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Differentiation Modulation of Adult Stem Cells in an Adipose System

Aditya Chaubey
Clemson University, achaube@clemson.edu

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DIFFERENTIATION MODULATION OF ADULT STEM CELLS IN AN ADIPOSE SYSTEM

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Bioengineering

by
Aditya Chaubey
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Accepted by:
Dr. Karen J.L. Burg, Committee Chair
Dr. Martine LaBerge
Dr. Ken Webb
Dr. Lawrence Grimes
ABSTRACT

The need for soft tissue reconstruction or augmentation has increased continuously over the years. This need is compounded by patients suffering from post-traumatic repair and congenital soft-tissue deformities. All the current options available to treat the soft tissue deformities have inherent difficulties associated with them. Hence, more research is needed to come up with a better solution to this problem which is only going to increase in magnitude.

Tissue engineering is a relatively new technique which has the potential to deliver a cell-based device which can overcome the problems associated with traditional therapies. However, before it becomes clinically viable we need to understand several key issues. The goal of these studies is to employ the principles of tissue engineering to better comprehend the interaction between cells and the implant on which they are seeded and their surrounding environment in order to facilitate the design of improved devices.

The long-term hypothesis, beyond the scope of the proposed work, is that through the control of the level of cellular differentiation in a tissue-engineered device, one can influence the viability of the device post-implantation. Specifically, the aim of these studies was to characterize different means by which the differentiation of adult stem cells could be modulated in a tissue engineered device.

The first two studies explore the possibility of using the surface texture of a scaffold to control the level of cellular differentiation. In the first study, polymer scaffold with microgrooves were employed to study the role of the defined surface texture in the differentiation process. In the second study, similar scaffolds were used to investigate the
onset of the differential rates of cellular differentiation in the adipose system and the amount of leptin secreted by these cells was compared. Polynomial models were proposed to model the leptin released over time.

The next study investigated the possibility of using the scaffold material and cell size to modulate the differentiation of adult stem cells. Different ECM molecules (laminin and collagen I) were evaluated for their efficacy in controlling cellular proliferation and differentiation. Laminin was found to enhance adipogenesis more effectively than collagen I. The role of cell size in determining the functionality of differentiated adipocytes was also explored in this study. Modified inkjet printer was used to create controlled width of adhesive area for cellular adhesion surrounded by non-adhesive area. It was found that printed adhesive areas did not bind strongly enough to the glass substrate to support the, rather large, differentiated adipocytes leading to detachment of the cell sheet.

The aim of the final study was to study the behavior of the differentiated adipocytes in a 3D environment. Three different scaffold types were investigated: collagen microcarriers, laminin-coated polylactide beads and control polylactide beads. It was found that cells seeded on collagen microcarriers accumulated the highest amount of lipid and that coating the surface of polylactide beads with laminin significantly enhanced their ability to affect adipogenesis and cellular adhesion. The proposed 3D system could also be use as a tunable, in vitro test system to study adipogenesis and its related pathologies.
ACKNOWLEDGMENTS

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xi</td>
</tr>
<tr>
<td>PREFACE</td>
<td>xv</td>
</tr>
<tr>
<td>BACKGROUND AND LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Tissue Engineering as a possible solution</td>
<td>2</td>
</tr>
<tr>
<td>Adipogenesis</td>
<td>3</td>
</tr>
<tr>
<td>Scaffold materials</td>
<td>6</td>
</tr>
<tr>
<td>Microtopography and cell behavior</td>
<td>11</td>
</tr>
<tr>
<td>Influence of ECM on cells</td>
<td>13</td>
</tr>
<tr>
<td>References Cited</td>
<td>14</td>
</tr>
<tr>
<td>SURFACE PATTERNING: TOOL TO MODULATE STEM CELL DIFFERENTIATION IN AN ADIPOSE SYSTEM</td>
<td>19</td>
</tr>
<tr>
<td>Introduction</td>
<td>19</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>21</td>
</tr>
<tr>
<td>Results</td>
<td>24</td>
</tr>
<tr>
<td>Discussion</td>
<td>45</td>
</tr>
<tr>
<td>Conclusions</td>
<td>48</td>
</tr>
<tr>
<td>References Cited</td>
<td>49</td>
</tr>
<tr>
<td>CHARACTERIZATION OF DIFFERENTIATION AND LEPTIN SECRETION OF D1 CELLS ON PATTERNED POLYLACTIDE FILMS</td>
<td>51</td>
</tr>
<tr>
<td>Introduction</td>
<td>51</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>53</td>
</tr>
<tr>
<td>Results</td>
<td>56</td>
</tr>
<tr>
<td>Discussion</td>
<td>66</td>
</tr>
<tr>
<td>Table of Contents (Continued)</td>
<td>Page</td>
</tr>
<tr>
<td>-----------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Conclusions</td>
<td>71</td>
</tr>
<tr>
<td>References Cited</td>
<td>71</td>
</tr>
<tr>
<td>EXTRA CELLULAR MATRIX COMPONENTS AS MODULATORS OF ADULT STEM CELL DEVELOPMENT IN AN ADIPOSE SYSTEM</td>
<td>75</td>
</tr>
<tr>
<td>Introduction</td>
<td>75</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>82</td>
</tr>
<tr>
<td>Results</td>
<td>88</td>
</tr>
<tr>
<td>Discussion</td>
<td>99</td>
</tr>
<tr>
<td>Conclusions</td>
<td>103</td>
</tr>
<tr>
<td>References Cited</td>
<td>104</td>
</tr>
<tr>
<td>MODULATING ADULT STEM CELL DIFFERENTIATION IN A TUNABLE 3D ADIPOSE SYSTEM</td>
<td>109</td>
</tr>
<tr>
<td>Introduction</td>
<td>109</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>114</td>
</tr>
<tr>
<td>Results</td>
<td>124</td>
</tr>
<tr>
<td>Discussion</td>
<td>130</td>
</tr>
<tr>
<td>Conclusions</td>
<td>135</td>
</tr>
<tr>
<td>References Cited</td>
<td>135</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>139</td>
</tr>
<tr>
<td>RECOMMENDATIONS</td>
<td>141</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Pairwise difference in means using Scheffe’s method for Oil Red O staining for the early time-point</td>
<td>29</td>
</tr>
<tr>
<td>2.2</td>
<td>Pairwise difference in means using Scheffe’s method for Oil Red O staining for the late time-point</td>
<td>30</td>
</tr>
<tr>
<td>2.3</td>
<td>ANOVA and polynomial regression for lactic acid released</td>
<td>36</td>
</tr>
<tr>
<td>2.4</td>
<td>Quadratic time trend model for lactic acid released</td>
<td>39</td>
</tr>
<tr>
<td>2.5</td>
<td>ANOVA and polynomial analysis for glucose consumed</td>
<td>42</td>
</tr>
<tr>
<td>2.6</td>
<td>Cubic time trend model for glucose consumed</td>
<td>44</td>
</tr>
<tr>
<td>3.1</td>
<td>ANOVA for testing the differences in time trend for released leptin</td>
<td>61</td>
</tr>
<tr>
<td>3.2</td>
<td>Linear time trend for leptin released</td>
<td>61</td>
</tr>
<tr>
<td>3.3</td>
<td>Outlier values for leptin released for the different scaffolds</td>
<td>62</td>
</tr>
<tr>
<td>3.4</td>
<td>ANOVA for leptin released after discounting the outlier values</td>
<td>62</td>
</tr>
<tr>
<td>3.5</td>
<td>Time trend for leptin released after discounting the outlying values</td>
<td>63</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Schematic representation of the patterned silicon wafer</td>
<td>25</td>
</tr>
<tr>
<td>2.2</td>
<td>SEM micrograph of a typical patterned PLLA film</td>
<td>26</td>
</tr>
<tr>
<td>2.3</td>
<td>(a) D1 cells on patterned PLLA film, day 1, 100x total magnification. The cells are elongated. (b), D1 cells on PS surface, day 3, 100x total magnification. The cells have formed a confluent monolayer. (c), D1 cells on PS surface, day 6, 320x total magnification. The cells are beginning to accumulate lipid droplets. (d), D1 cells on PS surface, day 28, 320x total magnification. The cells have accumulated large quantities of lipid droplets</td>
<td>26</td>
</tr>
<tr>
<td>2.4</td>
<td>Cells stained with ORO – early time point, 320x total magnification</td>
<td>30</td>
</tr>
<tr>
<td>2.5</td>
<td>Cells stained with ORO – late time point, 320x total magnification</td>
<td>31</td>
</tr>
<tr>
<td>2.6</td>
<td>Percent area occupied by lipid on the three scaffolds – early time point</td>
<td>32</td>
</tr>
<tr>
<td>2.7</td>
<td>Percent area occupied by lipid on the three scaffolds – late time point</td>
<td>32</td>
</tr>
<tr>
<td>2.8</td>
<td>Daily lactic acid released by cells on the three scaffolds</td>
<td>36</td>
</tr>
<tr>
<td>2.9</td>
<td>Cumulative lactic acid released by the cells on the three scaffolds</td>
<td>37</td>
</tr>
<tr>
<td>2.10</td>
<td>Quadratic trend of lactic acid released by the cells on the three scaffolds</td>
<td>38</td>
</tr>
<tr>
<td>2.11</td>
<td>Residuals for the quadratic trend data for lactic acid released</td>
<td>38</td>
</tr>
<tr>
<td>2.12</td>
<td>Daily glucose consumption by cells on the three scaffolds</td>
<td>42</td>
</tr>
<tr>
<td>2.13</td>
<td>Cumulative glucose consumption by cells on the three scaffolds</td>
<td>43</td>
</tr>
<tr>
<td>2.14</td>
<td>Cubic trend of glucose consumption by cells on the three scaffolds</td>
<td>43</td>
</tr>
<tr>
<td>2.15</td>
<td>Residuals for the cubic trend data for glucose consumption</td>
<td>44</td>
</tr>
</tbody>
</table>
List of Figures (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Percent area occupied by lipid on all the three surfaces. Each data point represents a mean of three values, error bars denote SEM</td>
<td>58</td>
</tr>
<tr>
<td>3.2 Cumulative lactic acid released by the cells on the different surfaces. Error bars denote SEM</td>
<td>58</td>
</tr>
<tr>
<td>3.3 Cumulative glucose consumed by the cells on different surfaces. Error bars denote SEM</td>
<td>59</td>
</tr>
<tr>
<td>3.4 Leptin concentration in the culture medium released by cells on different surfaces</td>
<td>60</td>
</tr>
<tr>
<td>3.5 Linear trend of leptin released based on the model proposed</td>
<td>64</td>
</tr>
<tr>
<td>3.6 Residuals for the linear trend of the leptin released</td>
<td>65</td>
</tr>
<tr>
<td>4.1 Differentiated adipocytes (Day 14) on collagen I substrate. Total magnification 320x. (b), Differentiated adipocytes (Day 14) on laminin substrate. Total magnification 320x. (c), Differentiated adipocytes (Day 14) on polystyrene substrate. Total magnification 320x</td>
<td>88</td>
</tr>
<tr>
<td>4.2 Lactic acid levels as a function of time (day) and substrate for low density coating. Each data point represents the mean of three values. The SEM is extremely low; thus, the error bars are not visible. Asterisks (*) denote differences (p&lt;0.05) in lactic acid level with respect to time; curves marked with similar symbols are significantly different at the designated time-point</td>
<td>92</td>
</tr>
<tr>
<td>4.3 Lactic acid levels as a function of time (day) and substrate for high density coating. Each data point represents the mean of three values. The SEM is extremely low; thus, the error bars are not visible. Asterisks (*) and pound signs (#) denote differences (p&lt;0.05) in lactic acid level with respect to time; curves marked with similar symbols are significantly different at the designated time-point</td>
<td>93</td>
</tr>
</tbody>
</table>
List of Figures (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4</td>
<td>Glucose levels as a function of time (day) and substrate for low density coating. Each data point represents the mean of three values; no significant difference in glucose levels was detected. The SEM is extremely low; thus, the error bars are not visible.</td>
<td>93</td>
</tr>
<tr>
<td>4.5</td>
<td>Glucose levels as a function of time (day) and substrate for high density coating. Each data point represents the mean of three values; no significant difference in glucose levels was detected. The SEM is extremely low; thus, the error bars are not visible.</td>
<td>94</td>
</tr>
<tr>
<td>4.6</td>
<td>Metabolic activity of D1 cells grown on different substrates. Each data point represents the mean of three values, and error bars denote SEM (in some cases, the SEM is extremely low; thus the error bars are not visible); the asterisk (*) and pound sign (#) denote differences (p&lt;0.05) in metabolic activity; data points marked with similar symbols are significantly different.</td>
<td>96</td>
</tr>
<tr>
<td>4.7</td>
<td>Optical density (OD) of differentiated D1 cells at 510 nm on different substrates. Accumulated lipid in the cells was stained with Oil Red O solution, extracted and its OD measured. Each data point represents the mean of three values, and error bars denote SEM (in some cases, the SEM is extremely low; thus the error bars are not visible); asterisks (*) denotes differences (p&lt;0.05) in OD; data points marked with similar superscripts are significantly different.</td>
<td>97</td>
</tr>
<tr>
<td>4.8</td>
<td>Light microscopy images of printed collagen pattern before cell seeding. (A) Total magnification: 50x, and (B) Total magnification: 100x.</td>
<td>98</td>
</tr>
<tr>
<td>4.9</td>
<td>Light microscopy images of printed line collagen pattern seeded with D1 cells having a confluent monolayer.</td>
<td>99</td>
</tr>
<tr>
<td>5.1</td>
<td>Metabolic activity of D1 cells on different substrates. Each data point represents the mean of three values, and error bars denote SEM; the bar with asterisk (*) denotes statistical differences (p&lt;0.05) in metabolic activity.</td>
<td>124</td>
</tr>
</tbody>
</table>
List of Figures (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2</td>
<td>Relative cell number for cells on the three scaffold-types. Each data point represents the mean of three values, and error bars denote SEM; bars with letters are significantly different (p&lt;0.05).</td>
<td>125</td>
</tr>
<tr>
<td>5.3</td>
<td>(a) Day 4 fluorescence micrograph showing cells attached on collagen microcarriers; Total magnification: 100x. (b), Day 4 fluorescence micrograph showing cells attached on laminin-coated PLLA beads; Total magnification: 100x. (c), Day 4 fluorescence micrograph showing cells attached on PLLA beads; Total magnification: 100x</td>
<td>126</td>
</tr>
<tr>
<td>5.4</td>
<td>Triglyceride analysis of differentiated D1 cells on the different scaffolds. Each data point represents the mean of three values, and error bars denote SEM; for each time-point, the group with letters are significantly (p&lt;0.05) different</td>
<td>129</td>
</tr>
<tr>
<td>5.5</td>
<td>Optical density (OD) of differentiated D1 cells at 510 nm on different substrates. Accumulated lipid by the cells was stained with Oil Red O solution, extracted and its OD measured. Each data point represents the mean of three values, and error bars denote SEM; bars with letters are significantly (p&lt;0.05) different</td>
<td>129</td>
</tr>
<tr>
<td>5.6</td>
<td>Evaluation of differential gene expression in 3D environment for cells on laminin-coated PLLA beads (Lam-PLLA), control PLLA beads (PLLA) and collagen microcarriers (Collagen) on day 16 of adipose differentiation by means of RT-PCR. Evaluation of characteristic fat genes aP2, PPARγ and internal standard β-actin was done in triplicate; one representative result is shown here</td>
<td>130</td>
</tr>
</tbody>
</table>
PREFACE

The current soft tissue reconstruction options available to treat patients recovering from breast cancer or for soft tissue reconstructions and augmentations, have affiliated problems such as soft tissue resorption or expensive and highly invasive follow-up surgeries. Tissue engineering offers a promising alternative to these options; however before this procedure can be considered clinically viable, various issues must be understood. One of the most important challenges is to develop a more thorough understanding of the interaction of the cells with the scaffold on which they are seeded, as this interaction profoundly affects the proliferation, functionality, and differentiation of the cells. Work detailed in this dissertation, describes different ways to modulate the differentiation of adult stem cells in an adipose system. Various studies were performed to evaluate the use of scaffold surface texture and scaffold material as means to control the rate of cellular differentiation. The cell-material interaction was also studied in a 3D environment, as a shift from 2D to 3D markedly affects all aspects of cell behavior.

This dissertation is comprised of five chapters, where the first provides a detailed literature review for the body of work presented. The review in Chapter 1 describes the relevance of the problem, the use of tissue engineering as an improved solution, adipogenesis, scaffold materials, microtopography and its effect on cell behavior, and the influence of extracellular matrix (ECM) on cellular behavior.

Chapters 2 through 5 represent individual studies and follow a format found in many scientific journals. A background begins each chapter, to provide additional and specific information not detailed in Chapter 1. The methods used and results obtained in
each study are then presented, followed by a discussion of the results. Conclusions and references appear at the end of each chapter. Separate sections provide the overall conclusions and recommendations of the whole work following Chapter 5.

The second and third chapters focus on the use of surface microtexture as a tool to modulate the differentiation of adult stem cells. Studies described in Chapter 2 detail the seeding of D1 cells onto textured polylactide films and discuss the utility of these films in controlling the rate of differentiation over a 21 day study. The work in Chapter 2 was published in the Journal of Biomedical Materials Research – Applied Biomaterials in 2007. Studies detailed in Chapter 3 addressed similar scaffolds and investigated the onset of the different rates of differentiation promoted by textured or plain polylactide scaffolds; the secretion of the protein leptin was also evaluated and polynomial models were proposed to predict the amount of leptin secreted by these cells over time. The work in Chapter 3 was presented at the Annual Biomedical Engineering Society (BMES) Fall Meeting in Baltimore, Maryland (2005).

The fourth chapter investigates the role of scaffold material and cell size in controlling the level of differentiation of adult stem cells. In Chapter 4 studies, laminin and collagen were used as scaffold materials and their effectiveness in modulating proliferation and differentiation of the cells was analyzed. Different densities of these ECM proteins were also studied to evaluate potential differences in resulting cellular functionality. The work presented in Chapter 4 was presented at the Annual Biomedical Engineering Society (BMES) Fall Meeting in Chicago, Illinois (2006). The secondary aim of Chapter 4 was to elucidate the role of cell size in influencing cellular adhesion,
proliferation, and differentiation. The cell size was controlled by seeding cells on printed collagen of controlled width (using a modified inkjet printer) surrounded by non-adhesive areas.

Chapter 5 studies investigated cellular behavior in a 3D environment. Three different scaffold types (collagen microcarriers, laminin-coated polylactide beads and control polylactide beads) were evaluated with respect to their ability to modulate the differentiation of D1 cells and affect cellular adhesion and proliferation over a 16-day period.

The work presented in this dissertation represents a preliminary effort in developing various techniques to modulate adult stem cell differentiation in 2D and 3D systems, with the goal of controlling the long-term viability of a cell-based device.
CHAPTER ONE
BACKGROUND AND LITERATURE REVIEW

Introduction

Adipose tissue was considered unattractive for detailed study among researchers and clinicians not too long ago; the general population also considered fat as a tissue of excess rather than a tissue worth investigation. However, in the last decade or so, adipose research has been gaining momentum. The main reason for this shift was the development of the immortal preadipocyte line by Green and colleagues [1, 2]. This line gave researchers the first tool to aid in understanding the developmental process and physiology of adipocytes. Another reason for the amplified interest is the increase in the incidence of breast cancer [3] as well as plastic/reconstructive surgery. Also, it is now known that fat secretes proteins that maintain energy homeostasis (leptin) [4, 5], coagulation (PAI -1) [6, 7], and immune function (TNFα, IL 6) [8, 9]. These findings establish that fat is also a very important endocrine organ. And finally, a dramatic rise in the rate of obesity and diabetes has led to a focused interest in adipocyte biology.

