Investigation of Dynamic Culture on Matrix-derived Microcarriers as a Strategy to Modulate the Pro-Regenerative Phenotype of Human Adipose-derived Stromal Cells

McKenna R. Tosh, The University of Western Ontario

Supervisor: Flynn, Lauren E., The University of Western Ontario

A thesis submitted in partial fulfillment of the requirements for the Master of Engineering Science degree in Biomedical Engineering

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Abstract

Pre-conditioning of adipose-derived stromal cells (ASCs) by tuning the cellular microenvironment during expansion has the potential to modulate their pro-regenerative functionality. The current study investigated the effects of microcarrier composition (decellularized adipose tissue versus collagen), oxygen tension (2% versus ~20% O₂) and stirring rate (static, 20, 40 rpm) on human ASCs cultured within spinner flask bioreactors. Dynamic culturing under 20% O₂ resulted in more consistent cell growth on both microcarrier substrates, leading to increases in microcarrier contraction and stiffness. Culturing on the microcarriers modulated the hASC immunophenotype, with varying CD90 and CD26 expression levels observed under the different culture conditions. Interestingly, the gene expression levels of a range of pro-angiogenic and immunomodulatory factors were enhanced in the hASCs cultured on the collagen microcarriers at 20 rpm and 2% O₂, supporting that the conditions within stirred bioreactors can be tuned to pre-condition hASCs for applications in vascular regeneration.

Keywords

Adipose-derived stromal cells; Pre-conditioning; Bioreactors; Decellularized adipose tissue; Collagen; Hypoxia; Dynamic culture; Microcarriers; Microenvironment; Paracrine factors; Tissue engineering; Vascular regeneration.
Summary for Lay Audience

Recent years have shown an increase in both researchers’ and the public’s interest in cell therapies for regenerative medicine applications. However, the most appropriate cell populations and methods for generating these cells for use in humans remain unclear, with billions of cells required for a single treatment. Found in human fat, adipose-derived stromal cells (ASCs) offer a possible solution. ASCs are abundant and easily accessible in the human body, in contrast to bone marrow-derived stromal cells (BMSCs), which are associated with a painful extraction process and low abundance. ASCs are known to secrete pro-regenerative proteins that can shift the immune response towards healing and encourage blood vessel growth, termed paracrine factors. Unfortunately, while more abundant than BMSCs, extracted ASCs do not meet the required cell numbers for clinical treatment without cell expansion, commonly done on tissue culture polystyrene (TCPS). With long-term expansion on TCPS, the capacity of ASCs to grow and secrete beneficial paracrine factors is reduced, thus diminishing their pro-regenerative properties. Evidently, a new expansion strategy is needed to maintain and ideally enhance the regenerative capacity of ASCs. Recognizing this need, this project focused on investigating the use of stirred bioreactor systems for expanding human ASCs. Within the bioreactors, culturing the ASCs on small scaffolds, termed microcarriers, designed to mimic the environment in which the cells reside within their native tissues was investigated as a promising approach to help maintain the desired cellular characteristics during expansion. Further, culturing ASCs under low oxygen levels and under varying stirring rates were explored as additional methods of tuning the cell culture environment to increase cell growth and augment paracrine factor expression levels. Overall, this study highlights strategies to augment the pro-regenerative capacity of ASCs during expansion, offering a potential alternative to cell growth on TCPS, with the aim of establishing improved culture methods to generate well-characterized therapeutic cell populations that could be applied in the future in clinical treatments.
Co-Authorship Statement

All experimental work included in this thesis was completed by McKenna Tosh, with the exception of the scanning electron microscopy imaging presented in Figure 3.3, which was performed by Dr. Todd Simpson on samples prepared by McKenna Tosh. Dr. Lauren E. Flynn contributed to the design and interpretation of all experiments and provided critical feedback during the writing of all chapters.
Acknowledgments

First and foremost, I would like to thank my supervisor, Dr. Lauren Flynn. Thank you for your guidance, insight, support, and patience. You have taught me how to think critically, nurtured me as a scientist and are an amazing role model in this daunting world of academia. Thank you for believing in me and my work. I am forever grateful to have been your student and will always value the time I spent in the Flynn lab.

