cells are increasing in proliferation and undergoing mitosis, or it is a precursor to apoptosis (cell death). Combining this data with views of the actual cell samples (Figure 9), along with a steady increase in cell numbers (Figure 10), it is likely that the small, circular nature of the cells on control is a sign of increased proliferation when compared to the cells on modified topography. On the other hand, the fact of cells on pits and posts seeming to be less prolific and at the same time large and irregular may be a sign that instead of proliferating they are being forced to adapt or, potentially, differentiating.

Our additional data seems to support these observations. Control cells show many large, defined actin fibers throughout the study (Figure 8), which would not be consistent with cells approaching apoptosis. These same cells showed very little change in overall morphology (Figure 7), not increasing in spreading nor in elongation. Posts and pits, on the other hand, showed a significant drop in actin fibers on Day 3 before increasing to match the actin definition of cells on the control by Day 7. Drops in actin definition are a sign of the cell depolymerizing the fibrous actin of the cytoskeleton in order to rebuild and rearrange the fibers in the cell. Again, this is often a sign of cells being forced to react and adapt to the environment around them or, with stem cells, potential differentiation.
Conclusion

This study has shown that hMSC behavior, measurable in cell morphological differences over time, is altered in response to different substrate topographical features. Cells on the control substrate showed a steady increase in actin fiber definition and proliferation. When cells are making changes to their structure, they cannot continue to proliferate until the remodeling is done. Stem cells usually remodel their cytoskeleton to adapt to their environment, either by restructuring and remaining stem cells or by differentiating into a new cell lineage. Cell samples grown on pits and posts both showed an initial drop in actin fiber definition and, at the same time, a drop in proliferation. This data shows that the cells on modified topography are indeed being forced to adapt in new ways that are not seen in the cells grown on the control.

Determining the mode of this adaptation should be explored in further studies. An analysis of focal adhesion changes in response to these new topographies would show how the cells are interacting with the physical features around them, e.g., do they show a special affinity for features or are the features avoided? To determine whether the cells are differentiating or not, a simple stain for calcium may be sufficient, as the rigidity of the substrate is expected to produce osteogenic lineages, noted for their calcium deposits. A more in-depth analysis (especially if calcium deposits are not present) could be done by running a western blot to determine gene activation and protein production within the cells.
We conclude that a change in the topographical features of a PDMS substrate results in significant changes, measurable in cell morphology. Cells grown on a control substrate are small, round, have consistently well-defined actin, and are prolific. Cells grown on posts or pits are large, noncircular, and have been observed to remodel their cytoskeleton structure resulting in delayed proliferation.

Further testing is needed to validate our results and to test our second hypothesis. A larger, more thorough sampling would hopefully provide greater statistical significance to the results already collected. In addition, more specific testing is needed to measure the direct interaction between the cells and the topographical features (analyzing focal adhesions, etc.) as well as confirm the possible differentiation of the cells and identify the lineage.

References


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