The primary focus in our lab is to use the principles of tissue engineering to provide a better alternative towards breast tissue reconstruction. The current treatment options for patients recovering from breast cancer include lumpectomy, mastectomy and/or therapeutic treatments (for example, hormone therapy and chemotherapy). Of the patients diagnosed with breast cancer, over 68,000 will select mastectomy as a treatment [10]. A breast reconstruction procedure can be performed immediately following the mastectomy or at a later time. Nearly 40% of all reconstruction procedures are performed
immediately following the mastectomy [10]. Also, breast-tissue reconstruction after breast cancer is not the only reason for soft-tissue reconstruction; it may also be required for the treatment of post-traumatic repair and congenital deformities.

Current reconstruction procedures include implants, tissue flaps and tissue transplantation. There are inherent problems associated with each of them. In the case of autologous tissue grafting, the survival of fat graft is low (on average, only 30% of the injected fat cells survive the implantation procedure [11], and the cell volume decreases by an additional 40-60% once relocated to the breast [11], generally displaying necrosis due to insufficient vascularization and resulting in resorption of the graft over time [11, 12] and fibrotic grafted tissue [12]. Tissue transplantation also leads to donor site complications, pain, and risk of infection. Also, the quantity of the fat available for grafting may be limited. Thus, patients who have had prior graft surgeries are not good candidates for autograft surgeries. In the case of synthetic replacement devices, such as silicone implants, there is a concern with implant longevity and leaching of the constituent particles. There is also a concern with immune response when using anything other than autografts. Clearly, there is a need for better technique to address these issues. The best possible solution appears to be to induce the formation of adipose tissue at the site of the defect.

**Tissue Engineering as a possible solution**

Tissue engineering is a relatively new field that is multidisciplinary in nature and holds considerable promise as an alternative to the above mentioned techniques. Tissue
engineering combines the principles of engineering, chemistry and biology for the development of replacement tissues and organs. The term “tissue engineering” was originally used in 1984 to describe the growth of an endothelial-like tissue on the surface of polymethylmethacrylate (PMMA) [13]. However, tissue engineering gained mainstream attention only in 1993 when Langer and Vacanti published its basic principles, scope and potential [14].

The basic principle of tissue engineering involves harvesting healthy cells from the patient’s body, growing and differentiating those cells in vitro on a scaffold (usually a biodegradable or absorbable polymer) and then implanting the cellular construct into the patient’s body at the defect site. Over time, the absorbable scaffold is broken down and degraded by the body; simultaneously, the cells on the matrix proliferate and lay down a healthy matrix, while maintaining their phenotype. The cells eventually replace the diseased tissue and conform to the shape of the defect site. However, for tissue engineering to be a viable alternative to current approaches, several issues need to be addressed. For any medical implant to be successful it has to be successfully integrated into the body; one of the most important aspects of the implant that dictates this occurrence is the implant interface with the surrounding tissue and, in the case of tissue engineering, the interaction of cells with the scaffold on which they are grown.

**Adipogenesis**

Adipocytes develop from multipotent mesenchymal stem cells that can also give rise to muscle, bone and cartilage [15, 16]. These cells become committed to adipocytic
lineage under the influence of cues that are still not completely understood. These cues might be hormonal interaction, or the result of cell-cell or cell-matrix interactions. This process results in a cell with fibroblastic morphology called a preadipocyte. However, there are no expression markers that absolutely identify a cell as a preadipocyte. Due to the inherent complexity of this system during \textit{in vivo} development, almost all work on adipogenesis has been done using predetermined clonal cell lines, such as 3T3-L1 and 3T3-F422A. Preadipocytes when cultured \textit{in vitro} start dividing till they form a confluent monolayer; these cells acquire a fibroblastic morphology which is maintained until they proliferate. Once the confluent monolayer is formed, the cell/cell contact at confluence leads to growth arrest and is a prerequisite for further preadipocyte differentiation [17]. After the growth arrest phase, when treated with appropriate stimuli (mitogenic and adipogenic), the cells undergo mitotic clonal expansion and then differentiate to mature adipocytes [18]; this state is characterized by the cells accumulating small lipid droplets [19] which eventually coalesce to form a single lipid vesicle.

Insulin increases the percent of cells that differentiate and also increase the amount of lipid that each fat cell contains [20]. Recent data has shown that neutralizing of insulin \textit{in vivo} stimulates the apoptosis of fat tissue [21], confirming the importance of insulin in adipocyte biology. Glucocorticoids have also been use for many years to induce optimal differentiation of cultured preadipocytes. It is not clear, however, if glucocorticoids are responsible for efficient differentiation adipogenesis \textit{in vivo}; since patients with Cushing’s syndrome (elevated circulating glucocorticoids) show visceral obesity but wasting of subcutaneous fat [22].
The differentiation process is regulated by exposure to differentiation inducers such as insulin, dexamethasone and 3-isobutyl-1-methylxanthine (IBMX) [17-19, 23-25]. Through a series of cellular and nuclear events, these inducers cause phenotype changes within the cell, producing a mature adipocyte. The differentiation process begins when insulin binds to the insulin receptor on the cell surface and activates G-proteins. The alpha G-protein then binds to adenylyl cyclase, which begins the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). Phosphodiesterase converts cAMP to AMP. IBMX, a phosphodiesterase inhibitor, inhibits the conversion of cAMP to AMP allowing an increase in the number of cAMP molecules in the cell. The cAMP molecules bind to protein kinase A, causing a conformational change in the protein kinase A molecule. The catalytic subunits of protein kinase A molecule separate and enter the nucleus, activating the transcription of targeted genes. Dexamethasone binds directly to glucocorticoid receptors, which are nuclear hormone receptors [19, 26]. Dexamethasone induces the expression of C/EPBδ, a key transcriptional gene in adipogenesis [19]. IBMX also increases the expression of C/EPBβ [19]. The activation of C/EPBδ and C/EPBβ stimulates the transcription of C/EBPα and PPARγ [18, 19, 26]. Both C/EBPα and PPARγ are needed to trigger the expression of adipocyte-specific fatty-acid-binding protein genes [18, 27], which leads to the terminal differentiation of the cell into mature adipocyte.

A variety of cytokines have been found to suppress adipocyte differentiation. TNFα, IL 1 and many other pro-inflammatory cytokines have this effect on most cultured preadipocyte lines., and in fact can de-differentiate already mature fat cells [28, 29]. In
addition, several growth factors are rather potent inhibitors of adipogenesis, including epidermal growth factor (EGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) [30]. Another environmental condition that affects adipogenesis is hypoxia, which inhibits the process. This effect seems to be mediated through hypoxia-inducible factor 1α (HIF-1α) [31].

Adipogenesis occurs in both the prenatal and postnatal states in humans and continues to happen throughout the lifetime of the organism. This adipogenesis occurs both as a consequence of normal cell turnover, and as a consequence of the requirement for additional fat mass that arises with significant calorie shortage and weight gain [32]. While the fat cell size varies with the amount of lipid stored, there is a limit to how large these cells can become. On the other hand, humans and other animals will continue to gain fat as long as energy intake exceeds nutritional requirements, demonstrating a theoretical requirement of de novo differentiation.

**Scaffold materials**

Tissue engineering scaffolds should encourage proliferation and differentiation of the particular cell-type. Various materials have been tried by researchers as possible candidates as a scaffold for soft tissue engineering applications; however there is no clear consensus as to the optimum material for supporting cell growth and differentiation in order to engineer breast tissue. Both preadipocytes and adipocytes are anchorage-dependent cells and need an appropriate scaffold surface for proliferation and
differentiation. Some of the desired qualities that a scaffold material must possess for tissue engineering applications are:

- biocompatibility, biodegradability and ability to support cell growth
- ability to provide structural integrity
- ease of manufacturing and reproducibility
- high surface to volume ratio
- ease of sterilization
- cost effectiveness

Engineered scaffolds can be fabricated from natural or synthetic materials. Synthetic materials that have been used as scaffolds include polymers like polylactides (PLLA), polyglycolides (PGA) and poly(lactide-co-glycolides) (PLGA). Ceramics like hydroxyapatite have also been used in tissue engineering applications. Natural materials include extracellular matrix (ECM) components, collagen, fibrin and chemically modified alginate.

Various materials have been tried as scaffold materials to support adipogenesis in vitro and in vivo. Gelatin microspheres containing basic fibroblast growth factor has been used as a carrier for preadipocytes to induce growth of adipose tissue in vivo [33]. Similarly, the in vivo viability of PGA and polyethylene glycol (PEG) microspheres in breast tissue engineering has been investigated [34]. PLGA discs have been researched extensively as scaffold material for adipose tissue engineering [12, 33, 35]. Collagen sponges have been used as scaffolds for preadipocytes to study adipogenesis in vitro and
in vivo [36]. Hyaluronic acid [12, 37] and alginate gels [38] have also been evaluated as potential candidates for scaffolds in soft tissue engineering.

Since there are no general rules that govern the interaction between a cell type and a polymer, a complete characterization of the bulk and surface properties of a polymer is absolutely necessary for a thorough understanding of the interaction between the two. PLLA, PGA and PLGA are among the best-characterized polymers and hence they are used extensively as staples, orthopedic devices and scaffold materials [39]. PLLA and PGA also have good mechanical properties, they can be easily processed, are well-characterized and are approved by the FDA for specific in-vivo applications and are particularly well-suited for tissue engineering applications as they are biodegradable. Polylactides have been used in various forms as scaffolds like foams [40], microbeads [41], hollow tubes [42], films [41], sponges [43] and woven mesh cylinders [44].

The body degrades PLLA, PGA and PLGA by hydrolyzing the polymer chains through a bulk degradation process. These biomaterials are processed for elimination by the tricarboxylic acid cycle (Krebs cycle). Eventually, both monomers are excreted as carbon dioxide via respiration or as water through urination. PLGA is highly crystalline, hydrophilic and generally loses its mechanical strength relatively fast (few weeks to months). PLLA is less crystalline, more hydrophobic and, depending on the molecular weight, has the potential to retain its strength comparatively longer (months to years). Degradation occurs via hydrolysis and hence PLLA has the potential to degrade at a slower rate than PLGA due to the presence of methyl group in PLLA. Generally speaking, higher molecular weight and crystallinity mean a slower rate of degradation.
A significant concern with the use of PLLA, PGA and PLGA is the local acidic environment created by the degradation of the polymer. If the rate of degradation is high it will lead to tissue necrosis due to high acidity. The initial rate of degradation of these polymers is low; however, the rate increases with time as the polymer is broken down into numerous chains of low molecular weight. The site of implantation can also affect the rate of degradation. Areas with low fluid flow can, for example, accelerate the rate due to the accumulation of the acidic products in the local environment.

Synthetic biomaterials that are biodegradable in nature have historically been the ideal choice as candidates for three-dimensional matrices for tissue engineering applications. However, these materials have inherent disadvantages that have led researchers to look elsewhere to answer some important questions. The most critical shortcoming of synthetic biomaterials is the lack of cell-recognition signals. The success of any tissue-engineered device is based on the attachment of cells to the solid substrate. In general, cell adhesion involves the recognition by cell surface receptors of molecules in the extracellular space called ligands. The presence of specific ligands greatly enhances and facilitates the process of cell adhesion to any surface. Since synthetic biomaterials do not have these receptors on their surfaces another class of biomaterials, called natural biomaterials, has become an interesting choice for tissue-engineered matrix.

Natural biomaterials are composed of polysaccharides, polypeptides, amino acids and their combinations. Some of the examples of natural biomaterials are collagen, fibronectin, fibrin, chitosan and gelatin. The advantages offered by them over synthetic
materials include superior physiological properties (for example, collagen offers superior cell adhesion because of the presence of adhesion ligands), similar structure to the surrounding tissue and biodegradability. However, they also have some disadvantages like infection, antigenicity, lack of large-scale production ability and uncontrolled degradation properties over an extended period of time. The process of designing a three dimensional scaffold from natural materials is also very challenging because of the difficulty associated with design parameters like architecture and controlled porosity.

Collagen is one of the most commonly used natural materials as a scaffold. It is composed of three chains wound into a triple helix, is the most abundant protein found in the body and is responsible for the structural integrity. There are more than twenty different kinds of collagen types available in the body and these different types of different properties. Type I collagen is the most common among all the types of collagen and is the chief component of tendons, cartilage and bones. Collagen is mainly synthesized by cells responsible for laying down the extracellular matrix (ECM), like fibroblasts, myofibroblasts, osteoblasts and chondrocytes. Since collagen has a high mechanical strength, encourages cell attachment, offers good resistance to degradation and has very low antigenicity it can be used in a wide variety of applications.

Another common natural material that is used fibronectin. Fibronectin is involved in many cellular processes including tissue repair, blood clotting and cell migration/adhesion. Fibronectin sometimes serves as a general cell adhesion molecule by anchoring cells to collagen or proteoglycan substrates. Fibronectin matrix formation is initiated by fibronectin binding to cell-surface receptors, followed by assembly and
reorganization of the cell-surface associated fibronectin into fibrils. Since conventional biodegradable scaffolds in cardiovascular tissue engineering applications exhibit toxic degradation and inflammatory reactions, Ye and co-workers [45] have suggested the use of three-dimensional fibrin gel scaffolds for vessel tissue engineering. They did not find any toxic degradation and inflammatory reactions in their experiments.

Gelatin is also a natural material comprised of denatured collagen which has been used as a support structure for cells. Bender and co-workers incorporated gelatin microcarriers into polycaprolactone to create hollow tubular scaffold; the interior surface of this scaffold served as a guide for neuron cell growth [46]. Chitosan, gelatin and hyaluronic acid were used to create a sponge scaffold that was capable of supporting a co-culture of fibroblasts and keratinocytes in vitro [47].

Many materials can serve as candidates for a scaffold for tissue engineering application. The choice of the ideal material depends on the intended application and feasibility of the situation. The success of any cell-based device ultimately depends on the interaction of the cells with the scaffold material used; hence, understanding this interaction and also that of the cells with the surrounding tissue is imperative for tissue engineering to become successful.

**Microtopography and cell behavior**

Tissues and the cells that comprise the tissue in the body are arranged in precise spatial patterns, and the type of patterning observed depends on the tissue-type and the functionality of the tissue. Chemical and physical cues are provided to the proliferating
cells to arrange themselves in the requisite patterns. It follows, then, that for a tissue-engineered device to be successful in vivo it must not only mirror the native tissue chemically but also spatially as the spatial form of an organ/tissue dictates its function.

Over the years many efforts have been made by researchers to study the interaction of cells with materials having a defined surface microtopography. Folch and co-workers [48] have published a review of microtopography and its influence on cellular interactions. It has been known for a long time that cells respond to surface features and react to them, leading to what is termed as “contact guidance”. The earliest efforts involved studying the effects of nerve cells cultured on spider webs and cover slips; or evaluating cell orientation on randomly scratched (grooved) mica substrates. These early studies have contributed to our basic understanding of cell interaction with microtopography. However, the studies fail to address, among other things, the issue of structural dimensions of the tissues and precise repeatability over areas comparable to those found in vivo.

Advances in microfabrication technology have opened new avenues for fabrication of substrates that have dimensional and chemical features similar to those found in the tissues. Utilization of microfabrication technology enables the control of cell-surface interactions at the micro- or nanometer scale. Several reports in the literature suggest that surfaces with microgrooves affect the orientation and migration of cells. However the mechanism and even the exact role of these microgrooves is poorly understood. Specific literature will be cited later in the appropriate section dealing with
the interaction between microtopography and cell behavior, in terms of proliferation and differentiation.

Influence of ECM on cells

As mentioned above, microfabrication techniques offer avenues for significant advances in the field of tissue engineering. These techniques applied to medical and biological research have been the basis for the initiation of the development of new microscale diagnostic devices. The substrates normally used in microfabrication techniques are glass, silicon and polymer. However, cells do not adhere or grow on these substrates as well as some of the natural materials. Extracellular matrix (ECM) is a natural substance that has been widely used for cellular adhesion. The cell interaction with ECM also promotes the cellular growth and differentiation of many cell types including epithelial cells, neurons, and hepatocytes [49, 50]. However, in the context of breast tissue reconstruction, there are few studies that investigate the influence of ECM on preadipocyte development and differentiation. During *in vivo* embryonic development, not only biochemical soluble signals, but macromolecular components of ECM may play an important role in defining and guiding process. The ECM, besides physical support, also displays an array of macromolecular cues, able to control and direct cell proliferation, migration and differentiation [51, 52].

Sponges from collagen I and surgical meshed coated with fibronectin have been proposed as scaffolds for adipose implants [36, 53]. However, there are conflicting data concerning the effects of ECM proteins on adipogenesis. The design of an adipose
implant is hindered by the absence of data on this topic. There are also several inconclusive studies on this topic. As one of the aims of this study is to further investigate the interaction between ECM components and preadipocytes the pertinent literature will be dealt in greater detail in a later section. The overall goal of the assembled studies is to assess the effects of microtopography and ECM components on cell behavior, proliferation and differentiation. These issues must be better understood before soft tissue engineering becomes a viable clinical option.

References


CHAPTER 2
SURFACE PATTERNING: TOOL TO MODULATE STEM CELL
DIFFERENTIATION IN STEM CELLS

Introduction

The need for soft tissue reconstruction or augmentation has increased continuously over the years. Breast cancer is the most common form of cancer affecting women [1]. The treatment options for breast cancer include lumpectomy, mastectomy and/or therapeutic treatments. Soft-tissue reconstruction is also crucial for the treatment of post-traumatic repair and congenital deformities. Current reconstruction procedures include implants, tissue flaps and tissue transplantation. There are inherent problems associated with each existing option. The survival of grafted fat is low, generally grafting results in necrosis due to insufficient vascularization and results in resorption of the graft over time [2]. Also, the volume of fat available for grafting may be limited. Longevity and leaching of the constituent particles is of utmost concern for synthetic replacement devices such as silicone implants, as is immune response to bulk and degradants.

Tissue engineering holds considerable promise as an alternative to existing options, by providing a biologically based solution. However, for any medical implant to be successful in the long-term, it must be successfully integrated into the body. Many strategies have been formulated to influence tissue-biomaterial interactions. For example, it has been shown that geometrical configuration of an implant [3] or the pore size employed [4] can influence the level of development of a capsule or an oriented fibrous attachment or its better integration with the body. It is well known that cells respond to surface features and react to them, leading to what is termed as “contact guidance”. It has
also been shown that the roughness of an implant can alter the affinity of cell types to hydrophobic and hydrophilic surfaces [5]. Porous surfaces can enhance tissue integration with the implant surface thus providing long term stability \textit{in vivo} [6]; the results of several studies detailed in the literature suggest that surfaces with microgrooves affect the orientation and migration of cells [7, 8]. Wan and co-workers [9] used microtopography to study the adhesion behavior of cells and found that cell adhesion was enhanced on PLLA and PS surface with nano-scale and micro-scale roughness compared to the smooth surfaces of the PLLA and PS. Parker and co-workers [10] evaluated tissue reaction around implants with different surface topographies and found that the application of microgrooves or random surface roughness to polymer implants apparently does not have beneficial effects on peri-implant tissue healing \textit{in vivo}. This indicates the contradictory results present in the literature regarding the role of microtopography.