Next, I would like to thank my advisory committee, Dr. David Hess, and Dr. Zia Khan. Thank you for your helpful suggestions and guidance throughout my master’s degree. Both of your additional support and dedication have greatly improved this project.

Thank you to all the past and present members of the Flynn Lab who I’ve had the pleasure of working with. Anna, Fiona, and JT, I am thankful for all of the time you have spent teaching me and offering advice. Sarah, Connor, and Agnes, I am so so grateful for all the exciting lunches, trivia nights, and making the lab an even more fun place to work. Last but not least, Julia, thank you for showing me best friends can still be made in these crazy times, you are my soul sister and have provided endless laughs, belays, gym spots and a shoulder to cry on, I am so glad to have met you.

Thank you to all the roommates I’ve had over these 2 years, especially Elizabeth and Anastasia, for always making me smile and endless hangouts and conversation. I value the friendship I’ve built with both of you. You are both amazing and inspiring women.

Thank you to my family, who without their moral support I never would have finished this degree. To my countless aunts, uncles and cousins who call constantly with happy thoughts and “you can do its”. To my sisters, Morgan, and Madison, who always listen and provide crazy antics near and far. To my boyfriend, Josh, who has endless support for me and will never stop believing I can do the impossible. And most importantly to my Mom, you are my rock, I don’t know how to express how much you mean to me and how much drive you’ve given me, but this project is truly all because of you.

Finally, to my cat, Tequila, your cuddles, kisses, and chatter have made all the difference.
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List of Abbreviations

2D Two-dimensional
3D Three-dimensional
ADAM A disintegrin and metalloproteinase
ALCAM Activated leukocyte cell adhesion molecule
ANOVA Analysis of variance
ASC Adipose-derived stromal cell
bFGF Basic fibroblast growth factor
BMSC Bone marrow-derived mesenchymal stromal cells
BSA Bovine serum albumin
CAM Chorioallantoic membrane
CCL2 Chemokine (C-C motif) ligand 2
CdM Conditioned media
COL Collagen
CXCL10 C-X-C motif chemokine ligand 10
CXCR4 C-X-C chemokine receptor type 4
DAT Decellularized adipose tissue
dH₂O Deionized water
DMEM/F12 Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMMB Dimethylmethylene blue
DNA Deoxyribonucleic acid
DNase Deoxyribonuclease
dsDNA Double-stranded DNA
ECM Extracellular matrix
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assays</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluorescence minus one</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>hASC</td>
<td>Human adipose-derived stromal cell</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>hypoxia inducible factor-1α</td>
</tr>
<tr>
<td>HMVEC</td>
<td>Human microvascular endothelial cell</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>IFATS</td>
<td>International Federation for Adipose Therapeutics and Science</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemical</td>
</tr>
<tr>
<td>IL1β</td>
<td>Interleukin 1β</td>
</tr>
<tr>
<td>IL4</td>
<td>Interleukin 4</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>IL10</td>
<td>Interleukin 10</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IPO8</td>
<td>Importin 8</td>
</tr>
<tr>
<td>ISCT</td>
<td>International Society for Cellular Therapy</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>LCA</td>
<td>Leukocyte-common antigen</td>
</tr>
<tr>
<td>LDF</td>
<td>LIVE/DEAD™ fixable</td>
</tr>
<tr>
<td>LDPI</td>
<td>Laser Doppler perfusion imaging</td>
</tr>
<tr>
<td>MCAM</td>
<td>Melanoma cell adhesion molecule</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MMR</td>
<td>Macrophage mannose receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stromal cell</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural stem cell</td>
</tr>
<tr>
<td>OCT4</td>
<td>Octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>OHP</td>
<td>Hydroxyproline</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PI GF</td>
<td>Placental growth factor</td>
</tr>
<tr>
<td>PSC</td>
<td>Pluripotent stem cell</td>
</tr>
<tr>
<td>PTGS</td>
<td>Prostaglandin-endoperoxide synthase</td>
</tr>
<tr>
<td>REX1</td>
<td>Reduced expression-1</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RPL13A</td>
<td>Ribosomal protein L 13a</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcriptase quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal cell-derived factor-1</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>sGAG</td>
<td>Sulphated GAG</td>
</tr>
<tr>
<td>SOX2</td>
<td>SRY (sex determining region Y)-box 2</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>SVF</td>
<td>Stromal vascular fraction</td>
</tr>
<tr>
<td>TCPS</td>
<td>Tissue culture polystyrene</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
Chapter 1