This study is a proof-of-concept to evaluate the effect of surface topography on the differentiation of D1 cells. Most of the studies in the literature have primarily evaluated the response of cells to the grooves in terms of orientation and cytoskeletal changes and the cell types used in those studies have a fibroblastic morphology. Due to the choice of these cell types, the majority of the studies cited in literature have employed grooves which are narrow and tightly spaced. In our study we wanted to evaluate the effect of the surface topography on differentiation and not orientation along the grooves. This involves cell-cell contact which might be altered due to the presence of the grooves. In addition, the size of an adipocyte, typically, ranges from around 20 µm to 120 µm. So,
for our study we used grooves which were separated by 100 µm which would allow the adult stem cells to differentiate to adipocytes and allow them to grow to their full size.

The focus of this study was to understand the interaction between adipocytes and patterned polymeric biomaterial surfaces in terms of proliferation and metabolism. The working hypothesis is that by controlling the level of \textit{in vitro} cellular differentiation in a tissue-engineered device, one can influence the viability of the device post-implantation. The specific purpose of this \textit{in vitro} study was to determine the behavior of D1 cells (in terms of lipid production, characteristic of functional adipocytes), compare their metabolism on patterned and plain PLLA films, and build statistical models to predict cellular behavior over time.

\textbf{Materials and Methods}

\textbf{Substrate fabrication}

Photolithography was employed by the Microelectronics division of the Electrical and Computer Engineering Department at Clemson University to etch micro-grooved patterns on a silicon wafer. The manufactured wafer had horizontal surface grooves of 3µm width over its surface and 100µm separation (pitch). The depth of each groove was 1.5µm and the diameter of the wafer was 7.62cm. The wafer was used as a template for patterned film fabrication. Figure 2.1 shows the schematic representation of template.

Polymer films were solvent-cast, using the silicon wafer template. Specifically, 1 gram of poly-L-lactide (molecular weight – 140000 g/mol) was mixed with 10 ml of dichloromethane to make a 10% polymer solution. The template was then covered with
10mL of the polymer solution. The solvent was allowed to evaporate slowly and, after the solvent evaporation was complete, the poly-L-lactide film was removed from the substrate. Unpatterned, control poly-L-lactide films were similarly made using a Petri dish as a template. Before using the films, selected specimens were examined by scanning electron microscope (SEM). Round discs (2 cm diameter) were punched from both the patterned and the plain films. These discs were placed in tissue culture treated multi-well plates which were coated with agarose to prevent the cells from preferentially adhering to the well-surface. A teflon ring was placed on each disc to prevent disc floatation. The tissue culture treated polystyrene (PS) surface in the multi-well plate was also used as a control in the experiment and each control well was also fitted with a teflon ring. The multi-well plates with discs and teflon rings was sterilized using ethylene oxide.

**Cell culture conditions**

D1 cells of passage 27 were seeded on all the scaffolds (textured films, plain films, and PS surfaces) at a density of $3.4 \times 10^4$ cells/well (20% confluence). The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with fetal bovine serum, fungizone and antibiotic-antimicotic; the supplemented medium hereafter will be referred to as DMEM-C. Cells were incubated at 37°C and 5% CO₂. The cells reached confluence in two days under these conditions; adipogenesis was induced by adding a differentiation induction cocktail of 0.5µM dexamethasone, 1µg/mL insulin and 0.5mM 1-methyl-3-isobutylmethylxanthene (IBMX) to DMEM-C.

Three 12-well plates were used in the 36-day experiment, two for histological staining using Oil Red O and one for lactic acid release and glucose consumption.
analysis. Each well plate contained six samples, one per well, including three poly-L-lactide patterned films and three plain poly-L-lactide film. Three additional wells per plate served as polystyrene controls.

**Qualitative assessment-phase contrast images**

Images showing lipid droplet accumulation on different surfaces were obtained using an inverted microscope in phase contrast mode and image acquisition software with a color digital camera. These images were used to monitor the morphological changes of the cell with time.

**Oil Red O staining**

To determine the percent area covered by lipid, Oil Red O was used to stain the cytoplasmic lipid droplets. This assay was performed at two different time-points: an early time-point (day 10 – i.e., ten days after introduction of differentiation inducers into the medium) and a late time-point (day 21) in order to determine the increase in the percent area covered by lipid. Image Pro® was used to quantify these values. Six images were taken per scaffold, each at a total magnification of 320x. Lipid appears red when stained with Oil Red O. For calculations red color was taken as the area of differentiated cells. Image Pro® was used to calculate the area of differentiated cells. This area was then divided by the total area and converted to a percentage. The total area represents the total cell population because cells were confluent in monolayer.
Lactic acid/glucose analysis

One hundred micro liters of medium was removed from each of the experimental and control wells before each medium change was performed. The amounts of lactic acid and glucose present in the extracted culture medium were subsequently measured every day (the medium was changed every two days) using a biochemistry analyzer (YSI).

Statistical analysis

Standard statistical analyses were performed to compare amounts of lipid formation on the three materials, and confidence intervals were provided for their pairwise differences, at each of the time points for which Oil Red O staining was applied. Metabolic activity measures (lactic acid release and glucose consumption) were modeled by appropriate cubic regressions, and differences between these measurements for the three scaffold materials were tested by analysis of variance methods. Detailed methodology will be described subsequently.

Results

Scanning Electron Microscopy of the patterned scaffolds

Scanning Electron Microscopy (SEM) was performed on patterned PLLA films to ensure that the pattern on the substrate was accurately reproduced on the film by the casting procedure. Figure 2.2 shows a SEM micrograph of the patterned film. As can be seen, uniform, horizontal grooves with the intended dimensions were replicated across the surface of the film.
**Qualitative assessment-phase contrast images**

Images illustrate the cell morphology as a function of the number of days in culture. When the cells were seeded they were initially fibroblast-like in appearance (Fig. 3a), gradually forming a confluent monolayer (Fig. 2.3b). The cells change their shape to a more rounded morphology following addition of the adipogenic cocktail. The next morphological change in the cells was the accumulation of small lipid droplets in the cytoplasm (Fig. 2.3c). Eventually, the small lipid droplets coalesced together to form a large, single lipid vesicle (Fig. 2.3d). As can be seen from these representative figures, the cells continued to accumulate lipid over the entire duration of the study. The morphological changes observed in the cells were the same on all three surfaces.

![Figure 2.1 – Schematic representation of the patterned silicon wafer](image-url)

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Figure 2.1 – Schematic representation of the patterned silicon wafer

1.5 µm groove depth

3 µm groove width

100µm pitch

3 µm groove width
Figure 2.2 - SEM image of a patterned PLLA film

Figure 2.3a - D1 cells on patterned PLLA film, day 1, 100x total magnification. The cells are elongated
Figure 2.3b - D1 cells on PS surface, day 3, 100x total magnification. The cells have formed a confluent monolayer.

Figure 2.3c – D1 cells on PS surface, day 6, 320x total magnification. The cells are beginning to accumulate lipid droplets.
The cells were stained with Oil Red O to evaluate the percent area occupied by the lipid droplets on all the three surfaces. Figure 2.4 shows the cells after 10 days of culture stained with Oil Red O; the cells seeded on all the surfaces have accumulated lipid. Figure 2.6 displays the sample mean values (over all wells and films) of percent area occupied by lipid for each of the three surfaces. The mean percent lipid area was highest for the PS (31%), followed by the patterned PLLA surface (25%) and then the plain PLLA surface (19%).

As can be seen from Table 2.1, the mean percent area occupied by lipid on the PS surface is significantly higher (p<0.05) than that of the patterned PLLA surface, which is
in turn significantly higher than that of the plain PLLA surface. In addition to the means, Figure 2.6 also displays error bars representing simultaneous 95% confidence intervals for each of the mean values; note that none of the error bars overlap.

Figure 2.5 shows images of the stained cells after 21-day culture and Figure 2.7 shows a summary of the. The data for the late time point shows a change in the trend from the early time point analysis. Here, the mean percent area occupied by lipid was highest on the plain PLLA film (49%) and lowest on the PS surface (41%); the mean for the patterned PLLA film is 42%. As can be seen from the Table 2.2, the mean percent area occupied by lipid on the plain PLLA surface is significantly higher (p<0.05) than that of the PS surface. However, the difference in means for the patterned PLLA and the PS surfaces is not statistically significant. (Note that the error bars in Figure 2.7 corresponding to the patterned PLLA and PS surfaces overlap.)

<table>
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<th>Simultaneous 95% confidence interval for difference in means</th>
<th>Difference is significant at level 5%</th>
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</thead>
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<tr>
<td>PS – Plain</td>
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<tr>
<td>Patterned – Plain</td>
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Table 2.1 - Pairwise difference in means using Scheffe’s method for Oil Red O staining for the early time-point
Figure 2.4 – Cells stained with ORO – early time point, 320x total magnification

PS surface

<table>
<thead>
<tr>
<th>Surfaces Compared</th>
<th>Difference in Means</th>
<th>Simultaneous 95% confidence interval for difference in Means</th>
<th>Difference is Significant at level 5%</th>
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<td>7.049</td>
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<td>Plain – PS</td>
<td>7.484</td>
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<td>Patterned – PS</td>
<td>0.434</td>
<td>(-6.312, 7.180)</td>
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</table>

Table 2.2 – Pairwise difference in means using Scheffe’s method for Oil Red O staining for the late time-point
Figure 2.5 – Cells stained with ORO – late time point, 320x total magnification
Figure 2.6 – Percent area occupied by lipid – early time point

Figure 2.7 – Percent area occupied by lipid – late time point
**Lactic acid released**

The daily value of lactic acid released is the increase in lactic acid from the previous day; that is,

$$LA\ released\ (j) = LA\ level\ (j) - LA\ level\ (j-1)$$

Figure 2.8 displays the daily values of lactic acid released for each of the three scaffolds. Two features stand out. First, there appears to be a large change in the magnitude of lactic acid released on day 21. The second notable feature is the “zig-zag” appearance of the plots, in which a relatively large value is generally followed by a smaller value and vice versa. This pattern exhibits that the measurements of lactic acid released are typically larger on days immediately following a medium change. In general, the values for the three scaffolds follow similar patterns. However, the values of LA released for the PS scaffold tend to be higher than the other two scaffolds.

In order to smooth the effects of the medium change (the “zig-zag” appearance) and investigate the differences due to scaffold further, cumulative values of lactic acid released were considered. Specifically,

$$Cumulative\ LA\ released\ (j) = LA\ released\ (1) + LA\ released\ (2) + \ldots + LA\ released\ (j)$$

Figure 2.9 displays the cumulative values of lactic acid released. As the length of the study increases, lactic acid released appears to increase at an increasing (quadratic) rate. While there is very little difference between the plain and patterned plots, the PS
values are noticeably higher. Polynomial regressions were performed to both model the trend and also investigate the difference due to scaffold. Note that as the cocktail was first administered after the day 3 measurement, only data from day 4 was included in the regression. Table 2.3 shows the analysis of variance (ANOVA) for lactic acid released.

It can be seen from Table 2.3 while the data can be fitted by an overall quadratic time trend, there are significant differences in the growth rates for the different surfaces. Coefficients of the corresponding regression give estimates of the differences. First, in order to uniquely estimate the parameters of the model, certain restrictions on the model coefficients must be imposed. This step is accomplished by setting the coefficients corresponding to the Plain values equal to 0. Thus, the overall intercept, Day, and Day² coefficients will model the trend in Plain values. The trend in the Patterned and PS values is modeled by adjusting the coefficients from this baseline. Table 2.4 summarizes the results of the quadratic time trend model.

The coefficients for Patterned and PS in Table 2.4 represent the adjustments from the baseline Plain values. These coefficients translate into the following model:

Plain: \[\text{Cumulative LA Released} = 0.427 - 0.076\text{Day} + 7.458 \times 10^{-3}\text{Day}^2\]
Patterned: \[\text{Cumulative LA Released} = 0.645 - 0.103\text{Day} + 8.063 \times 10^{-3}\text{Day}^2\]
PS: \[\text{Cumulative LA Released} = 0.796 - 0.143\text{Day} + 1.070 \times 10^{-2}\text{Day}^2\]

Note that the large p-values corresponding to the Patterned coefficients indicate that these coefficients are not significantly different from 0. Therefore, while there are different numerical values of the parameter estimates for the plain and patterned data,
there is no significant difference in the trend for these two surfaces. However, the trend for the PS data does remain significantly different.

Figure 2.10 displays the data along with the quadratic trend; overall $R^2$ of the regression is 0.997. While the quadratic trend provides a rough approximation, the residual plot in Figure 2.11 uncovers some flaws in the model. As mentioned previously, lactic acid release is generally higher immediately following a medium change. This pattern is exhibited by the “up-down” pattern of the residuals, particularly after day 20. Also, the residuals prior to day 20 are mostly positive, indicating that the fitted trend underestimates lactic acid release in the early days of the experiment. Nevertheless, the above quadratic model provides a simple description of the growth in the amount of lactic acid released. (A cubic trend was also fitted to the data, but there was no significant improvement over the quadratic trend.)
Figure 2.8 - Daily lactic acid released

<table>
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<th>p-value</th>
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<td>8.0</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
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<td>2</td>
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</table>

Table 2.3 – ANOVA and polynomial regression for lactic acid released
Figure 2.9 – Cumulative lactic acid released
Figure 2.10 – Quadratic trend of lactic acid released

Figure 2.11 – Residuals for the quadratic trend data for lactic acid released
<table>
<thead>
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</tr>
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<td>N/A</td>
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<tr>
<td>PS</td>
<td>0.369</td>
<td>0.145</td>
<td>0.0124</td>
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<td>Day * Plain</td>
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<td>N/A</td>
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<td>2.824 x 10^{-4}</td>
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</tr>
<tr>
<td>Day² * Patterned</td>
<td>6.051 x 10^{-4}</td>
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<td>0.1333</td>
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<tr>
<td>Day² * Plain</td>
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<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Day² * PS</td>
<td>3.237 x 10^{-3}</td>
<td>3.994 x 10^{-4}</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Table 2.4 – Quadratic time trend model for lactic acid release for

**Glucose consumed**

The daily value of glucose consumed is the decrease in glucose level from the previous day; that is,

\[ \text{Glucose consumed} (j) = \text{Glucose level} (j-1) − \text{Glucose level} (j) \]

Figure 2.12 displays the daily values of glucose consumed for each of the three scaffolds. One notable feature is the prevalence of negative values – i.e. indicating that glucose increased from the previous day. This is especially true for the plain scaffold; almost all of the values prior to day 20 are negative. The plot also exhibits a “zig-zag” feature like that observed in Figure 2.8 for lactic acid released. Here, the amount of glucose consumed is typically larger on days immediately following a media change.
There is less similarity between the scaffolds in the glucose consumed data than there was for lactic acid release. In general, glucose consumed tends to be highest for PS, followed by Patterned and then Plain.

In order to smooth the effects of the medium change (the “zig-zag” appearance) – and further investigate the differences due to scaffold – cumulative values of glucose consumed were considered. Specifically,

\[
\text{Cumulative Glucose Consumed (j)} = \text{Glucose consumed (1) + Glucose consumed (2) + \ldots + Glucose consumed (j)}
\]

Figure 2.13 displays the cumulative values of glucose consumed. For the first 20 days, glucose consumed remains fairly constant for the Patterned and PS data. This result is due to the presence of negative values canceling out the positive values. Also, the prevalence of negative values for the Plain data prior to day 20 causes an overall decrease in the amount of glucose consumed. After day 20, the cumulative values increase, roughly linearly. Also, after day 20, the increasing trend is fairly similar across the three scaffolds.

Polynomial regressions were performed to both model the trend and also investigate any difference due to scaffold. Again, as the cocktail was first administered after the day 3 measurement, only data from day 4 and later was included in the regression. Table 2.5 summarizes the analysis of variance (ANOVA) of the results

Table 2.5 shows that while the data can be fit by an overall cubic time trend, there are significant differences in the growth rates for the different surfaces. Coefficients of
the corresponding regression give estimates of the differences. First, in order to uniquely estimate the parameters of the model, certain restrictions must be imposed on the model coefficients. The restrictions are implemented by setting the coefficients corresponding to the Plain values equal to 0. Thus, the overall intercept, Day, Day$^2$, and Day$^3$ coefficients will model the trend in Plain values. The trend is modeled in the Patterned and PS values by adjusting the coefficients from this baseline and the results are shown in Table 2.6.

The coefficients for Patterned and PS in Table 2.6 represent the adjustments to the intercept due to the overall difference in surface means. These coefficients translate into the following model:

Plain: \[ \text{Cum. Glu. Cons.} = -0.312 + 0.207\text{Day} - 0.027\text{Day}^2 + 6.359 \times 10^{-4}\text{Day}^3 \]
Patterned: \[ \text{Cum. Glu. Cons.} = 0.184 + 0.073\text{Day} - 0.011\text{Day}^2 + 3.782 \times 10^{-4}\text{Day}^3 \]
PS: \[ \text{Cum. Glu. Cons.} = 0.671 - 0.112\text{Day} - 2.286 \times 10^{-4}\text{Day}^2 + 6.359 \times 10^{-4}\text{Day}^3 \]

(The above cubic model is a significant improvement over a quadratic model. One reason for this is that the cubic model is better able to account for the change in curvature prior to day 20, particularly for the plain film data.)

Figure 2.14 displays the data along with the cubic trend; overall $R^2$ of the regression is 0.996. The cubic trend provides a good approximation of the overall trend, but it does not account for the “up-down” effects of the media change, as exhibited by the residual plot in Figure 2.15. Also, the cubic time trend attempts to model the data as a whole. It is clear from the plots that there are different patterns before and after day 20. The cubic trend can accommodate this feature, yet it might be “overfitting” the data. In
particular, if only the data from day 20 on are considered, a quadratic or possibly even linear trend might be a good approximation.

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<th>p-value</th>
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<tr>
<td>Day³</td>
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</tr>
<tr>
<td>Day³ * Surface</td>
<td>2</td>
<td>1.3</td>
<td>&lt; 0.0001</td>
</tr>
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</table>

Table 2.5 – ANOVA and polynomial analysis for glucose consumed

Figure 2.12 – Daily glucose consumption
Figure 2.13 – Cumulative glucose consumption

Figure 2.14 – Cubic trend of glucose consumption
Figure 2.15 – Residuals for the cubic trend data for glucose consumption

<table>
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<tr>
<th>Parameter</th>
<th>Parameter Estimate</th>
<th>Standard Error</th>
<th>p-value</th>
</tr>
</thead>
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<td>0.1015</td>
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<td>Patterned</td>
<td>0.496</td>
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<td>0.0663</td>
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<td>Plain</td>
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<td>N/A</td>
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<tr>
<td>PS</td>
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<td>0.0004</td>
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<td>Day</td>
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<td>Day * Patterned</td>
<td>-0.134</td>
<td>0.052</td>
<td>0.0114</td>
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<tr>
<td>Day * Plain</td>
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<td>N/A</td>
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<tr>
<td>Day * PS</td>
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</tr>
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<td>N/A</td>
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<td>Day^3 * PS</td>
<td>-4.063 x 10^-4</td>
<td>4.748 x 10^-5</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Table 2.6 – Cubic time trend model for glucose consumed
Discussion

The aim of this study was to evaluate the lipid production and metabolism of D1 cells when cultured on patterned PLLA films. Qualitative assessment using phase contrast microscopy showed that cellular differentiation into adipocytes may be manipulated using appropriate surface topography stimuli. Oil Red O staining and image quantification was employed at two different time points to observe the amount of lipid produced at the early and the late stages of the study. At the early time point (day 10), the cells cultured on plain PLLA films produced the least amount of lipid and those cultured on the PS surface produced the highest amount. However, at the late time point the picture was reversed, with cells cultured on plain PLLA films producing the highest amount of lipid and those cultured on PS surface producing the lowest. Since the cells seeded on all the surfaces reached confluence at the same time, this finding suggests that the material property itself plays a regulatory role in the production of lipid by the cells. Although the initial lipid production by the cells on the PS surface is very high, the amount tapers off, as evidenced by a mean percentage change of about 40% in between the two time points. The cells on plain PLLA film show a much higher mean percentage increase between the two time points (about 163%) and the cells on the patterned surface show an intermediate increase (about 72%). One possible explanation is that, in the early stages, the positively charged tissue culture treated polystyrene encourages the proliferation and differentiation of cells more than the hydrophobic PLLA. However, with increasing time this effect diminishes. Because the adipocytes have differentiated faster on the PS surface they tend to dedifferentiate or detach from the surface faster too.
as compared to the PLLA surface. Our hypothesis is that the cells in all systems that we studied differentiate and then dedifferentiate (i.e., we are still dealing with a 2-D surface); the texture simply changes the rate of differentiation and dedifferentiation, but not the occurrence of either. According to the measured numbers, the cells on polystyrene differentiate the fastest, then dedifferentiate; the cells on patterned surface follow suit next, the cells on the plain surface ones last. So it appears that eventually all the systems reach the same endpoint, but we can control the timing of these events and that can be of critical importance with a tissue-engineered device.

It has been reported [11] that cells are more proliferative on a natural material like gelatin than PLLA. This behavior is attributed to the surface chemistry and roughness and due to the lack of functional surface groups or peptides on polylactide. The cells cultured on the patterned PLLA films consistently displayed a moderate differentiation rate as compared to the cells on the other two surfaces. This indicates that surface microtopography may be useful in controlling the rate of differentiation of different cell types in different construct materials.

Another aspect of these results warranting further investigation is the effect of surface texture on the process of cell differentiation. There are numerous reports in the literature [8, 12] that detail the use of microgrooved substrates in assessing the efficacy of microgrooves in influencing cell alignment. Two other crucial aspects of microtextured substrates are groove width and groove depth – both affect cell alignment differently. It has been reported [13], for example, that cell alignment is significantly affected by differences in groove depth but that difference in groove width is not as influential on cell
alignment. Also, studies [8, 12, 14] have shown demonstrated lack of correlation between microtexture and cell proliferation, although it has also been reported that actin filaments and focal adhesions of osteoblast-like rat bone marrow cells align with microgrooves. One specific groove size and depth was assessed in our laboratory; it is imperative to experiment with various combinations of the size and depth to optimize the groove dimensions. Our results show significant differences between the response of D1 cells on patterned and plain surface at the level of dimensions used in this on the differentiation of D1 cells. More work is being done in our lab to investigate the role of lower groove spacing on differentiation of D1 cells.

A hormone cocktail was employed in our study to induce the D1 cells to differentiate into adipocytes as we wanted to investigate the effect of surface topography on the differentiation while controlling for the effect of hormones; however the independent role of the surface texture towards the differentiation of cells also needs to be studied further. It has been reported [15] that bone cell differentiation and proliferation is affected by the interaction between hormones, growth factors and surface microtopography. There are various reports in the literature [16, 17] that suggest that surface roughness and topography play an important role in determining the proliferation and differentiation of cells. Although the role of surface texture has been studied in some detail in osteoblasts and chondrocytes, few studies have investigated its role in soft-tissue regeneration. Our focus was to study the interplay between surface texture, scaffold material and hormones in the context of soft-tissue; further work will be conducted to better elucidate this relationship.
Lactic acid released and the glucose consumed by the cells was also analyzed as it indicates the metabolic activity of the cells. As exhibited by the polynomial models, cells metabolized nutrients from the medium at an overall increasing rate on all surfaces. Cells seeded on the PS surface showed significantly higher lactic acid release than those on the other surfaces. There were no significant differences in lactic acid release between the plain and patterned PLLA surfaces. Cells seeded on the PS surfaces also showed significantly higher overall glucose consumption than those on the patterned PLLA surface, which in turn showed significantly higher overall glucose consumption than those on the plain PLLA surface. The data reveal many negative values of daily glucose consumed, most prevalently on the plain PLLA films. This observation is consistent for the entire duration of the study and for one scaffold type, likely attributable to instrument variability. As the process used to measure the concentration of glucose is enzymatic in nature, the result might be due to the presence of a confusing molecule in the sample.

The long-term goal of the research in our lab is to provide a better and more viable device for breast tissue reconstruction. This study serves as a starting point and highlights the use of adult marrow cells in tissue engineering and the manipulation of surface topography in cell differentiation for soft-tissue regeneration. The findings raise some additional concerns about the selection of scaffold material and characterization of target cell population.

**Conclusions**

Patterned PLLA films were used to compare the proliferation and differentiation of D1 cells on plain PLLA films and tissue culture-treated PS surface. This investigation
was completed by comparing the percent area occupied by lipid produced by the cells at two different time-points. The results indicated that microtopography can be used as a tool to modulate the differentiation of D1 cells to adipocytes. Cells cultured on patterned PLLA films showed an intermediate rate of lipid production at both the early and late time-points. At early time-point cells on PS surfaces had significantly higher percent lipid area than cells on plain PLLA films and this trend was reversed on the late time-point. Polynomial mathematical models describing changes in cell activity over time provide a good approximation of cellular glucose consumed and lactic acid release.

References


CHAPTER 3
CHARACTERIZATION OF THE DIFFERENTIATION AND LEPTIN SECRETION PROFILE OF D1 CELLS ON PATTERNED POLYLACTIDE FILMS

Introduction

The need for soft tissue reconstruction or augmentation has increased continuously over the years. Breast cancer is the most common form of cancer affecting women [1]. The treatment options for breast cancer include lumpectomy, mastectomy and/or therapeutic treatments. Soft-tissue reconstruction is also crucial for the treatment of post-traumatic repair and congenital deformities. Current reconstruction procedures include implants, tissue flaps and tissue transplantation. Inherent problems exist with each option. The survival of grafted fat is low; generally grafting results in necrosis due to insufficient vascularization and results in resorption of the graft over time [2]. Additionally, the volume of fat available for grafting may be limited. Longevity and leaching of the constituent particles in synthetic replacement devices such as silicone implants is of the utmost concern, as is immune response to the bulk and degradant materials.

Tissue engineering holds considerable promise as an alternative to existing options, and provides a biologically based solution. However, for any medical implant to be successful in the long-term, it must be successfully integrated into the body. Many strategies have been formulated to influence tissue-biomaterial interactions. The results of several studies detailed in the literature suggest that surfaces with microgrooves affect the orientation and migration of cells [3, 4]. Wan and co-workers [5] used microtopography to study the adhesion behavior of cells and found that cell adhesion was
enhanced on poly-L-lactide (PLLA) and polystyrene (PS) surfaces with nano-scale and micro-scale roughness compared to the smooth surfaces of the PLLA and PS. Parker and co-workers [6] evaluated tissue reaction around implants with different surface topographies and found that the application of microgrooves or random surface roughness to polymer implants did not have beneficial effects on peri-implant tissue healing \textit{in vivo}. Thus, the role of microtopography in the healing response is not clear. Microtopography may therefore play an important role in guiding adipocyte function in a tissue engineered device.

Adipocytes act as more than just energy reservoirs; they also exert significant regulatory influence on the brain in controlling body weight. The protein secreted by the adipocytes which plays a key role in this regulatory process is leptin. Leptin was discovered in 1994 by Zang and co-workers, is the product of \textit{ob} gene coding for 167 amino acid protein and 21 amino acid signal peptide [7], and acts via the hypothalamus region of the brain [8]. Leptin is also secreted by bone marrow adipocytes and stimulates myeloid and erythroid development. The leptin receptor is also expressed in hematopoietic stem cells [9].

Leptin provides the brain with information about the amount of fat present in the body and thereby acts as a part of a feedback mechanism to control the amount of fat present. The majority of leptin circulates in the bound form in lean individuals and in the free form in obese subjects [10]. When the size and number of adipocytes increase, the cells produce leptin which is then released into plasma. Studies have demonstrated that plasma leptin concentration correlates with the amount of body fat present [11]. Insulin
has been shown to increase the leptin production in rat and human adipocytes in vitro [12]. Leptin also counteracts insulin-mediated activation of glucose transport, glucose synthesis, and lipogenesis in isolated rat adipocytes [13]. Both in vivo and in vitro studies in rodents and humans have shown that glucocorticoids enhance leptin gene transcription and leptin levels [14]. Leptin levels are also elevated in rats treated with dexamethasone [14]. Hence, the amount of leptin present in the cell culture medium was used in this study as an indicator to assess the functionality of adipocytes present on different PLLA films. This proof-of-concept study evaluated the effect of surface topography on the differentiation of D1 cells, evaluating changes in lipid production and leptin levels in response to topographical feature changes.

Materials and Methods

Substrate fabrication

Photolithography was employed by the Microelectronics division of the Electrical and Computer Engineering Department at Clemson University to etch micro-grooved patterns on a silicon wafer. The manufactured wafer had horizontal surface grooves of 3µm width over its surface and 100µm separation (pitch). The depth of each groove was 1.5µm and the diameter of the wafer was 7.62cm. The wafer was used as a template for patterned film fabrication.

Polymer films were solvent-cast, using a silicon wafer template. Specifically, 1 gram of poly-L-lactide (Boehringer Ingelheim, molecular weight – 140000 g/mol) was mixed with 10 ml of dichloromethane (VWR) to make a 10% polymer solution. The
template was then covered with 10mL of the polymer solution. The solvent was allowed to evaporate slowly and, after the solvent evaporation was complete, the poly-L-lactide film was removed from the substrate. Unpatterned, control poly-L-lactide films were similarly made using a Petri dish as a template; select specimens were examined by scanning electron microscope (SEM). Round discs (2 cm diameter) were punched from both the patterned and the plain films. These discs were placed in tissue culture treated multi-well plates (Corning) which were coated with agarose (Sigma) to prevent the cells from preferentially adhering to the well-surface. A teflon ring (Fluoro-Plastics) was placed on each disc to prevent disc flotation. The tissue culture treated polystyrene (PS) surface in the multi-well plate was also used as a control in the experiment; for consistency, each control well was also fitted with a teflon ring. The multi-well plates with discs and teflon rings were sterilized using ethylene oxide (Anderson Sterilizers).

**Cell culture conditions**

D1 cells (ATCC) of passage 29 were seeded on all the scaffolds (textured films, plain films, and PS surfaces) at a density of 3.4 x 10⁴ cells/well (20% confluence). The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM; ATCC) supplemented with fetal bovine serum (Mediatech), fungizone (Invitrogen) and antibiotic-antimicotic (Invitrogen), the supplemented medium hereafter will be referred to as DMEM-C. Cells were incubated at 37°C and 5% CO₂. The cells reached confluence in two days under these conditions; adipogenesis was induced by adding a differentiation
induction cocktail of 0.5µM dexamethasone (Sigma), 1µg/mL insulin (Sigma) and 0.5mM 1-methyl-3-isobutylmethylxanthene (IBMX; Sigma) to DMEM-C.

Eight 12-well plates were used in this experiment, seven for histological staining using Oil Red O and one for measuring lactic acid released/glucose consumed and leptin released by the cells. Each well plate contained six samples, one per well, including three poly-L-lactide patterned films and three plain poly-L-lactide film. Three additional wells per plate served as polystyrene controls.

**Oil Red O staining**

To determine the increase in the percent area covered by lipid over a two week span, Oil Red O was used to stain the cytoplasmic lipid droplets. This analysis was performed at seven different time-points: Days 2, 4, 6, 8, 10, 12, 14. Image Pro® was used to quantify the amount of lipid. Six images were taken [using a Zeiss inverted microscope] per scaffold per time-point, each at a magnification of 320x, thereby resulting in a total of eighteen images per scaffold-type per time-point. Red color was considered the area of differentiated cells. Image Pro® was used to calculate this area. The total area represents the total cell population, because the cells were confluent in monolayer.

**Lactic acid/glucose analysis**

One hundred micro liters of medium was removed from each of the experimental and control wells every day. The amounts of lactic acid and glucose present in the
extracted culture medium were subsequently measured using a biochemistry analyzer (YSI).

**Leptin measurement**

The leptin ELISA was performed according to the manufacturer’s instructions (R&D Systems). Medium was isolated from all the samples and the controls daily and stored (at -20°C) until the end of the experiment, after which the ELISA was performed. Values are expressed as picogram of leptin per construct (scaffold).

**Statistical analysis**

Standard statistical analyses were performed to compare amounts of lipid formation on the three materials, and confidence intervals were provided for their pairwise differences, at each of the time points for which Oil Red O staining was applied. Leptin secretion was modeled by appropriate regression analysis, and differences between these measurements for the three scaffold materials were tested by analysis of variance methods. Detailed methodology will be described subsequently.

**Results**

Oil Red O staining was performed on cells seeded on the patterned and plain PLLA films and on the PS surface over seven different time-points (Day 2, 4, 6, 8, 10, 12 and 14) in order to quantify the increase in the percent area occupied by lipid droplets over the two week period on all the surfaces. Statistical analysis revealed an overall
significant difference in the lipid accumulated between the three scaffold-types. Also, the amount of lipid accumulated by the cells on plain and patterned films was significantly different on Days 6, 8, 10, 12 and 14 (p<0.01). Figure 3.1 shows the percent area occupied by the lipid droplets on all the surfaces over the period of two weeks. The cells on the patterned surface accumulated the highest amount of lipid and the cells on the plain PLLA surface had the lowest amount of lipid.

**Lactic acid released and glucose consumed**

Lactic acid is a product of cellular anaerobic metabolism, and glucose is the primary energy source for cellular metabolism. Metabolic activity was assessed to investigate if indeed any surface induced differences existed. No significant difference (p>0.05) was found between the amounts of lactic acid released by the cells on any of the surfaces (Figure 3.2) or between the amounts of glucose consumed by the cells on any of the surfaces (Figure 3.3).
Figure 3.1 – Percent area occupied by lipid on all the three surfaces. Each data point represents a mean of three values, and the error bars denote standard error of the mean (SEM).

Figure 3.2 – Cumulative lactic acid released by the cells on the different surfaces over the 14 day study. Error bars denote SEM.
Figure 3.3 – Cumulative glucose consumed by the cells on different surfaces over the 14 day study. Error bars denote SEM.

Leptin release

The amount of leptin, a protein hormone characteristic of adipocytes, released from D1s was measured over a period of twenty days. Measurement of leptin concentration in the culture medium of the constructs revealed that the cells released significantly higher amount of leptin on the polystyrene surface (p<0.05) as compared to the patterned and plain surfaces; however, no significant difference was found in the leptin released by the cells on plain versus leptin released by those on patterned surfaces. The amount of leptin released by the D1 cells was 3.92 ng of leptin per plain film, 4.07 ng of leptin per patterned film, and 4.56 ng of leptin per polystyrene surface, respectively. Figure 3.4 shows the progression of daily leptin values with time.
Figure 3.4 – Leptin concentration in the three culture media with time.

Figure 3.4 shows the leptin released by the cells on the different surfaces. The PS scaffolds generally exhibit the highest level of leptin secretion, while the levels for the Plain and Patterned scaffolds are similar. The PS scaffolds also exhibit a somewhat higher level of variability than the other two scaffolds. Leptin secretion increases linearly over time.

Analysis of covariance was performed to test for differences in the time trend due to the scaffold. The following table (Table 3.1) summarizes the results.
Table 3.1 – ANOVA: differences in time trend for released leptin due to the scaffold type

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<th>p-Value</th>
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<td>4,273</td>
<td>0.6896</td>
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After accounting for a linear time trend, a significant difference in the overall mean leptin values across the different scaffolds exists, which can be incorporated in the model by adjusting the intercept of the linear trend. However, after accounting for the difference in mean due to scaffold, allowing for the different slopes in the time trend does not significantly contribute to the model. The table below (Table 3.2) summarizes the parameter estimates.

Table 3.2 – Linear time trend for leptin released

<table>
<thead>
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<th>Parameter</th>
<th>Parameter Estimate</th>
<th>Standard Error</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
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<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Patterned</td>
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<td>10.09</td>
<td>0.3802</td>
</tr>
<tr>
<td>Plain</td>
<td>0.00</td>
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<td>N/A</td>
</tr>
<tr>
<td>PS</td>
<td>19.36</td>
<td>10.39</td>
<td>0.0644</td>
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<tr>
<td>Day</td>
<td>34.99</td>
<td>0.73</td>
<td>&lt; 0.0001</td>
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</tbody>
</table>

To estimate the parameters uniquely, the coefficient for the Plain scaffold is set to 0. The coefficients for Patterned and PS represent the adjustment to the intercept of the trend from the baseline Plain values. Note that the coefficient for the Patterned scaffold is not statistically significant; that is, there is no significant difference in the intercept of
the linear trend between the Plain and Patterned scaffolds. These parameter estimates translate into the following model:

Plain: \( \text{Leptin secreted (Day)} = 125.05 + 34.99\text{Day} \)
Patterned: \( \text{Leptin secreted (Day)} = 116.17 + 34.99\text{Day} \)
PS: \( \text{Leptin secreted (Day)} = 144.41 + 34.99\text{Day} \)

The above model is based on all data observations. However, several observations (see Table 3.3) were identified as either outliers or influential in this analysis.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Observations removed (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain</td>
<td>11, 18</td>
</tr>
<tr>
<td>Patterned</td>
<td>2, 3, 11</td>
</tr>
<tr>
<td>PS</td>
<td>2, 4, 11, 15, 16</td>
</tr>
</tbody>
</table>

Table 3.3 – Outlier values for leptin released for the different scaffolds

The analysis of covariance was performed again with these observations excluded, and Table 3.4 summarizes the results.

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>1</td>
<td>6,012,738</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Scaffold</td>
<td>2</td>
<td>9,856</td>
<td>0.0241</td>
</tr>
<tr>
<td>Day*Scaffold</td>
<td>2</td>
<td>7,351</td>
<td>0.0609</td>
</tr>
</tbody>
</table>

Table 4 – ANOVA for leptin released after discounting the outlier values
There is still a significant contribution due to the overall mean for each scaffold. Yet now there is also some evidence of a significant difference in the slope of the time trend due to differences across the scaffold. Inclusion or exclusion of the observations in Table 3 significantly affects the resulting model. Table 3.5 summarizes the parameter estimates for the revised model.