1 Introduction

1.1 Adipose-derived Stromal Cells

Mesenchymal stromal cells (MSCs) are a heterogeneous population of multipotent cells that play important roles in regenerating adult tissues damaged by disease or injury [1]. Fundamentally, the ability to both self-renew and differentiate are necessary for a cell population to be classified as stem cells [2], [3]. While MSCs were initially referred to as mesenchymal stem cells, the field has moved away from this description as they have not been proven to have the capacity to self-renew in vivo. Further, a growing body of evidence supports that MSCs primarily stimulate regeneration in vivo through indirect mechanisms, rather than through long-term engraftment and differentiation [2], [4].

MSCs were initially referred to as ‘stromal’ cells in 1985 by Owen et al., after they were first discovered by Friedenstein et al. in 1966 in the bone marrow of mice [2], [5], [6]. Since their discovery, MSCs have been identified in many human tissues, including the bone marrow, lungs, heart, peripheral blood, and adipose tissue [7], [8]. The most common sources of MSCs used in tissue-engineering research are derived from bone marrow and adipose tissue [9].

Adipose-derived stromal cells (ASCs) offer several advantages compared to other MSC sources, including that they are easily obtainable and abundant in the human body [10]. ASCs can be readily isolated from subcutaneous fat stores, which can be extracted through minimally-invasive surgeries such as liposuction, or collected from tissues discarded during elective breast and abdominal reduction surgeries [11]. Notably, adipose tissue contains a higher frequency of MSCs in comparison to the bone marrow. More specifically, evidence suggests that up to 2% of total nucleated cells in adipose tissue are multipotent MSCs, as compared to only 0.002% in the bone marrow [12], [13].
1.1.1 Origin and Characterization

ASCs were first identified by Zuk et al. in human adipose tissue obtained by liposuction in 2001, as an alternative cell source to bone marrow-derived mesenchymal stromal cells (BMSCs) [14]. ASCs are believed to reside throughout the stroma of subcutaneous adipose tissue, close to the vasculature within a region referred to as the perivascular niche [15]. However, there is a lack of consensus in the field on whether the perivascular niche is the precise location of ASCs within adipose tissue [16]–[18]. This uncertainty is due to the lack of definitive markers for identifying ASCs in vivo [19]. In order to classify a cell population as ASCs, the International Federation for Adipose Therapeutics and Science (IFATS) established three defining criteria in collaboration with the International Society for Cellular Therapy (ISCT) [20]. First, the cell population must be isolated from adipose tissue and be able to adhere to tissue culture polystyrene (TCPS). Second, the cell population must show multipotent differentiation capacity in vitro towards the adipogenic, chondrogenic, and osteogenic lineages. Lastly, the cell population must have an immunophenotype that is >80 % positive for at least two of the primary positive markers outlined in Table 1.1, as well as <2 % positive for at least two of the primary negative markers [20].

There are several different techniques that are used to isolate ASCs from adipose tissue [21]. Standard protocols typically involve a combination of mechanical processing, enzymatic digestion, and separation through centrifugation to obtain the stromal vascular fraction (SVF) [22], [23], which contains a heterogenous mixture of fibroblasts, pericytes, endothelial progenitor cells, macrophages, blood cells, more highly committed adipose progenitors, and ASCs [24]. Following separation from the mature adipocytes, the SVF is purified by expansion on TCPS to obtain the adherent cell population, which includes the multipotent ASCs [21], [23].

ASCs are a heterogenous cell population, and as such can vary in cell surface marker expression due to differences in factors such as donor variability, isolation techniques, tissue harvesting sites, and culture conditions [25], [26]. The immunophenotype of ASCs can also change across passages; in particular, CD34 and CD146 have shown higher
levels of expression in primary isolates, with decreased expression observed with successive passaging [27]. Opposite trends have been seen with CD29, CD90, CD73, CD105, and CD166, where increased expression has been shown as the cells are passaged on TCPS [28].

Table 1.1: ASC immunophenotype [29], [30]

<table>
<thead>
<tr>
<th>CD markers</th>
<th>Descriptive names</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD13</td>
</tr>
<tr>
<td></td>
<td>CD29</td>
</tr>
<tr>
<td></td>
<td>CD44</td>
</tr>
<tr>
<td>Primary positive markers</td>
<td>CD73</td>
</tr>
<tr>
<td>(&gt; 80 %)</td>
<td>CD90</td>
</tr>
<tr>
<td></td>
<td>CD105</td>
</tr>
<tr>
<td></td>
<td>CD166</td>
</tr>
<tr>
<td></td>
<td>CD10</td>
</tr>
<tr>
<td>Secondary positive markers</td>
<td>CD36</td>
</tr>
<tr>
<td></td>
<td>CD49e</td>
</tr>
<tr>
<td></td>
<td>CD26</td>
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<tr>
<td></td>
<td>CD34</td>
</tr>
<tr>
<td></td>
<td>CD49d</td>
</tr>
<tr>
<td></td>
<td>CD49f</td>
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<tr>
<td>Variable markers</td>
<td>CD146</td>
</tr>
<tr>
<td></td>
<td>CD200</td>
</tr>
<tr>
<td></td>
<td>CD271</td>
</tr>
<tr>
<td>Primary negative markers</td>
<td>CD31</td>
</tr>
<tr>
<td>(&lt; 2 %)</td>
<td>CD45</td>
</tr>
<tr>
<td></td>
<td>CD235a</td>
</tr>
<tr>
<td></td>
<td>HLA-DR</td>
</tr>
</tbody>
</table>
### Secondary negative markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>T-cell co-receptor</td>
</tr>
<tr>
<td>CD11b</td>
<td>αM-integrin</td>
</tr>
<tr>
<td>CD19</td>
<td>B-lymphocyte surface antigen B4</td>
</tr>
<tr>
<td>CD163</td>
<td>Scavenger receptor cysteine-rich type 2 protein</td>
</tr>
<tr>
<td>PODXL</td>
<td>Podacallyxin-like protein 1</td>
</tr>
</tbody>
</table>

#### 1.1.2 Paracrine Mechanisms of Pro-Regenerative Function

ASCs are known for their pro-regenerative function; however, until recently the main mechanism through which they contributed to regeneration was thought to be based on their ability to differentiate. While these cells have been shown to have the capacity to differentiate towards multiple lineages *in vitro, in vivo* studies provide limited evidence that the differentiation of delivered ASCs leads to host regeneration [31], [32]. In contrast, the primary mechanism through which ASCs promote regeneration *in vivo* is now thought to be through their secretion of paracrine factors [33], [34]. These paracrine factors include both growth factors and cytokines, which can have a broad range of effects including stimulating angiogenesis, promoting cell survival, and modulating the inflammatory response [35]–[37]. There have been several studies that have investigated the secretome of human adipose-derived stromal cells (hASCs), focusing on immunomodulation and angiogenesis. Identified factors secreted by hASCs and their associated functions are summarized in Table 1.2. To develop a deeper understanding of paracrine-mediated regeneration, studies have investigated the effects of ASC-conditioned media (CdM) or direct or indirect co-culturing of ASCs with various cell types, such as macrophages or endothelial cells [38].
**Table 1.2: Regenerative effects of paracrine factors secreted by ASCs**