<table>
<thead>
<tr>
<th>Parameter Estimate</th>
<th>Standard Error</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>118.54</td>
<td>13.83</td>
</tr>
<tr>
<td>Patterned</td>
<td>-21.39</td>
<td>21.36</td>
</tr>
<tr>
<td>Plain</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>PS</td>
<td>53.22</td>
<td>21.20</td>
</tr>
<tr>
<td>Day</td>
<td>35.64</td>
<td>1.19</td>
</tr>
<tr>
<td>Day*Patterned</td>
<td>1.02</td>
<td>1.75</td>
</tr>
<tr>
<td>Day*Plain</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>Day*PS</td>
<td>-3.18</td>
<td>1.77</td>
</tr>
</tbody>
</table>

Table 3.5 – Linear time trend for leptin released after discounting the outlying values

Again, the coefficients for the Plain scaffold are set to 0. The coefficients for Patterned and PS above represent the adjustments to the slope and intercept from the baseline Plain values. Note that the coefficients for the Patterned scaffold are not statistically significant and thus there is no significant difference in the linear trend (slope and intercept) between the Plain and Patterned scaffolds. These parameter estimates translate into the following model:

Plain: \( \text{Leptin secreted (Day)} = 118.54 + 35.64 \text{Day} \)

Patterned: \( \text{Leptin secreted (Day)} = 97.14 + 36.66 \text{Day} \)

PS: \( \text{Leptin secreted (Day)} = 171.76 + 32.46 \text{Day} \)
Figure 3.5 displays the estimated model along with the daily mean leptin values. Figure 3.6 displays the corresponding residuals. The main feature of the residual plot is the “within day” correlation between the residuals. Figure 3.7 shows the cumulative amount of leptin released for all scaffolds.

Figure 3.5 – Linear trend of leptin released, based on the model proposed
Figure 3.6 – Residuals for the linear trend of the leptin released

Figure 3.7 – Cumulative leptin released by D1 cells on different surfaces. Error bars denote standard error of mean (SEM)
Discussion

The aim of this study was to examine the differentiation of D1 cells into adipocytes and to assess their functionality, i.e. the cellular response, in terms of lipid production, leptin secretion and metabolism (lactic acid released and glucose consumed) when cultured on PLLA films with defined surface topographies. Surface topography considerably affects the proliferation and differentiation of cells and hence microtopography is a useful tool to better understand the cell-material interaction in a carefully designed system. The size of an adipocyte typically ranges from around 20 µm to 120 µm; hence we assessed cell response to grooves which were separated by 100 µm.

Oil Red O staining was performed on the D1 cells to determine the amount of lipid accumulated by the cells and the trend of lipid production by the cells on the different surfaces over a two week period. The D1 cells accumulated lipid droplets on all surfaces, the amount of which increased significantly over the period of two weeks. The area covered by lipid was significantly different on each surface. Our results indicated that the patterned surface was involved in the differentiation of D1 cells as the cells cultured on the patterned surface accumulated significantly higher amount of lipid from Day 6 to the end of the last time-point (Day 14). No difference in accumulated lipid was noted on Days 2 and 4, which is not surprising as the cells do not start differentiating until after a few days of treatment with the cocktail. Also, a more linear trend of lipid accumulation by the cells cultured on patterned PLLA films was observed throughout the period of the study, as compared to the cells cultured on the other two surfaces. This
trend might be attributed to a more controlled and gradual differentiation (and hence lipid accumulation) due to the surface microtexture.

Studies in the literature [4, 15] suggest that microgrooves influence cell alignment, where two critical factors are groove width and depth. Clark and co-workers [16] have shown that cell alignment is significantly affected by differences in groove depth, but that differences in groove width is not very critical in influencing cell alignment. No clear consensus defines the optimum groove width or depth, as this value depends on several factors such as the cell-type or the in vitro conditions of the experiments conducted. Results from preliminary studies in our laboratory have shown that groove width does affect differentiation of D1s to adipocytes. In the current experiment, however, one combination of groove width and depth was used; further studies with various groove sizes are needed to better understand and quantify the significance of these parameters. Many studies have employed microtopography as a tool to study alignment of cells of osteoblastic lineage [17, 18]; however, there are very few studies that investigate the effect of microtopography on soft-tissue differentiation and functionality. In this study a hormone differentiation inducer was used in all cases, thus differences in cellular behavior were attributed to surface microtopography. However, the role of the substrate and its surface texture on the differentiation of cells also needs to be studied further. It has been reported [19] that bone cell differentiation and proliferation is affected by the interaction between hormones, growth factors, and surface microtopography.
We observed a very linear trend (and significantly higher amount of lipid) in the lipid accumulation by the cells on patterned PLLA films as compared with cells cultured on other surfaces; as all the parameters were same for the cells seeded on the three surfaces, it can be inferred that the patterning of the PLLA films does have an effect on the cellular behavior. Although the results indicate that this particular set of dimensions of the patterned surface is capable of influencing the differentiation, further work must be completed to pinpoint the exact mechanism by which this linear trend is orchestrated.

Cellular metabolism as a function of lactic acid released and glucose consumed was also analyzed. There was no significant difference in either of the parameters between the cells cultured on PLLA films and those on tissue culture treated polystyrene surface, suggesting that PLLA can serve as a good scaffold material. However, literature reports [20] state that cells are more proliferative on a natural material like gelatin than PLLA. Studies in the literature have focused on adipocytes seeded on gelatin microcarriers, hence the findings could be due to differences between surface chemistry or roughness. Another reason for the difference in results could be due to the fact that cells cultured on three dimensional scaffolds (beads) tend to proliferate and differentiate differently than on two dimensional surfaces (films). Further investigation is required to better quantify and compare cell behavior on natural and synthetic polymers.

In the past decade, since its discovery in 1994, leptin has received tremendous attention in the field of obesity research. Leptin is a key protein secreted by adipocytes and is also a marker used to assess the functionality of adipocytes. To the best of our knowledge, this is the first study that has characterized the leptin secretion profile of D1
cells which have differentiated into adipocytes. The results show that D1 cells cultured on all the surfaces visually display the adipocyte phenotype. Several studies have examined the process of adult stem cell commitment and differentiation to osteogenic lineage; however, much less is known about the conditions favoring adipogenic differentiation. Our results further highlight the adipogenic potential of multipotent mouse bone marrow stromal precursor (D1) cells. We found that the cells seeded on tissue culture treated polystyrene surface released the highest amount of leptin into the culture medium as compared to the cells seeded on the polymer films. This result suggests that PLLA has an inhibitory influence on the leptin secretion ability of D1 cells. More work is needed to better elucidate the leptin secretion ability of these cells on different polymeric materials (for example, synthetic versus natural).

Once the cells became confluent, they were consistently maintained in the adipogenic cocktail; however, results from the literature [21] suggest that extended “resting periods” in the absence of adipogenesis inducers (after the onset of differentiation) enhances the differentiation process and increases the leptin mRNA levels of human mesenchymal stem cells. It is of interest to investigate if there is a similar pattern observed in D1 cells. Two key transcription factors in adipocyte differentiation, PPARγ and C/EBPα, affect leptin gene expression in opposite ways. While PPARγ decreases leptin expression [21-23], C/EBPα induces leptin gene expression [24-26]. The presence of multiple mechanisms suggests that the level of circulating leptin is controlled by a complex set of signals. Also, cAMP has been shown to decrease the leptin level in rodents [27]. We employed a hormonal cocktail, IBMX, to induce differentiation. IBMX,
which is a component of the cocktail, increases the production of cAMP and hence should serve to decrease the production of leptin. Leptin has also been implicated in regulating body-weight, in fetomaternal signaling [28] and in haemopoiesis [29].

In a recent study it was shown that physiological concentrations of leptin (25-100ng/mL) can stimulate the proliferation of both normal and malignant breast epithelial cells [30]. The authors also demonstrate that leptin enhanced anchorage-independent growth of select breast cancer cell lines but did not similarly enhance the growth of normal cell lines. It has also been shown that leptin might play a role in the development of mammary tumors [31]. Elevated serum protein levels associated with obesity may also promote breast cancer development. One of the major focus areas in our lab is breast tissue engineering; these findings make it imperative to address more profoundly the role of leptin, which is a key regulatory adipocyte protein, in tissue-engineered constructs for breast reconstruction.

Preadipocytes are the standard, current model system for studying adipogenesis. However, the inherent disadvantages posed, such as the limited number of cells that can be obtained from an individual and limited life cycle, necessitate further research with marrow stromal cells, which do not have these limitations. There is a growing interest in natural polymers as well as the evaluation of D1 cell behavior on natural polymers like collagen. Our study offers a functional characterization of D1 cells and investigates their behavior (differentiation and metabolism) when cultured on polymers with a defined topography. The long-term goal of the research in our lab is to provide a better and more viable alternative towards breast-tissue reconstruction using tissue engineering. This
study offers a starting platform toward a better understanding of the interaction between synthetic scaffold and the cellular component of a tissue engineered device.

Conclusions

Patterned PLLA films were used to compare the behavior of D1 cells on plain PLLA films and tissue culture-treated PS surface by assessment of lipid production, metabolism and leptin secretion. Lipid accumulation was significantly different for cells on the patterned scaffolds when compared to plain scaffolds. No differences were detected in the metabolism of the cells on any of the scaffolds, as measured by lactic acid released and glucose consumed. Polynomial models were proposed to predict the amount of leptin released by the cells as a function of time.

References


Introduction

Increasingly, soft-tissue implants have been used to contour tissue in reconstructive surgery, e.g., following oncological resection [1]. The traditional choices for these procedures include transplanted autologous adipose tissue and implanted synthetic materials; however, these options have many disadvantages. A promising alternative is an adipose implant prepared from a patient’s own cells and seeded within a biocompatible scaffold. The preferred cell source for such an implant may include stromal-vascular (SV) cells harvested from the adipose tissue rather than the post-mitotic adipocytes [2]. Adult stem cells harvested from the bone-marrow offer an attractive alternative to adipocytes because of their plasticity and relative ease of extraction. Undifferentiated adult stem cells in the implant would proliferate within the scaffold to amplify tissue mass and differentiate into adipocytes, providing replacement tissue. A regenerated implant eliminates the issue of transplantation related donor site morbidity, thus reducing the duration and the cost of surgery.

Extracellular matrix (ECM) is a natural substrate that has been widely used in cell culture to enhance cellular adhesion. Cellular adhesion to ECM proteins is a fundamental feature of development, maintenance of tissue organization, and many pathological conditions; adipogenesis necessarily involves the adhesion of preadipocytes to substrates. It is therefore crucial to understand the adhesion process in order to develop viable soft-
tissue implants that will not resorb in the long term. The cellular interaction with ECM also promotes cellular growth and differentiation and, although this interaction is well characterized in select cell types like neurons, hepatocytes, and vascular endothelial cells [3, 4], it is not clearly understood in the case of adipocytes. Very few studies address the interaction of ECM components with adipocytes [5], and many discrepancies are reported in the literature. Most of the reported studies focused on primary S-V cells [6-8] and indicate that ECM enhances adipogenesis in these cells. ECM from corneal endothelial cells, for example, has been shown to inhibit preadipocyte differentiation [9]. Although the molecular mechanisms of preadipocyte-to-adipocyte differentiation is well understood [10, 11], the initial adherence of preadipocytes to different substrates has not been thoroughly investigated. Since it is apparent that preadipocytes play a prominent role in pathological adipogenesis conditions of obesity [12-14] and diabetes [14, 15] and are essential in the creation of breast tissue via tissue engineering strategies, the development of therapeutics for obesity, diabetes, and engineered replacement breast tissue is dependent on the understanding of preadipocyte attachment and differentiation on various substrates.

With this study we wanted to address two aspects critical towards the development of a tissue-engineered adipose implant. Firstly, we wanted to evaluate the efficacy of ECM proteins in controlling the proliferation and differentiation of adipocytes. Secondly, we wanted to explore the feasibility of using an ECM protein to control the cell size /footprint, which has been shown to profoundly affect cell function, especially in cells like adipocytes which have an inherent storage capacity associated
with their functionality. We will first present the literature associated with ECM proteins and their effect on adipocyte differentiation and then move on to the importance of cell size in regulating cell function.

Many strategies have employed ECM components as scaffolds for preadipocytes. Sponges of collagen I and surgical mesh coated with fibronectin have been proposed as scaffold materials for preadipocytes. It was reported that the transplantation of preadipocytes on collagen sponges gives rise to a well-vascularized adipocyte-like tissue and also leads to enhanced formation of ECM [16]. Reports also indicate that scaffolds made of hyaluronic acid modified by esterification (HYAFF 11) are superior to collagen sponges with respect to scaffold cellularity post implantation [17]. Most of the reports in the literature addressing the role of fibronectin are conflicting or inconclusive. Kral and co-workers reported that monofilament-expanded polytetrafluoroethylene coated with fibronectin has enhanced adhesion properties as compared with the same material coated with collagen [18]. However, it was also reported that a fibronectin substrate inhibits adipogenic conversion of mouse 3T3-F442A cells [19] but has no influence on primary cultures of porcine SV cells [6]. Collagen I is reportedly a better candidate for a scaffold material than fibronectin for SV cells [20]. These diverse observations demonstrate the ambiguity of results with reference to our understanding of attachment and differentiation of preadipocytes; these contradictory results, coupled with a paucity of studies on this subject, have limited our understanding of the attachment and differentiation process.

Many biochemical studies of established preadipocyte cell lines have indicated that changes in the level and type of ECM components occur during adipocyte
differentiation. During adipocyte differentiation, the expression of collagen I and III decreases [21], while the synthesis of collagen IV increases [22]. Also, while the production of laminin remains constant, the extracellular laminin increases [22]. The amount of pericellular and cellular synthesis of fibronectin decreases during adipocyte differentiation [23]. ECM also has a functional importance in adipocyte differentiation. The ECM components associated with the cytoskeleton via receptors such as integrins play an important role in the morphological change in the preadipocytes during differentiation [9, 24]. It has been shown that fibronectin has an inhibitory effect on preadipocyte differentiation [19], and that the inhibition of collagen synthesis by ethyl-3-4-dihydroxybenzoate (EDBH) prevents preadipocytes from differentiating [25]. It has also been reported that laminin and collagen IV promote fat cell differentiation [26].

Laminin mediates adhesion events that are critical to a number of biological mechanisms [27, 28]. Hausman and co-workers showed that preadipocyte attachment and spreading in porcine stromal-vascular (SV) cultures is enhanced on laminin substrata [6, 8]. It has also been shown that rat preadipocytes can be recruited to microenvironments consisting of Matrigel and basic fibroblast growth factor (bFGF) in vivo, leading to de novo adipogenesis [29, 30]. Matrigel is composed primarily of laminin, while the balance is comprised of collagen IV and proteoglycans; laminin may be the component responsible for the Matrigel-enhanced adipogenesis [6]. In addition, it has been observed that laminin is expressed in the extracellular space in bovine intramuscular adipose tissue [31]. Furthermore, immunocytochemical studies indicate that laminin is present around small differentiating adipocytes in fetuses [32].
The relationship between cell volume and cell function has been previously investigated (a review on this topic can be found in the literature [33]) with particular focus on cells that exhibit swelling and shrinkage due to extracellular and/or intracellular osmotic perturbations. For example, it has been suggested that hyper- and hypotonicity changes in liver cells may play an important role in regulating hepatic glucose metabolism by altering gene expression [34]. Cells with important storage capacities, such as hepatocytes and adipocytes, undergo changes in volume of greater amplitudes than those observed after osmotic changes. Adipocyte cell footprint may vary greatly in physiological conditions. In healthy human beings, adipose cell volume also increases by 20-30 times (a three-fold increase in the diameter is equivalent to a $3^3$-fold increase in the volume); these changes are even greater in obesity syndrome, which is mainly characterized by fat cell hypertrophy [35]. Several metabolic functions are subject to change with adipocyte size in obese animals or humans. These functions include storage and mobilization of lipids [36, 37] as well as secretion of both leptin [38] and TNFα [39].

Adipocyte-like cells, which have associated storage capacity, as well as other kinds of cells with no intrinsic storage capacity, are also affected by their size. For example, it has been shown that cell size correlates with cell differentiation phenotypes and proliferative capacity in human corneal epithelial cells [40]. In one study documented in the literature, cells were sorted in four different categories depending on their size, ranging from 10 to 31 micron in diameter, and it was found that the smallest cells (10 microns) seemed to be enriched for putative stem cells. They concluded that small size may represent one of the important properties of adult corneal stem cells. Jouvion and co-
workers [41] evaluated the effect of morphometric characteristics of muscle-derived cells (MDC) behavioral features. The morphometric analysis showed that MDC showed wide cell size diversity (4µm to 10 µm). Their results showed that cell size was associated with specific expression of myogenic markers, revealing different commitment levels; and that in vitro, the smallest MDC exhibited limited myogenic activity while larger MDC displayed a myogenic potential that increased with their size. They concluded that the size of MDC is indicative of their respective progression towards myogenic differentiation lineage. It has been demonstrated that capillary endothelial (CE) cells seeded on fibronectin (FN)-coated adhesive islands that restricted cell size underwent apoptosis, whereas cells on islands that permitted spreading were able to enter the S-phase [42]. Bluher and co-workers found that protein expression patterns are regulated in adipocytes, both as a function of cell size and as a function of impaired insulin signaling [43]. There are other reports in the literature [44, 45] that show that size of the adipocytes affects functional properties of the cell. In the proposed study, we will compare functional characteristics of two batches of adipocytes that vary in size.

Modified inkjet printers have been shown to be an effective tool to print cells. Previously developed methods of constraining cellular pattern include soft lithography [46], laser-directed cell-writing [47], photolithographic techniques [48], and dip-pen nanolithography [49]. Soft lithographic techniques include microcontact printing and microfluidic channel flow patterning [50]. Of the above mentioned methods microcontact printing is the most commonly used technique. The primary benefit of this method is the
high-resolution capability to create features on the scale of 2-500 µm [51], allowing for the production of patterns on the scale of single cell.

In contrast to the above methods we have used a modified inkjet printer to print protein of controlled width to create adhesive area. Inkjet printing and computer-aided design (CAD) techniques have already been used in the field of biomaterials. They have previously been applied for biosensor development [52] and free-form fabrication techniques to create a cellular polymeric scaffolds [53] among other applications. The use of inkjet printing is an attractive option as it is fast, relatively cheap, has a high throughput and can be used on any shaped scaffold. Modified ink-jet printers have been used by researchers to create chemical patterns [54, 55].

Our long-term hypothesis is that by controlling the level of cellular differentiation in vitro one can affect the viability of a tissue-engineered device in vivo. To provide foundational information regarding this theory, we evaluated the ability of select ECM molecules to control the level of differentiation of adult stem cells to adipocytes. Laminin and collagen were selected as candidates for this study. Collagen was selected as it one of the most ubiquitous and best-characterized ECM proteins, while laminin was chosen because of the evidence in the literature that it is extensively involved in the differentiation of adipocytes from a very early stage. The objective of this study was to compare collagen I and laminin as possible biomaterial candidates for adipose tissue engineering and to evaluate their role in modulating D1 cell spreading and adipocyte differentiation. This study also assessed two different densities of collagen I and laminin coatings to investigate their possible density-dependence. We also hypothesize that by
printing adhesive areas of controlled widths, surrounded by anti-adhesive areas, we will be able to study the behavior of adipocytes of different size/footprint.

**Materials and Methods**

**Objective (A)**

**Surface preparation**

Surface studies were conducted in 12-well plates. The wells were coated with bovine collagen I (Inamed; Fremont, CA) and laminin (BD Biosciences; San Jose, CA) at two different densities: a low density (LD) of 5 µg/cm² and a high density (HD) of 50 µg/cm². These matrices were prepared as a thin layer on tissue culture surface according to manufacturer instructions. After incubation at room temperature under sterile conditions, the precoated plates were washed with serum-free medium to remove excess coating solution. The uncoated polystyrene wells were used as controls.