<table>
<thead>
<tr>
<th>Paracrine Factor</th>
<th>Regenerative function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>angiogenin</td>
<td>Stimulates angiogenesis by binding to endothelial cells to promote invasiveness; stimulates endothelial cell proliferation and tubule formation.</td>
<td>[39], [40]</td>
</tr>
<tr>
<td>angiopoietin-1</td>
<td>Modulates endothelial cell-to-cell interactions, capillary stability, and tubule formation.</td>
<td>[41]</td>
</tr>
<tr>
<td>basic fibroblast growth factor (bFGF)</td>
<td>Regulates cell proliferation, survival, migration, and differentiation; promotes endothelial cell tubule formation.</td>
<td>[41], [42]</td>
</tr>
<tr>
<td>granulocyte-macrophage colony stimulating factor (GM-CSF)</td>
<td>Also known as colony-stimulating factor-2 (CSF-2). Involved in the recruitment of monocytes, macrophages, neutrophils, and eosinophils.</td>
<td>[42]</td>
</tr>
<tr>
<td>hepatocyte growth factor (HGF)</td>
<td>Promotes angiogenesis and wound healing, aiding new tissue growth or development. Stimulates cell migration and the branching of endothelial cells into tube-like structures.</td>
<td>[43], [44]</td>
</tr>
<tr>
<td>insulin-like growth factor-1 (IGF-1)</td>
<td>Important in wound healing and angiogenesis. Stimulates the proliferation and migration of fibroblasts and acts as a chemotactic agent for endothelial cells.</td>
<td>[41], [45]</td>
</tr>
<tr>
<td>interleukin 1β (IL1β)</td>
<td>Mediator of the pro-inflammatory response; involved in monocyte activation.</td>
<td>[41]</td>
</tr>
<tr>
<td>interleukin 4 (IL4)</td>
<td>Inflammatory mediator; promotes differentiation of T cells to Th2 cells and macrophages towards pro-wound healing M2-like polarization.</td>
<td>[46]</td>
</tr>
<tr>
<td>interleukin 6 (IL6)</td>
<td>Pro-inflammatory cytokine that attracts phagocytic cells to clear cellular debris from damaged tissues; regulates balance between pro-inflammatory (M1-like) and pro-wound healing (M2-like) macrophage polarization.</td>
<td>[43]</td>
</tr>
<tr>
<td>interleukin 8 (IL8)</td>
<td>Pro-inflammatory cytokine that attracts and activates neutrophils to sites of tissue damage.</td>
<td>[43]</td>
</tr>
<tr>
<td><strong>interleukin 10 (IL10)</strong></td>
<td>Immunosuppressive cytokine; regulates T cell function, reduces maturation of dendritic cells and promotes M2-like macrophage polarization.</td>
<td>[43]</td>
</tr>
<tr>
<td><strong>monocyte chemotactic protein-1 (MCP-1)</strong></td>
<td>Recruits monocytes, dendritic cells, and T cells. Also known as chemokine (C-C motif) ligand 2 (CCL2).</td>
<td>[47]</td>
</tr>
<tr>
<td><strong>platelet-derived growth factor (PDGF)</strong></td>
<td>Helps stimulate endothelial and mesenchymal cells leading to blood vessel repair and cell proliferation.</td>
<td>[48]</td>
</tr>
<tr>
<td><strong>prostaglandin E2 (PGE2)</strong></td>
<td>Immunosuppressive, reduces maturation of dendritic cells and helper T cells after activation, reducing inflammation.</td>
<td>[43]</td>
</tr>
<tr>
<td><strong>placental growth factor (PlGF)</strong></td>
<td>Has strong mitogenic and chemoattractant effects on endothelial cells.</td>
<td>[41]</td>
</tr>
<tr>
<td><strong>stromal cell-derived factor-1 (SDF-1)</strong></td>
<td>Homing factor for circulating stromal cells, also plays roles in inflammation and angiogenesis.</td>
<td>[41]</td>
</tr>
<tr>
<td><strong>transforming growth factor beta (TGF-β)</strong></td>
<td>Promotes angiogenesis and wound healing, aiding new tissue growth or development. Modulates proliferation and differentiation of lymphocytes.</td>
<td>[43]</td>
</tr>
<tr>
<td><strong>tumor necrosis factor alpha (TNF-α)</strong></td>
<td>Pro-inflammatory cytokine that attracts phagocytic cells that clear cellular debris from sites of injury.</td>
<td>[43]</td>
</tr>
<tr>
<td><strong>vascular endothelial growth factor (VEGF)</strong></td>
<td>Promotes angiogenesis and wound healing, aiding new tissue growth or development. Destabilizes and increases the permeability of blood vessels, allowing for endothelial cell activation.</td>
<td>[43]</td>
</tr>
</tbody>
</table>

Recently, the ability of ASCs to promote angiogenesis through paracrine-mediated mechanisms has become of great interest in the field of tissue engineering [49]. The effects of hASC CdM on endothelial cells have been demonstrated using *in vitro* models, with one study showing that the migration and tubule formation of human microvascular endothelial cells (HMVECs) was increased after 3 days of culture in hASC CdM, compared to culture in control medium [46]. In a study using an *in vivo* murine model of hindlimb ischemia, the intramuscular injection of CdM from hASCs was compared to the
injection of control medium over 8 weeks [50]. The study showed increased expression of genes related to angiogenesis, including inducible nitric oxide synthase (iNOS), IL-1β, and TNFα, along with increased restoration of blood flow in the hindlimbs of the mice that were treated with the hASC CdM [50]. In addition, the effects of transplanting mouse ASCs versus control medium into ischemic and control fat pads in transgenic mice have been investigated [51]. In the study, mouse ASCs transplanted in ischemic fat pads were shown to induce a higher vascular density within the fat pads at 28 days. Further, increased levels of angiogenic growth factors including HGF and VEGF were detected in vivo in the cell treatment group, as well as in later in vitro experiments with hASC CdM [33], [51].

ASCs have also been shown to have effects on the immune system, as cytokines secreted by ASCs can have immunomodulatory and pro-wound healing activities [46], [52]. For example, when ASCs and macrophages are indirectly co-cultured, growth factors secreted by the ASCs can influence the phenotype of the macrophages, referred to as macrophage polarization [53]. During macrophage polarization, non-activated naïve “M0” macrophages can be activated towards a pro-inflammatory “M1-like” macrophage phenotype or towards a more pro-wound healing/anti-inflammatory “M2-like” macrophage phenotype [54]. Although the terminology “M1-like” and “M2-like” are common nomenclature in the literature, it is important to note that there is a broad spectrum of macrophage phenotypes, which are involved in different aspects of tissue repair and regeneration [53], [54]. Additionally, there is evidence that macrophage polarization can occur in already activated macrophages as opposed to only in non-active naïve M0 macrophages, in response to growth factors or external cues such as cytokines, resulting in changes between macrophage phenotypes (from M1-like to M2-like or vice versa) [55].

ASCs are thought to promote a more M2-like macrophage phenotype, which has been shown to be beneficial for wound healing [56]–[58]. In an in vivo wound healing mouse study, the effects of delivering hASCs on collagen scaffolds to full-thickness wounds was compared to collagen scaffolds alone [59]. The study demonstrated the capacity of hASCs to promote macrophage polarization from an M1-like phenotype that expressed
iNOS to an M2-like phenotype that expressed macrophage mannose receptor (MMR) in the mouse wound model. Moreover, hASC delivery accelerated wound healing, with 75% of hASC-treated mice having wounds entirely healed on day 21 in comparison to only 25% of control mice [59]. Similarly, a study conducted in the Flynn lab showed enhanced angiogenesis, as well as increased host cell recruitment and a more pro-regenerative M2-like macrophage phenotype, when decellularized adipose tissue (DAT) bioscaffolds were seeded with rat ASCs and implanted subcutaneously in an immunocompetent rat model, compared to unseeded DAT bioscaffolds [60]. More specifically, immunohistochemical (IHC) staining revealed that a significantly higher fraction of the infiltrating CD68+ macrophages expressed the pro-regenerative M2-like macrophage marker CD163 in the rat ASC-seeded group in comparison to the control group at 12 weeks [60].