**Cell culture and differentiation induction**

Each well was seeded with D1 cells (ATCC; Manassas, Va), of passage 27 at a density of 3.4 x 10⁴ cells/well (20% confluence) on Day 0. The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM; ATCC; Manassas, Va) supplemented with fetal bovine serum (Mediatech; Herndon, Va), fungizone (Invitrogen; Carlsbad, CA) and antibiotic-antimicotic (Invitrogen; Carlsbad, CA); the supplemented medium hereafter will be referred to as DMEM-C. The cellular systems were incubated at 37°C and 5% CO₂, and the cells reached confluence in two days under these conditions (Day 2). Adipogenesis was induced by adding a differentiation induction cocktail, on Day 2, of
0.5µM dexamethasone (Sigma; St. Louis, MO), 1 µg/mL insulin (Sigma; St. Louis, MO), and 0.5 mM 1-methyl-3-isobutylmethylxanthene (IBMX; Sigma; St. Louis, MO) to the DMEM-C.

Four 12-well plates were used in the study, two for Oil Red O (ORO) early time-point analyses (Day 7, after addition of the cocktail) and two for late time-point ORO analyses (Day 14, after addition of the cocktail). Two 12-well plates were designated for each time point, one contained wells with low density collagen and laminin coatings and the other contained wells with high density collagen and laminin coatings. Each well plate contained six samples, one per well, including three collagen coated wells and three laminin coated wells. Three additional wells per plate served as uncoated polystyrene controls.

**Qualitative assessment – phase contrast images**

Cells were monitored for lipid accumulation and change in morphology due to the different ECM components using an inverted microscope (Carl Zeiss; Thornwood, NY) in phase contrast mode and a color digital camera (Diagnostic Instruments; Sterling Heights, MI). Pictures were taken at a total magnification of 100x until the cells formed a confluent monolayer, and at 320x total magnification from confluence onward to better monitor the accumulation of lipid and the accompanying morphological changes.
Metabolic activity

Metabolic activity of cells on the different ECM components was measured using an alamarBlue (Biosource; Camarillo, CA) assay. Unlike some other assays (for example, CellTiter 96® assay which uses the MTS reagent and which is an end-point assay), almarBlue dye is minimally toxic to cells; thus, it can be used to assess the same population of cells at different time-points. Reduction of the alamarBlue dye from a non-fluorescent blue to a fluorescent red occurs by the uptake of the dye in metabolically active cells. Values of the metabolic activity may vary due to the number of cells present in different phases of cell cycle. Furthermore, only relative comparisons of metabolic activity are useful with this assay, since surfaces used to normalize activity to cell number are 2-D and therefore do not allow perfect extrapolation to 3-D systems, where cellular metabolic activity may be vastly different.

The assay was performed on cells seeded on collagen I, laminin, and the uncoated polystyrene control, at both (high and low) densities at 4 hours post seeding and 48 hours post seeding. Following the addition of 1.5 mL of fresh DMEM-C to each well, 150 µL of the alamarBlue reagent was added to each well. The assay plates were placed in the incubator for a period of 2 hours. After incubation, 100 µL of the samples was transferred in triplicate to a 96-well plate. Fluorescence values were immediately obtained using a fluorometric plate reader (GMI; Ramsey, MN) with an excitation filter of 544 nm and an emission filter of 590 nm.
Lactic acid and glucose levels

The amounts of lactic acid and glucose present in the medium were measured every time the medium was changed (Days 2, 4, 6, 8, 10, 12, 14, 15 and 16) using a biochemistry analyzer (YSI; Yellow Springs, OH). Note that medium was changed on Day 15 also because the medium was very acidic due to high levels of cellular activity. Five hundred microliters of medium was removed from each well before medium changes were performed. Lactic acid is the product of cellular anaerobic metabolism only; therefore, the level of lactic acid in the medium cannot be used to make conclusions regarding total metabolic activity. Glucose is the primary energy source for cellular metabolism, so a lower value of glucose remaining in the medium indicates a higher level of glucose consumption in cellular metabolism. Thus, the results from assays designed to measure metabolic activity and the levels of lactic acid and glucose in the culture medium should be used in combination to gain an overall assessment of the metabolic activity in a cell culture study.

Oil Red O staining

Intracellular lipid accumulation was measured using Oil Red O (ORO) analyses. This assay was performed at two time-points – an early time point (Day 7) and a late time-point (Day 14). ORO solution was made as described previously [56]. Briefly, a stock solution was made by dissolving 0.5 gm of the ORO powder in 100 mL of isopropanol and then filtering. To prepare the working solution, 6 mL of stock solution was mixed with 4 mL of distilled water, left for 1 hour at room temperature, and then
filtered prior to use. Cell cultures were washed twice with phosphate buffered saline (PBS) and fixed with 10% neutral formalin for at least 1 hour at room temperature. The cells were washed with water twice before staining for 2 hours with the ORO working solution, and then washed exhaustively with water. Excess water was evaporated by placing the stained cultures at 37°C. The dye was then extracted with 500 µL isopropyl alcohol per well by gentle pipetting, and the absorbance was measured immediately at 510 nm.

**Statistical analysis**

All quantitative assays were performed in triplicate. Values presented are mean ± standard error of mean (SEM). Using SAS® statistical software, three-factor ANOVA was conducted with the Least Squares Means (LSMEANS) statement to form pairwise comparisons between the effects of different ECM components (laminin and collagen I) at different densities and time-points. The significance level for all comparisons was p<0.05.

**Objective (B)**

**Substrate preparation**

Glass microscope cover slips (Fisher Scientific, Pittsburg, PA), of 22sq.mm. dimension, were used as substrates in the study. The cover slips were cleaned in highly acidic pirhana solution to obtain a very clean hydrophilic surface that was amenable to
cell attachment and proliferation; these cover slips were then dip coated with an autoclaved 0.15% solution of type II agarose (to prevent unhindered attachment of cells) and then dried and UV sterilized for at least 2 hours in a laminar flow hood.

**Substrate patterning**

Two different models of modified Hewlett-Packard (HP) ink-jet printers (340 series and 3900 series) were employed to print a single line of type I collagen on these cover slips. The stock solution of type I collagen used for printing was diluted with Dulbecco’s phosphate buffered saline (PBS) to a final concentration of 1 mg/mL. The patterns were designed using Microsoft Word application. Depending on the model of the printer used we printed single lines of type I collagen with an associated width of 55 µm (3900 series) and 110 µm (340 series). After printing the patterns were dried in aseptic conditions and then rehydrated prior to cell seeding.

**Cell culture**

D1 cells of passage 28 were seeded on the patterned cover slips. The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with fetal bovine serum, fungizone and antibiotic-antimicotic; the supplemented medium hereafter will be referred to as DMEM-C. Cells were incubated at 37°C and 5% CO₂. The patterned cover slips were placed in the 36 mm well of a 6 well-plate. Adipogenesis was induced by adding a differentiation induction cocktail of 0.5µM dexamethasone, 1µg/mL insulin and 0.5mM 1-methyl-3-isobutylmethylxanthene (IBMX) to DMEM-C. The
medium was changed every 1-2 days and the growth of cells was monitored daily via light microscopy.

**Results**

**Objective (A)**

**Qualitative assessment – phase contrast images**

![Differentiated adipocytes (Day 14) on collagen I substrate. Total magnification 320x](image)

Figure 4.1(a) – Differentiated adipocytes (Day 14) on collagen I substrate. Total magnification 320x
Figure 4.1(b) – Differentiated adipocytes (Day 14) on laminin substrate. Total magnification 320x

Figure 4.1(c) – Differentiated adipocytes (Day 14) on polystyrene substrate. Total magnification 320x

D1 cells grew well on all surfaces and showed fibroblastic morphologies in the early stages, before reaching confluence. Cell-to-cell contact at confluence and contact
inhibition is an essential requirement for the cells to stop dividing and start differentiating. Differentiation inducing cocktail was added on Day 2, after which the cellular morphology changed markedly to a more rounded state. No qualitative differences in the shapes of the cells grown on various substrates (collagen I, laminin and polystyrene control) were observed. Figure 4.1(a-c) shows the phase-contrast images of adipogenesis occurring on the different substrates. As can be seen from the images, cells on all the substrates accumulated lipid but the volume of lipid accumulated was dependent on the substrate.

**Lactic acid and glucose levels**

Lactic acid and glucose levels were measured to assess the metabolic activity of the cells on different surfaces; net values were reported in order to isolate the amount of lactic acid/glucose resulting from cellular activity from that measured in the unconditioned medium. In general, the amount of lactic acid released by the cells on all surfaces increased with respect to time. The amount of lactic acid released by cells on polystyrene dropped significantly (p<0.05) on Day 14 as compared to that of cells on the low density (5 µg/cm²) collagen I and laminin coatings (Figure 4.2). The lactic acid release was significantly (p<0.05) different on Day 14 for the three surfaces. On Day 15, the amount of lactic acid released by the cells on collagen I was significantly higher than that released by the cells on polystyrene control; there was no difference in the amount of lactic acid released on Day 16 by cells on any of the surfaces. Also, the mean amount of lactic acid released by the cells on low density collagen I was significantly higher than that of cells on low density laminin.
The lactic acid released by the cells on all high density (50 µg/cm²) surfaces of laminin and collagen I followed a similar pattern as described above (Figure 4.3), where the levels generally increased as a function of time. Figure 4.3 shows the significant differences in the levels of lactic acid released by the cells on different surfaces. The amount of lactic acid released by the cells on collagen I was significantly higher than those on polystyrene (p<0.05) on Day 15, and the difference between the amount of lactic acid released by the cells on collagen I and laminin approached significance (p=0.06). The amount of lactic acid released by the cells on polystyrene was significantly lower than on laminin and collagen I (p<0.05) on Day 16; again, the difference in the amount of lactic acid on collagen I and laminin approached significance (p=0.08). Also, the level of lactic acid released by the cells seeded on collagen I at the two different densities was significantly different on Days 14 and 16 (p<0.05), with high density collagen coatings displaying higher levels as compared to low density collagen coatings. No such difference was found for laminin coatings.

Statistical analysis revealed no significant differences in the amount of glucose consumed by cells seeded on collagen I or laminin at either of the densities (Figures 4.4 and 4.5). However, cells seeded on high density collagen coating consumed a significantly lower amount of glucose than those on low density coatings on Day 16 (p<0.05).
Figure 4.2 – Lactic acid levels as a function of time (day) and substrate for low density coating. Each data point represents the mean of three values. The SEM is extremely low; thus, the error bars are not visible. Asterisks (*) denote differences (p<0.05) in lactic acid level with respect to time; curves marked with similar symbols are significantly different at the designated time-point.
Figure 4.3 – Lactic acid levels as a function of time (day) and substrate for high density coating. Each data point represents the mean of three values. The SEM is extremely low; thus, the error bars are not visible. Asterisks (*) and pound signs (#) denote differences (p<0.05) in lactic acid level with respect to time; curves marked with similar symbols are significantly different at the designated time-point.

Figure 4.4 – Glucose levels as a function of time (day) and substrate for low density coating. Each data point represents the mean of three values; no significant difference in glucose levels was detected. The SEM is extremely low; thus, the error bars are not visible.
Figure 4.5 - Glucose levels as a function of time (day) and substrate for high density coating. Each data point represents the mean of three values; no significant difference in glucose levels was detected. The SEM is extremely low; thus, the error bars are not visible.

Metabolic activity

Metabolic activity of cells grown on collagen I and laminin at two different densities was assessed at 4 hours and 48 hours post-seeding; the measured values are shown in Figure 4.6. No differences were found in the metabolic activity of cells seeded on low density collagen I or laminin after 4 hours. Similarly, for the cells seeded on high density collagen I and laminin, no significant differences in metabolic activity were found. However, the metabolic activity of cells seeded on low density laminin was higher, approaching significance (p=0.07), than cells seeded on low density collagen I after 4 hours.

Figure 4.6 also shows the metabolic activity of cells grown on the different substrates 48 hours post-seeding. At this time point, cells seeded on low density laminin
showed significantly higher metabolic activity than cells seeded on low density collagen I (p<0.05). However, both cellular systems had significantly lower metabolic activity than cells seeded on polystyrene control surfaces (p<0.05). At the same time point, cells seeded on high density laminin did not exhibit significantly higher metabolic activity as compared to cells seeded on high density collagen I. However, when comparing the metabolic activity of cells grown on the same ECM component at different densities, several significant differences were noted. For example, cells grown on low density collagen I showed a significantly higher metabolic activity than cells grown on high density collagen I (p<0.05). Similarly, cells grown on low density laminin exhibited significantly higher metabolic activity than cells grown on high density laminin density (p<0.05).

**Oil Red O staining**

Cells grown on all the substrates were stained with ORO to detect intracellular lipid, representative photographs are shown in Figure 4.7. As can be seen from Figure 4.7, the amount of lipid accumulated by cells grown on low density laminin at Day 7 was significantly lower than that of cells grown on high density laminin (p<0.05). Cells grown on different densities of collagen I did not show statistically different levels of lipid accumulation. Also, cells grown on high density collagen I and on the control (PS) surfaces showed significantly lower amounts of lipid accumulation at Day 7 than cells grown on high density laminin (p<0.05). Similarly, cells grown on low density collagen I and on the control showed significantly lower amount of lipid accumulation than cells grown on low density laminin (p<0.05) on Day 7.
Cells grown on high density laminin accumulated significantly higher amounts of lipid than cells grown on low density laminin (p<0.05) over the first 14 days of culture. No differences were found between the amount of lipid accumulated by cells grown on low and high density of collagen I. Cells grown on high density collagen I and on the control (PS) showed significantly lower amount of lipid accumulation than cells grown on high density laminin (p<0.05) at Day 14. Similarly, on Day 14, cells grown on low density collagen I and on the control showed significantly lower amounts of lipid accumulation than cells grown on low density laminin (p<0.05).
Figure 4.7 – Optical density (OD) of differentiated D1 cells at 510 nm on different substrates. Accumulated lipid in the cells was stained with Oil Red O solution, extracted and its OD measured. Each data point represents the mean of three values, and error bars denote SEM (in some cases, the SEM is extremely low; thus the error bars are not visible); asterisks (*) denotes differences (p<0.05) in OD; data points marked with similar superscripts are significantly different.

Objective – (B)

Protein pattern analysis

Soluble collagen I solution was printed onto substrates to form protein pattern of controlled widths. The patterned substrates were analyzed after protein deposition using an inverted microscope in phase contrast mode and a color digital camera. Figure 4.8 shows the pattern of printed 1 mg/mL collagen solution prior to rehydration and cell seeding.
Cell pattern analysis

After cell seeding on the protein pattern, the cultures were monitored daily via light microscopy. Prior to first medium change many floating cells were detected because of the lack of adhesive area present and were rinsed away. The cells that settled on collagen adhered and proliferated till a confluent monolayer was formed. Figure 4.9 shows cells having formed a monolayer on the printed collagen pattern.
Figure 4.9: Light microscopy images of printed line collagen pattern seeded with D1 cells having a confluent monolayer

Figure 4.9 shows very few cells outside of the printed collagen pattern, and the few cells that are there have a rounded morphology indicating that they were non-viable. These results demonstrate the efficacy of printed collagen as means to control the cell size. However, since adipocytes after differentiating increase markedly in volume they tended to detach before any functional assays could be performed. Different substrates (plastic cover slips, cover slips with negative surface charge to enhance the bonding strength of collagen to it) were tried along with other chemicals (than agarose) to coat the substrate (SigmaCote) but none of the modifications increased the number of days the differentiated adipocytes remained attached.

Discussion

Qualitative assessment using phase contrast microscopy showed that cells grown on all the substrates differentiated into adipocytes; however, the level of cytoplasmic
lipid accumulation varied and was influenced by the substrate. No morphological difference was observed between cells grown on the different substrates. The results indicated that the metabolic activity of D1 cells was greatly affected by the choice of the ECM substrate and the density of the ECM protein coating, with low density laminin coating being more effective in increasing the metabolic activity of D1 cells. The density results are contrasted with those of Viravaidya and co-workers [56], the latter which showed that high density Matrigel (100 µg/cm²) and collagen moderately enhanced the proliferation of 3T3-F422A adipocytes as compared to regular Matrigel. The two obvious differences in the two studies are the different cell-types used and the use of laminin instead of Matrigel. The adult stem cells used in our study have different characteristics than the adipocytes used by Viravaidya and co-workers, and they already have acquired a unique phenotype. Matrigel, unlike laminin, contains Collagen IV and proteoglycans which may affect the proliferation of cells. The increased metabolic activity of the adult stem cells on low density laminin coating can be manipulated to provide a steady source of proliferating cells since, in vivo, preadipocytes are distributed throughout the adipose depots, alongside mature adipocytes. Although most of the studies reported in the literature have employed primary cultures of adipose tissue stromal-vascular cell (SV), it is likely that a cell-material construct which uses the patient’s own stem cells will be more likely to result in a viable implant. Hausman and co-workers [6] demonstrated that laminin preferentially enhances preadipocyte spreading and that collagen I does not have any influence on the attachment and spreading. We similarly found laminin to be much more effective in promoting the metabolic activity of D1 cells. Laminin has been shown
to decrease fibroblast adhesion and causes distinct morphological changes, depending on the cell type [57], thus suggesting that laminin has a major role in cell proliferation and should be studied in more detail.

The level of lactic acid released and glucose consumed by the cells on different substrates were also examined. The overall release and uptake profiles of lactic acid and glucose were similar for cells on all the substrates at both densities. However, in general lactic acid released by the cells on low density collagen I was significantly higher than that of cells grown under all other conditions. Since lactic acid is the product of cellular anaerobic metabolism, it can be argued that cells grown on laminin were perhaps better assimilated than cells grown on collagen. This observation is consistent with the observations of metabolic activity, which showed that cells on low density collagen I coating were less proliferative than cells on low density laminin coating. The nutrient uptake profile (in terms of glucose consumed) of the cells was not affected either by the choice of the substrate or by the coating density.

The results for Oil Red O staining showed that laminin was more effective than collagen for inducing adipogenesis; also, the effect was amplified by the high-density laminin coating. These results are consistent with those reported in the literature [6, 56], however we have found only one other study [33] which investigates the role of the effect of density of ECM coating (Matrigel, in their case) on adipogenesis. This finding is interesting in the context of adipose tissue engineering as it provides a tool to increase the amount of lipid in an adipose implant and that increases the suppleness of the adipose tissue [20].
The composite results of this study indicate that laminin is a better substrate for enhancing adipogenesis as compared to collagen I and that laminin has a similar effect on D1 cells as on S-V cells. Hausman and co-workers [6], for example, showed that laminin increases adipocyte number and size as well as the proportion of adipocytes among SV cells, while O'Connor and co-workers [20] demonstrated that Matrigel (which is a combination of laminin and collagen IV) induced higher levels of differentiation of SV cells to adipocytes as compared to collagen and fibronectin.

Another aspect of this study was the comparison of two different densities of collagen I and laminin. The results suggested that the different densities of collagen I had no impact on the differentiation of D1 cells, in contrast to previous reports where hydrated collagen gel has been shown to promote growth and differentiation of 3T3-F442A preadipocytes at a higher rate than monolayer cultures grown on polystyrene [58]. Thus, a higher concentration of collagen I or collagen gels may be needed to increase the differentiation of D1 cells. Laminin, on the other hand, was far more effective in increasing adipogenesis at the higher density. Results reported in the literature have demonstrated the enhanced ability of high densities of Matrigel coatings to increase lipid content in cells over that resulting from growth on manufacturer recommended density coatings of Matrigel [56].

Due to the contradictory results on the effect of density on proliferation and differentiation, it can be argued that, in the adipocyte scaffold optimization process, tradeoffs must be considered with respect to differentiation and proliferation. Kral and co-workers demonstrated that fibronectin greatly enhances the spreading of preadipocytes...
It has also been shown that fibronectin inhibits adipogenesis [19]. These studies further demonstrate the inverse relationship between proliferation and differentiation. Finally, since the modulation of cell-matrix rather than cell-cell contact appears to be central to adipocyte differentiation [59], and since laminin seems to be the most important ECM component with respect to adipocyte development, there is a great need for deeper, focused studies, including pre-adipocytes and other relevant primary cultures, in order to better understand this relationship.

We have also shown that modified inkjet printers can be used to seed cells in an extremely selective fashion/controlled area, potentially affecting the functionality of various cell types, especially the determination of stem cell fate, and providing a means to accomplish this assessment in a much more cost-effective and high-throughput manner. This system could be much more useful in studying cell-types that do not substantially increase in dimension after differentiation (smooth muscle cells, neurons etc.). Other methods, e.g. photolithography or microcontact printing, may provide better tools for studying the effect cell size in adipogenesis.

**Conclusions**

Under the conditions specified in this study, laminin enhances the differentiation of D1 cells to adipocytes, and is much more effective than collagen I in this regard. Laminin coating density also has an influence on adipogenesis, with the higher density tested resulting in higher levels of adipogenesis than the low density tested. No such effect was evident in the case of collagen I. Lastly, the results indicate that conditions for
proliferation are different than conditions for differentiation and that for a cell-based implant to be optimized for adipocytes a balance will be necessary.

References


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CHAPTER 5
MODULATING ADULT STEM CELL DIFFERENTIATION IN A TUNABLE 3D ADIPOSE SYSTEM

Introduction

Adult stem cells are ideal candidates for various cell-based tissue engineering applications because of their plasticity and easy access. However, their use in therapeutic applications is restricted by our limited understanding of their behavior in three-dimensional (3D) tissue constructs. This is mainly due to conventional cell-culture approaches which investigate cellular characteristics on two-dimensional (2D) substrates resulting in anomalous cellular behavior, morphology and physiology [1].

Recent studies have suggested that different tissue development when grown in 3D scaffolds compared to conventional 2D systems. Cukierman and co-workers [2, 3] have shown that adhesion complexes differed when cell were cultured on 3D substrates. This affected tyrosine phosphorylation, thereby influencing signaling and other cellular processes. Other groups have demonstrated that variations in cell spatial organization within a matrix influence both their proliferation and function [4, 5]. This underscores the complexity of the interplay between a particular cell, its neighbors and the extracellular matrix (ECM) environment [6]. Undifferentiated stem cells secrete several ECM proteins [7] as well as various cytokines [8] and thus play an important role in regulating their specific microenvironment. Understanding the development of this ECM environment in 3D systems and its influence on the adult stem cell proliferation and differentiation is extremely important in developing engineered tissue constructs.
It has been reported that a change in the environment from 2D to 3D causes marked changes in the phenotype and functional behavior of cells. For example, in a study involving osteoblasts it has been shown that static and dynamic mechanical stresses, in concert with 3-D collagen matrices, played a significant role on the phenotypic modulation of osteoblast-like cells [9]. A better understanding of the mechanism of adipose tissue differentiation is of paramount importance in the development of therapeutic strategies for the treatment and prevention of obesity and type 2 diabetes mellitus. Optimal results using tissue culture models can be expected only when the *in vitro* adipocyte closely resembles adipose tissue *in vivo*. Several functional aspects of adipocytes change in three dimension as compared to a two dimensional environment. For instance, it has been shown that the emergence of glycerophosphate dehydrogenase activity is delayed in 3D scaffolds when compared to cells seeded in 2D environment [10]. Kang and co-workers found that adipocytes matured faster on 3-D constructs and even before complete differentiation, secreted leptin at levels that were different that of fully differentiated adipocytes in 2-D conventional cell cultures [11]. In an *in vivo* study involving preadipocytes isolated from human fat tissue which were suspended with the gelatin microspheres containing bFGF and incorporated into a collagen sponge of cell scaffold and then transplanted subcutaneously in the back of a nude mice, the authors found that the combination of gelatin microspheres containing bFGF and preadipocytes with the collagen sponge was essential to achieve tissue engineering of fat tissue [12]. These findings highlight the importance of 3D scaffold changing the dynamics and effectiveness of adipocyte differentiation.
Our long-term hypothesis is that by controlling the level of cellular differentiation
in vitro we can affect the viability of a tissue-engineered device in vivo. Consequently,
we believe that the development of 3D in vitro systems in which the level of cellular
differentiation can be modulated will be of utmost importance in developing clinically
viable cell-material constructs. Adipogenesis involves the differentiation of preadipocytes
into mature adipocytes and the sequence of events include determination of preadipocyte
cell fate, growth arrest at confluence, clonal expansion and terminal differentiation [13-
15]. Preadipocytes are distributed throughout the adipose depots, alongside mature
adipocytes and changes in their numbers in adipose tissue are largely achieved through a
complex interplay between proliferation and differentiation. This lends support to our
hypothesis that the control of the level of cellular differentiation and the presence of
adequate number of undifferentiated, proliferating cells is one of the most important
factors in the development of cell-based devices. Understanding this process is also
important in assessing the role of adipocytes in metabolic diseases.

The current in vitro models of adipogenesis are limited in nature because, in many
instances, cells fail to express adipocyte-specific genes [16]; moreover planar 2D
monolayer does not represent the complex 3D architecture of adipose tissue in vivo. Fat is
organized into a 3D tissue composed of preadipocytes and differentiated adipocytes,
interstitial cells, and a microvascular system entwined within a highly organized ECM
consisting of various ECM proteins [17]. Results that can be compared to the in vivo
setting can be expected only when the in vitro fat cells resemble adipose tissue as closely
as possible. Advances in tissue engineering have demonstrated the feasibility of using 3D
scaffolds to direct cell differentiation and organization the approximate *in vivo* architecture and function [18]. Bissell and co-workers [19] and other groups [2] have demonstrated that biological function can be profoundly governed by 3D geometry.

Adipose tissue engineering is a relatively new area focused mainly on breast reconstruction and soft tissue defects. But, in addition to this an *in vitro* 3D model of fat could be instrumental in assembling a novel test system for obesity and type 2 diabetes research. It has already been speculated that the greatest impact of tissue engineering will be the design of *in vitro* physiological models or test systems to study disease pathogenesis and to develop molecular therapeutics [20].

We have previously shown that scaffold microtexture and various ECM proteins can be used to control the level of cellular differentiation. The purpose of this study was to examine adhesion, proliferation and differentiation of D1 cells (multipotent, mouse bone marrow stromal precursor cells) in a 3D environment and, to evaluate if we can modulate the differentiation of D1 cells on natural and synthetic 3D scaffolds. We chose polylactide beads as scaffolds to assess synthetic polymers as polylactide is one of the most commonly used and best characterized synthetic polymer and also to maintain a level of consistency as we have used the same polymer in previous studies. We also used commercially available collagen microcarriers in this study as collagen is one of the most ubiquitous ECM proteins and has been used in a variety of tissue engineering applications; also, it is also known to be implicated in adipogenesis. Polylactide beads have previously been used in our lab for other applications and the results have suggested that the cellular attachment to these beads was not too strong and that the cells tended to
peel-off the bead surface in a relatively short amount of time. To overcome this problem we decided to coat the bead surface with laminin with the idea being that laminin would enhance the cell attachment on the beads as it mediates adhesion events that are critical to a number of biological mechanisms [21, 22]. There is a lot of indirect evidence that suggests that laminin is a key ECM protein involved in preadipocyte cell function [23, 24]. It has also been shown that rat preadipocytes can be recruited \textit{in vivo} to microenvironments consisting of Matrigel and basic fibroblast growth factor (bFGF) leading to \textit{de novo} adipogenesis [25, 26]. Furthermore, immunocytochemical studies indicate that laminin is present around small differentiating adipocytes in fetuses [27]. The cumulative indirect evidence alludes to the role of laminin in the differentiation of adipocytes.

In addition, adsorption of serum proteins present in the culture medium is the first that occurs when cells are seeded on a material, and the adsorbed protein layer influences cell adhesion, spreading and proliferation [28]. As a result, coating of surface with cell adhesion mediators like laminin or fibronectin or other ECM components is becoming a regular practice to enhance cell attachment [29]. There is also very little known about how the adsorbed protein layer affects the cell phenotype.

To summarize, the objective of this study was to evaluate the behavior of D1 cells in terms of adhesion, proliferation and differentiation when cultured on 3D laminin adsorbed-polylactide beads and collagen microcarriers and to assess if polymer scaffolds (natural and synthetic) can modulate the level of differentiation of D1 cells to adipocytes.
Materials and Methods

Polylactide Beads

Polylactide beads were prepared by a single emulsion process. Four grams of PLLA mesopolymer pellets (Cargill; Minneapolis, MN) was dissolved overnight in 20 mL dichloromethane (Mallinckrodt; Phillipsburg, NJ) to produce a 20% (w/v) PLLA solution. Two additional working solutions were made one day prior to forming the beads. Three grams polyvinyl alcohol (PVA) (Aldrich Chemical; Milwaukee, WI) with a M\text{w} range of 13 to 23 kDa was dissolved in 1 L distilled water to make a 0.3% (w/v) PVA solution; the PVA solution acts as an emulsifier in the process. A 2% (v/v) isopropanol solution was made by mixing 60 mL isopropanol (VWR; West Chester, PA) in 2.94 L distilled water; the isopropanol solution extracts the solvents from the beads, allowing them to harden.

A cylindrical, 10 L glass tank was elevated in a chemical hood to protect a bottom mounted drain valve. The drain valve opening in the tank was covered with a brass mesh screen (Paragona; Sweden) to prevent bead loss during solution removal. Tygon® tubing (Saint-Gobain Performance Plastics; Akron, OH) was used to transfer waste solution to a drain. The entire PVA solution was pored into the tank, and an additional 5 L of distilled water was added to create 6 L of 0.05% (v/v) PVA solution. A stainless steel paddle stirrer attached to a EUROSTAR Power Control-Visc electronic overhead stirrer (both from IKA Works; Wilmington, NC) was positioned in the center of the tank at a depth of approximately 2 inches from the bottom. The PVA solution was stirred at 200 rpm for 5
minutes to ensure thorough mixing, and the speed was increased to 275 rpm for bead processing.

The PLLA solution was poured quickly into a 20 mL glass syringe (Popper & Sons; New Hyde Park, NY) equipped with a 1.5 inch, 16-gauge needle (Becton Dickinson; Franklin Lakes, NJ). After inserting the plunger, slight pressure was maintained, and the syringe was inverted to remove air bubbles. The needle was submerged at 45° angle near the vortex of the PVA solution, and gentle pressure was applied to the syringe to inject the PLLA solution. Beads of PLLA formed rapidly, and they were stirred for 10 minutes to obtain a desirable size of approximately 1 mm. After stirring was stopped, the beads settled to the outer edge of the tank. Approximately 2/3 of the PVA solution was drained from the tank, and 2 L of the 2% isopropanol solution was added to the tank. The solution was stirred again at 200 rpm for 5 minutes. Stirring was stopped, and all but approximately 1 L of the solution was drained from the tank.

The beads were transferred to a 1 L Pyrex® glass bottle (Corning; Corning, NY) using a vacuum pump and Tygon® tubing. After aspirating the remaining solution, 200 mL of fresh isopropanol was added to the bottle. The bottle was placed in on an orbital shaker (IKA Works; Wilmington, NC) overnight at 150 rpm. The PLLA beads were dried the next day by transferring the beads and solution to a Buchner filter funnel apparatus (Fisher Scientific; Hampton, NH) lined with a cellulose filter paper (Whatman; Florham Park, NJ). The apparatus was placed in a 500 mL Erlenmeyer flask (Chemglass; Vineland, NJ) attached to house air at low pressure. The PLLA beads dried in 2 hours and they were sieved using a Cellector® Tissue Sieve System (PGC Scientifics; Frederick,
MD). Sieves with 10- and 30-mesh screens were used to collect beads with particle sizes in the range of 0.52 – 1.91 mm. The PLLA beads were stored in glass vials under vacuum (500mm Hg).

**Collagen Microcarriers**

Collagen microcarriers (Cultispher-S) (HyClone Laboratories; Logan, UT) were also used in the study. These collagen microspheres were rehydrated in calcium- and magnesium-free phosphate buffered saline (PBS) in the ration of 50 mL of PBS for 1 gm of the collagen microspheres for at least one hour and the beads were allowed to settle down. They were then autoclaved and the PBS was removed as much as possible. Fresh medium was finally added and the beads were stored at 4°C until further use.

**Cell Seeding and Culture Conditions**

The polylactide beads to be used in the study were sterilized at room temperature using a 12-hour ethylene oxide (EtO) infiltration cycle in an AN74i sterilization chamber (Anderson Products, Haw River, NC). Degassing was performed automatically in the chamber for two hours. Following removal of samples, degassing was performed for an additional 48 hours under vacuum (500 mm Hg) in a desiccator. Laminin (BD Biosciences; San Jose, CA) was adsorbed onto PLLA beads to evaluate if laminin adsorption on the polylactide beads affected cellular proliferation and differentiation. Specifically, 2.5 gm of PLLA beads were soaked in 50 μg/mL of laminin overnight at room temperature under sterile conditions. Control polylactide beads were
soaked in Dulbecco’s Modified Eagle Medium (DMEM; ATCC; Manassas, Va) supplemented with 10% fetal bovine serum (Mediatech; Herndon, Va), fungizone (Invitrogen; Carlsbad, CA) and antibiotic-antimicotic (Invitrogen; Carlsbad, CA); the supplemented medium will be referred to as DMEM-C.

All three substrate types were transferred to 24-well ultra low attachment plates (Corning) on the day of cell seeding, using 2 mL previously modified and EtO sterilized disposable pipets. The low attachment well plates contain a proprietary hydrogel coating that inhibits cell attachment to the culture chamber. A total of 0.1 mL of each substrate was transferred to each well with the modified pipets. After all the substrates were distributed, the spent medium was aspirated and fresh medium was added to each well. Each well was seeded with D1 cells (ATCC; Manassas, Va) of passage 28, at a density of $3 \times 10^5$ cells/well on Day 0. The cells were maintained in DMEM-C supplemented with adipogenic supplements, consisting of 0.5µM dexamethasone (Sigma; St. Louis, MO), 1µg/mL insulin (Sigma; St. Louis, MO), and 0.5mM 1-methyl-3-isobutylmethylxanthene (IBMX; Sigma; St. Louis, MO). The cellular systems were incubated at 37°C and 5% CO$_2$ and maintained on an orbital shaker (IKA® Works) at 50 rpm.

Six 12-well plates were used in the study - one for metabolic activity and cell number assays (Days 3 and 4, respectively), one for Live/Dead assay (Day 4), two for triglyceride accumulation assay (Days 7 and 16), one for oil red O staining assay (Day 16), and one for Revere Transcription – Polymerase Chain Reaction (RT-PCR) analysis (Day 16). Each well plate contained six samples, one per well, including three collagen
microspheres (Collagen) and three laminin-adsorbed PLLA beads (Laminin). Three additional wells per plate housed polylactide bead (PLLA) control groups.

**Metabolic activity**

Metabolic activity of cells on the three groups was measured using an alamarBlue (Biosource; Camarillo, CA) assay. Unlike other assays (for example, CellTiter 96® assay which uses MTS reagent and which is an end-point assay), alamarBlue dye is minimally toxic to cells; thus, it can be used to assess the same population of cells at different time-points. Reduction of the alamarBlue dye from a non-fluorescent blue to a fluorescent red occurs by the uptake of the dye in metabolically active cells. Values of the metabolic activity may vary due to the number of cells present in different phases of cell cycle. Furthermore, only relative comparisons of metabolic activity are useful with this assay, since 2-D surfaces used to normalize activity to cell number do not allow perfect extrapolation to 3-D systems, where cellular metabolic activity may be vastly different.

The metabolic activity assay was performed on Day 3. Following the addition of 1.5mL of fresh DMEM-C to each well, 150µL of the alamarBlue reagent was added to each well. The assay plates were placed in the incubator for a period of 2 hours. After incubation, 100µL of the samples was transferred in triplicate to a 96-well plate. Fluorescence values were immediately obtained using a fluorometric plate reader (GMI; Ramsey, MN) with an excitation filter of 544nm and an emission filter of 590nm.
**PicoGreen® Assay**

PicoGreen® dsDNA Quantitation kit (Molecular Probes; Eugene, OR) is a fluorescent nucleic acid stain used to measure double stranded deoxyribose nucleic acid (DNA) in solution. A volume of 100 µL (1X) of Triton-X-100 (Fisher) was added to each sample. The samples were subjected to repeated (at least 3 times) freeze/thaw cycles (-20°C to 37°C water bath). PicoGreen working reagent and 1X TE reagent were prepared according to manufacturer instructions. A volume of 100 µL of PicoGreen working reagent was added to each sample, and 200 µL of the sample was then transferred to a black Costar 96® well plate (Corning). The samples were incubated for 5 minutes at room temperature, and the fluorescence was then read on the Fluoroskan Ascent FL (Thermo Electron Corporation, Milford, MA) with an excitation filter of 480 nm and an emission filter of 520 nm. Again, due to the normalization issue, only relative comparisons are meaningful.

**Cell Viability**

Cell viability was observed on Day 4 using a LIVE/DEAD® Viability/Cytotoxicity kit (Molecular Probes; Eugene, OR). The cells were fluorescently labeled using the calcein AM and ethidium homodimer dyes in the kit according to the manufacturer instructions. Live cells retain a dye that produces bright green fluorescence (excitation and emission of 495 nm and 515 nm, respectively), while dead cells fluoresce a bright red (excitation and emission of 495 nm and 635 nm, respectively). Observations
were made by using an inverted microscope (Carl Zeiss; Thornwood, NY) in phase contrast mode and a color digital camera (Diagnostic Instruments; Sterling Heights, MI).

**Oil Red O staining**

Intracellular lipid accumulation was measured using Oil Red O (ORO) analyses. This assay was performed on Day 16. ORO solution was made as described previously [30]. Briefly, a stock solution was made by dissolving 0.5gms of the ORO powder in 100mL of isopropanol, then filtering the solution. To prepare the working solution, 6mL of stock solution was mixed with 4mL of distilled water, left for 1 hour at room temperature, and then filtered prior to use. Cell cultures were washed twice with phosphate buffered saline (PBS) and fixed with 10% neutral formalin for at least 1 hour at room temperature. The cells were washed with water twice before staining for 2 hours with the ORO working solution, and then washed exhaustively with water. Excess water was evaporated by incubating the stained cultures at 37°C. The dye was then extracted with 500µL isopropyl alcohol per well by gentle pipetting, and the absorbance was measured immediately at 510nm.

**Triglyceride Determination Assay**

This assay was performed on Days 7 and 16. The total triglyceride concentration was determined using a differentiation assay protocol (Zen-Bio, Inc; Research Triangle Park, NC). The scaffolds were washed with 1 mL of PBS, the PBS was aspirated, and the cells were lysed using 1 mL of a 0.5% solution of Triton-X-100 (Fisher). The cells were
then incubated at room temperature for 30 minutes. Aliquots of 80 µL of PBS and 20 µL of lysate were pipetted into 96-well plate wells in triplicate. Next, 100 µL of Infinity® Triglyceride Reagent (Thermo Electron Corp; Melbourne, Australia) was added to each well. The plates were incubated for another 15 minutes at room temperature. The absorbance was read at 490 nm (Dynex Technologies, Chantilly, Va). Again, only relative comparisons of the results are considered meaningful.