1.1.3 Barriers to Clinical Translation

Despite their regenerative potential, there remain several barriers that limit the broad-scale clinical translation of ASCs. An initial limitation is the relatively low yield of ASCs isolated from adipose tissue in comparison to the high doses that will be needed for clinical treatments. More specifically, it is estimated that the number of cells required will be up to 1x10^9 per dose for each patient [61], [62], with the potential for multiple doses to be required. To address this need, ASCs must be expanded in vitro following their isolation to generate sufficient cell numbers for clinical use.

The most common method of expanding ASCs involves two-dimensional (2D) planar culture on TCPS substrates, due to its simplicity and relatively low initial costs. However, these conventional culture techniques have associated limitations, which may be attributed to the fact that they are highly dissimilar to the native cellular microenvironment within tissues [63], [64]. Recent cell biology studies highlighting three-dimensional (3D) culture models have shown large differences in cell survival, migration, cell-cell interactions and differentiation in comparison to 2D culture models [65]. Moreover, 2D in vitro expansion has been found to diminish ASC proliferation and multilineage differentiation potential with increased passaging [66], [67]. In addition, the
ASC secretome can be altered with increased passaging, with one study reporting a decrease in the angiogenic potential of hASCs at higher passage numbers [68]. Other limitations of 2D static culture on TCPS include little control over culture parameters, as well as the costs associated with the prolonged culture required to generate the large number of cells needed for clinical doses [69], [70]. Further, enzymatic treatments used for successive passages can lead to altered cell properties [71]. For example, the use of trypsin to dissociate ASCs from TCPS can be harmful to the cells, resulting in increased cell death, especially if overexposure occurs [72], [73]. Additionally, the static nature of this growth method is known to lead to gradients in pH, dissolved oxygen, and nutrients within the culture medium, resulting in a heterogeneous environment that can influence cell growth [74].

Another barrier to the clinical translation of ASCs is the small number of cells which reach or survive at the target site following cell delivery in saline [75]. Target sites for ASC delivery are often hostile environments of hypoxic stress, inflammation, and insufficient blood supply, which can lead to low ASC survival rates [76], [77]. Low cell survival and retention within the first few days following ASC injection is believed to be attributed to both the injection itself and the harsh environment [78], [79]. To enhance ASC survival, as well as the effectiveness of ASCs for clinical applications, there is increasing interest in the development of pre-conditioning strategies to try to augment cell survival and pro-regenerative function following in vivo transplantation by manipulating the culture microenvironment during the in vitro expansion process [80]. There is a growing body of evidence that pre-conditioning can modulate the angiogenic and immunomodulatory potential of ASCs [81]. The following section will explore different pre-conditioning strategies of current interest and their various effects on ASCs or other MSC populations.

### 1.2 Pre-conditioning of ASCs

Pre-conditioning of MSC populations including ASCs has become an emerging topic of interest within the cell therapy field as a potential strategy to augment the biological function of cells, including cell survival, proliferation capacity, paracrine factor secretion,
and/or differentiation [82]. More specifically, pre-conditioning involves growing cells ex vivo within controlled microenvironments using different physical and/or chemical stimuli that can alter cell phenotype and function [83]. A variety of different pre-conditioning strategies have been investigated within both 2D static culture systems and 3D bioreactors, including the use of biomaterial scaffolds [76], [84], [85], dynamic culture [86]–[88], culturing under reduced oxygen tension [89]–[91] and stimulation with soluble factors or cytokines [92], [93]. Multiple pre-conditioning strategies can be combined, with the potential for synergistic effects [67]. While more complex to implement, bioreactors can enable greater control over culture parameters, such as pH, oxygen tension, nutrients and temperature [83].

1.2.1 Pre-Conditioning with Biomaterial Scaffolds

Biomaterial scaffolds are often used for ASC expansion in bioreactors, which in turn is a method of cell pre-conditioning. As adult stromal cells reside within a 3D microenvironment in their native tissues, biomaterial scaffolds can help to mimic the natural environment in vitro, and therefore, influence cellular behaviour via physical and biochemical cues [76], [94]. The integration of biomaterial scaffolds within culture systems has the potential to promote cell proliferation and/or differentiation [76]. In particular, substrate stiffness has been shown to modulate both cell attachment and differentiation of BMSCs [95] and ASCs [95]–[97], as well as the proliferation and differentiation of embryonic stem cells (ESCs) [98], [99] and neural stem cells (NSCs) [100], [101]. It should be noted that the terms soft and stiff are relative and are both material and application dependent.