**Gene Expression**

RT-PCR was performed on Day 16 to determine the expression of various adipocyte-specific genes. The primers selected for use were mouse cadherin 11 [31], PPAR γ [32] and aP2 [33]. Mouse beta-actin (β-actin) was used as the control gene [34].

**Ribonucleic Acid (RNA) Isolation**

Following manufacturer protocol, RNA was isolated from the cells on Day 16 using RNeasy® Mini Kit (QIAGEN; Valencia, CA). Cell membranes were lysed by adding 600 µL of Buffer RLT to each well; cells were not released from the PLLA beads prior to membrane disruption. Each sample was homogenized by pipetting the lysate onto a QIAshredder spin column in a 2 mL collection tube; the tubes were centrifuged at 13,000 rpm for 2 minutes. Subsequently, 600 µL of 70 % ethanol was added to each homogenized lysate aliquot, and the solution was triturated thoroughly. A volume of 600 µL of the sample solution was added to the RNeasy mini column in a new 2 mL collection tube, and the mixture was centrifuged at 13,000 rpm for 2 minutes. The
balance of the sample solution (600 µL) was loaded onto the same column, and the centrifugation repeated. The flow-through was discarded after centrifuging each time, leaving the sample RNA on the column membrane. Volumes of 700 µL Buffer RW1 and 500 µL of Buffer RPE were then added to the column in separate steps, and the tubes were centrifuged at 13,000 rpm for 15 seconds to wash the RNA on the column; the flow-through was discarded after each step, and a new 2 mL collection tube was used to dry the column membrane with Buffer RPE. A volume of 500 µL of Buffer RPE was added to the column, and the tube was centrifuged at 13,000 rpm for 2 minutes. RNA solutions of 100 µL were obtained eluting the RNA through the column using two successive steps. For each step, 50 µL of RNase free water was added to the RNeasy column in a 1.5 mL tube, and the tube was centrifuged at 13,000 rpm for 1 minute. All samples were stored at -80°C.

**RNA Analysis**

The purity and concentration of the isolated RNA was analyzed using the RNA 600 Nabo Assay Kit (Agilent Technologies, Inc; San Jose, CA), RNA 6000 ladder (Ambion, Inc; Austin, TX), RNA 6000 Nano LabChip® (Caliber Technologies Corp; Mountain View, CA), Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.), and 2100 Expert Software (Agilent Technologies, Inc.). The software calculates the RNA concentration and the ribosomal RNA ration (28s:18s) in each sample. The isolated RNA was stored at -80°C until RT-PCR was performed.
**RT-PCR**

Each 50 µL RT-PCR was formulated according to manufacturer instructions and consisted of 10 µL of 5X PCR Buffer, 2 µL of 10 mM dNTP solution, 2 µL of Qiagen Enzyme Solution, 1 µg of RNA and varying amounts of RNase free water. A sense primer (IDT) and corresponding antisense primer (IDT) were added to each solution at a concentration of 0.5 µM. Reverse transcription and the polymerase chain reaction sequences were completed using a Mastercycler gradient thermocycler.

**Gel Electrophoresis**

Agarose gels of 2% were made by mixing 2 gm of agarose (Sigma) with 100 mL of 1X Tris-borate EDTA (TBE) buffer. Ethidium bromide was used to stain the gel at a concentration of 0.5 µg/mL. The agarose solution was poured into an electrophoresis tray and allowed to cool for an hour. The tray was placed in the electrophoresis chamber and covered with 1X TBE.

A volume of 3 µL of 6X loading buffer was added to each cDNA sample. The cDNA samples and a 1 kilobase DNA ladder (Promega; Madison, WI) were loaded into separate lanes in the agarose gel. Gel electrophoresis was conducted for 2 hours at 5 volts per centimeter (Model 250, Life Technologies, Inc; Rockville, MD). The gel was then analyzed using UV light (Fotodyne Inc; New Berlin, WI). Pictures were captured using a GelCam (DS 34 Polaroid Corporation, Boston, MA).
Statistical Analysis

All statistical analyses were performed using SAS 9.1 (SAS Institute Inc; Cary, NC). The significance level used was $\alpha = 0.05$.

Results

Metabolic Activity

Metabolic activity was evaluated using an alamarBlue assay for the cells on each of the scaffold types (laminin-coated PLLA beads, collagen beads and PLLA beads) on Day 3 after cell seeding. Statistical analysis revealed that cells on collagen microspheres had significantly higher metabolic activity than the other two groups ($p<0.005$). There was no significant difference in the metabolic activity of cells on laminin-coated PLLA beads and control PLLA beads. The results are shown in Figure 5.1.

Figure 5.1: Metabolic activity of D1 cells on different substrates. Each data point represents the mean of three values, and error bars denote standard error of mean (SEM); the asterisk (*) denotes a statistical difference ($p<0.05$) in metabolic activity
**PicoGreen Assay**

PicoGreen measures the number of viable cells present by measuring cells with intact double-stranded DNA. This assay was performed on Day 4 (Figure 5.2) for cells on all the scaffolds. Statistical analysis revealed that both collagen microcarriers and PLLA beads coated with laminin had significantly higher number of cells than control PLLA beads (p<0.05). There was no significant difference between the number of cells present on collagen microcarriers and those on PLLA beads coated with laminin.

![Figure 5.2](image_url)

Figure 5.2: Relative cell number for cells on the three scaffold-types. Each data point represents the mean of three values, and error bars denote SEM; the letter denotes significantly different value (p<0.05)
Cell Viability Assay

Live/Dead assay was conducted to qualitatively evaluate the cell viability on the three scaffolds. Figure 5.3 (a-c) shows that the cells were viable on all three scaffold types on Day 4.

Figure 5.3(a): Day 4 fluorescence micrograph showing cells attached on collagen microcarriers, total magnification: 100x

Figure 5.3(b): Day 4 fluorescence micrograph showing cells attached on laminin-coated PLLA beads, total magnification: 100x
Figure 5.3(c): Day 4 fluorescence micrograph showing cells attached on PLLA beads, total magnification: 100x

Qualitatively, it can be seen that, although the cells are viable on all the scaffolds, the cell density is decidedly higher for cells on collagen microspheres, followed by those on laminin-adsorbed PLLA beads with the lowest density on the control polylactide beads.

**Triglyceride Analysis**

As the cells differentiate to adipocytes, they accumulate triglyceride. The amount of triglyceride accumulated by the cells was measured for all the scaffolds and the results are shown in Figure 5.4. This was conducted at two time-points (Days 7 and 16). Cells on collagen microcarriers accumulated significantly higher amounts of triglyceride than cells on laminin-coated PLLA beads and control PLLA beads (p<0.05) on Day 7. No difference was noted between the amount of triglyceride accumulated by the cells on laminin-coated PLLA beads and those on control PLLA beads.
Cells on collagen microcarriers accumulated significantly higher amount of triglyceride than cells on laminin-coated PLLA beads and control PLLA beads (p<0.05) on Day 16. At this time-point cells on laminin-coated PLLA beads also had a significantly higher amount of triglyceride than cells on control PLLA beads (p<0.05).

**Oil Red O Staining**

Intracellular lipid of cells grown on all the substrates was stained on Day 16 with ORO, and the optical density of the dye absorbed by the cells was measured (Figure 5.5). Statistical analysis revealed that cells on collagen microcarriers accumulated significantly higher amount of lipid than cells on laminin-coated PLLA beads and control PLLA beads (p<0.05). Also, cells on laminin-coated PLLA beads had significantly higher amount of lipid than cells on control PLLA beads (p<0.05).

![Figure 5.4: Triglyceride analysis of differentiated D1 cells on the different scaffolds. Each data point represents the mean of three values, and error bars denote SEM; within each time-point, the group with letters are significantly (p<0.05) different](image-url)
Figure 5.5: Optical density (OD) of differentiated D1 cells at 510 nm on different substrates. Accumulated cellular lipid was stained with Oil Red O solution, extracted and its OD measured. Each data point represents the mean of three values, and error bars denote SEM; bars with letters are significantly (p<0.05) different.

**RT-PCR**

With the objective of investigating the differential expression of key adipocyte genes on the different scaffolds, RT-PCR was conducted on cells from all the groups. As can be seen from Figure 5.6, cells seeded onto collagen microcarriers showed enhanced band intensities for both the genes expressed. The next greatest intensity levels were associated with the cells on the laminin-coated PLLA beads; the cells on control PLLA beads showed a very faint band for PPARγ.
Figure 5.6: RT-PCR evaluation of differential adipose gene expression in 3-D environment for cells on laminin-coated PLLA beads (Laminin), control PLLA beads (PLLA) and collagen microcarriers (Collagen) on Day 16. Evaluation of characteristic fat genes aP2, PPARγ and internal standard β-actin was repeated in triplicate; one representative result is shown in this Figure.

Discussion

Although much progress has been made in adipose tissue engineering in recent years, the reconstruction of viable adipose tissue is still extremely challenging and not very successful. One of the primary reasons for the remaining challenges is the lack of proper understanding of the behavior of adipocytes in 3-D environments. The molecular biology of preadipocyte differentiation is characterized very well in 2-D systems through obesity and diabetes research [35, 36]; however the behavior of mature adipocytes in 3-D systems is still poorly understood. Scaffold material selection is one of the most important aspects of a successful tissue-engineered construct, but there is no clear indication about the efficacy of a specific scaffold material in adipose tissue engineering. In addition to providing reconstruction options, another aspect of tissue engineering that is extremely important is the development of in vitro engineered test-systems to study
developmental aspects of adipogenesis with great control and to provide a platform for pathologic evaluation and drug discovery.

Thus, the aim of this study was to evaluate the response of adipocytes on collagen microcarriers and laminin-adsorbed PLLA beads and to investigate if the differentiation of the cells could be tuned by the choice of scaffold thereby aiding in the development of an in vitro test system with which to study adipogenesis. We have previously shown that scaffold microtexture and various ECM proteins can be used to control the level of cellular differentiation in a 3-D cellular scaffold. The purpose of the current study was to examine adhesion, proliferation and differentiation of D1 cells (multipotent, mouse bone marrow stromal precursor cells) in a 3-D environment and, to evaluate if one might modulate the differentiation of D1 cells on natural and synthetic 3-D scaffolds. Polylactide beads were selected to study as polylactide is one of the most commonly used and best characterized synthetic absorbable polymers clinical application. We also used commercially available collagen microcarriers in this study; since, collagen is one of the most ubiquitous ECM proteins, has been used in a variety of tissue engineering applications, and is known to be implicated in adipogenesis. Polylactide beads have previously been used in our lab for other applications; cellular attachment is low and cells tend to detach from the bead surfaces in a relatively short amount of time in vitro. To overcome these issues and enhance cell attachment to the beads, we coated the bead surfaces with laminin, a known mediator of adhesion events. We used D1 cells in the study since they will, under controlled stimuli, differentiate into adipocytes. Despite the fact that these cells are derived from a cell line and therefore may not actually reflect the
true \textit{in vivo} characteristics they serve as a homogeneous, proliferative cell source and avoid the major drawbacks of primary cells such as cellular heterogeneity, low proliferation rates, and donor-dependent differentiation capacity [37]. The D1s exhibit similar features, such as lipid accumulation, gene expression, and functionality, as their \textit{in vivo} counterparts.

To evaluate cellular proliferation, we examined relative cell number and relative metabolic activity indices. Our results indicate that cells on collagen microspheres were significantly more metabolically active than the cells on the other scaffolds, which is not surprising, as collagen is a natural protein. Previous studies in our lab showed that when preadipocytes are seeded onto PLLA beads, they detach in a relative short period of time (i.e., a few days). To prevent this scenario, we coated the PLLA beads with laminin. We chose laminin because it is one of the ECM glycoproteins that mediates adhesion events that are critical to a number of biological mechanisms [21, 22]. Indirect evidence also suggests that laminin is a key ECM protein involved in preadipocyte cell function. For instance, Hausman and co-workers demonstrated that preadipocyte attachment and spreading in porcine stromal-vascular (SV) cultures is enhanced on laminin substrata [23]. Laminin is also known for its role in embryogenesis and has been implicated in cell survival, proliferation and induction [38]. We have previously shown that laminin is a profound regulator of adipogenesis in 2-D systems [39]. The results of the current study indicate that there was a significantly higher amount of intact dsDNA in the cellular laminin-coated PLLA bead systems than in the cellular control beads, which, in turn, suggests a higher cell number. This result is in general agreement with the results
presented by Kral and co-workers [40], who found that coating a synthetic polymer with ECM components enhanced preadipocyte spreading. The process of cell adhesion (governed by integrins and adaptor proteins) also regulates tissue morphogenesis. It has been shown that [2] the molecular composition of adhesion structures is affected by the composition, three-dimensionality and pliability of the substrates. Our results agree with this observation as we found different degree of cell adhesion on the three different substrates tested.

Adipocyte differentiation was also assessed by Oil Red O staining and triglyceride accumulation measurements. Triglyceride analysis showed that collagen microcarriers induced the highest amount differentiation. This result is in agreement with other published results [41] which have shown that collagen functions as an adequate carrier for adipose tissue engineering. We also found that by Day 16, cells on laminin-coated PLLA accumulated significantly higher amount of triglyceride than the cells on the control PLLA samples. This result could either indicate that the adsorbed laminin on the bead surface plays a direct role in the differentiation enhancement of D1 cells to adipocytes or it could also be due to the fact that the adsorbed laminin increases the number of cells attached to the bead surface for an extended time and thereby resulting in increased triglyceride content. Further work needs to be done to firmly clarify this point.

In either case, the observation that an ECM coating on a 3-D scaffold can enhance differentiation is important as it provides a tool with which to control the level of cellular differentiation in a cell-based device. Cell-ECM interactions within in a 3-D matrix have been generally known to influence tissue properties. Specifically, ECM components have
been shown to play a regulatory role in adipogenesis by facilitating morphological changes [37, 42] and their synthesis is required for terminal differentiation [43]. More importantly, function and relation within different ECM components in the adipose system are still not understood [37]. The Oil Red O staining results supported the triglyceride analysis; the cells on collagen microcarriers accumulated significantly higher amounts of lipid than the cells on the other two groups, and the cells on laminin-coated PLLA beads had higher accumulated levels than the cells on the control PLLA beads.

Gene expression results from our study concurred with Oil Red O staining and triglyceride analysis results, and indicated that collagen microspheres as a scaffold provide the highest amount of differentiated cells and that surface modification of PLLA beads leads to an increase in differentiation attenuation when compared to control PLLA beads.

A successful implant will likely need to have a population of proliferating cells and cells at various levels of differentiation. An in vitro 3-D system in which the level of cellular differentiation can be controlled will provide a useful tool toward this end. One important tissue engineering application is the development of a tunable test system for understanding adipogenesis and the various pathologies associated with it. With further investigation, the 3-D system described in this study can also be used to study the endocrine functionality of adipocytes as it has been reported factors responsible for protein expression are absent in 2-D systems. For example, the levels of TNF α and leptin found in vivo far exceed the levels that are seen 2-D systems in vitro [44, 45]. The system
described in this study can also be used to study the cell-ECM interactions and cell-cell interactions by co-culturing adipocytes with other cells types found in the tissue.

We believe that our studies give us insight toward the development of such tissue systems, by presenting us with options to control both the level of cellular differentiation and proliferation in a 3-D environment. The resulting intentional scaffold design will provide optimal environments for heterogeneous populations of cells to develop into complex tissue structures.

Conclusions

Three-dimensional scaffold design and development plays an important role in tissue engineering. We evaluated proliferation and differentiation of adult stem cells on three different 3-D scaffolds. Our results indicate that collagen microspheres provide the highest rate of cellular differentiation among the scaffolds tested. We also found that coating the surface of polylactide beads with laminin enhanced cell adhesion to the surface as compared to control polylactide beads and also led to higher differentiation levels. Finally, the results of this study will help in the development of a tunable in vitro 3-D test system which can be used to study adipogenesis and its related pathology in a controlled environment.

References


CONCLUSIONS

Regarding the role of surface texture in modulating the differentiation of adult stem cells in an adipose system,

- Defined microtexture on a polymer scaffold can be used as a tool to modulate the differentiation;
- Polynomial models can be used to predict the metabolic activity as a function of time;
- The microtextured scaffold starts affecting the rate of differentiation from day 6 onwards after the addition of the hormonal cocktail; and
- The amount of leptin secreted by the differentiated adipocytes is not affected by the microtextured surface.

Regarding the role of ECM components to modulate the differentiation of adult stem cells in adipose system,

- Laminin enhances the differentiation of adipocytes more than collagen I;
- Laminin coating density also has an influence on its ability to promote adipogenesis;
- An increase in the coating density of collagen did not affect its ability to promote adipogenesis; and
- Conditions for proliferation are different than conditions for differentiation.

Regarding the use of modified inkjet printers to evaluate the role of cell size on the differentiation of adult stem cells in adipose system,

- Modified inkjet printers can be used to print adhesive areas of controlled width surrounded by non-adhesive areas;
- Different models of modified inkjet printers can be used to print collagen strips of varying widths; and
The printed collagen is not bound strongly enough to the glass cover slip to prevent cells from detaching before the differentiation is attenuated and can be studied in detail.

Regarding the role of 3D scaffolds to modulate the differentiation of adult stem cells in an adipose system,

- Cells attach to all scaffolds evaluated (collagen microspheres, laminin-coated PLLA beads and control PLLA beads);
- Collagen microspheres provide the highest amount of differentiated cells among the scaffolds tested; and
- Coating the surface of polylactide beads with laminin enhanced cell adhesion to the surface as compared to control polylactide beads and also led to higher differentiation levels.
RECOMMENDATIONS

- One set of groove dimensions was assessed to better understand the role of groove dimension in modulating differentiation. Additional experiments should be conducted with grooves of varying dimensions.

- A hormonal cocktail was employed while assessing the role of surface microtexture in the differentiation process. More work should be completed without the cocktail to investigate how microtexture affects the differentiation in the absence of the hormonal cocktail.

- Scaffolds with different surface roughnesses should be examined in additional studies.

- Additional studies with different densities of laminin and collagen should be conducted to determine if a “saturation” point exists, beyond which the increase in density does not enhance adipogenesis.

- The composition of the lipid should be thoroughly analyzed using Mass Spectroscopy to determine if there is a compositional change in the accumulated lipid due to the interaction of the adipocytes with a particular ECM protein.

- Other ECM proteins should be examined to see how they affect adipogenesis.

- HEPES buffer should be incorporated in the culture medium to reduce the high extracellular pH levels when working with the ECM components.

- Select other ECM components must be employed to print a controlled line width as collagen I does not bind strongly enough to a glass substrate.
• Different substrates must be examined to see if they may provide a better bond strength with the printed ECM components to support the fully differentiated adipocytes.

• Alternate means beyond modified inkjet printing, e.g. photolithography, should be employed to create controlled adhesive areas and study the role of cell size in affecting differentiation.

• Complete surface characterization should be performed on the polylactide beads.

• More cell proliferation assays should be performed to better understand the relationship between proliferation and differentiation.

• After extensive in vitro studies have been performed, in vivo animal studies should be conducted to evaluate scaffold performance.