Tissues within the human body have a wide range of stiffnesses, with subcutaneous adipose tissue having a Young’s modulus in the range of ~ 1-3 kPa [102]. For human BMSCs grown on collagen I and polyacrylamide hydrogels of varying stiffnesses, softer substrates ranging between ~ 1-3 kPa were shown to produce weaker cell adhesion and lower proliferation rates than stiffer substrates of > 15 kPa [103]. In another study, human BMSCs cultured on stiffer hydrogel matrices made of the polymer poly(acrylamide-co-acrylic acid) with Young’s moduli of approximately 47.5 kPa
showed enhanced cell adhesion and greater proliferation, with increased cell spreading and the formation of well-aligned stress fibers [104], [105]. In terms of ASC differentiation, stiffer substrates with Young’s moduli of 1 MPa have been shown to promote osteogenesis in favor of adipogenesis in studies using polydimethylsiloxane substrates of varying thicknesses with mouse ASCs [96]. Similarly, other studies suggest that stiffer matrices with Young’s moduli of ~15 kPa can stimulate differentiation towards the chondrogenic and osteogenic lineages, while softer matrices with Young’s moduli of ~1 kPa promote differentiation towards the adipogenic lineage [103], [106]. Interestingly, studies have also reported increases in the angiogenic potential of mouse ASCs cultured on stiffer bioscaffolds with Young’s moduli of ~195 kPa, shown through the upregulation of pro-angiogenic factors such as VEGF, in comparison to controls of relatively softer bioscaffolds with Young’s moduli of ~15 kPa [107].

In addition to matrix stiffness, the biochemical composition of the material can also influence cell growth and behaviour. As such, it is important to look at the cell-instructive effects of the extracellular matrix (ECM), being the natural cellular microenvironment. The tissue-specific composition and organization of the ECM play important roles in directing cell phenotype and function within tissues [108], which will be discussed in more detail in Section 1.4. Briefly, the integration of ECM proteins within biomaterials has been studied with the goal of developing platforms that can harness the pro-regenerative capacities of hASCs. In a study by Sawadkar et al., hydrogel biomaterials comprised of collagen, elastin or fibrin were investigated in terms of their effects on the survival, proliferation, and phenotype of hASCs in vitro, as well as adipose regeneration in vivo [109]. All three formulations were shown to support hASC viability and proliferation, with the collagen scaffolds showing the highest proliferative activity [109]. Additionally, all three scaffolds seeded with hASCs actively supported adipose regeneration in vivo. The data suggested that the different platforms mediated regeneration through different mechanisms, with collagen supporting cell proliferation and adipogenesis, elastin maintaining a balance between tissue degradation and formation, and fibrin supporting angiogenesis [109].
Studies have also reported that pre-conditioning cells on biomaterials can alter paracrine activity in comparison to traditional 2D cell culture methods [110]–[114]. In one study, a significant increase in the secretion of anti-inflammatory and pro-angiogenic factors, such as TGF-β and VEGF, was reported when rat ASCs were grown on electrospun polycaprolactone scaffolds in comparison to conventional culture on TCPS over 2 days. Further, CdM from the rat ASCs cultured on the electrospun scaffolds accelerated wound healing, along with increasing macrophage recruitment and polarization towards a pro-wound healing M2-like macrophage phenotype, in comparison to CdM generated by rat ASCs cultured on TCPS in a rat excisional wound healing model [110]. Similarly, a study by Yang et al., showed that culturing rat BMSCs on collagen-based hydrogels enhanced messenger ribonucleic acid (mRNA) and protein expression of multiple immunomodulatory factors including HGF, prostaglandin-endoperoxide synthase (PTGS) and TGF-β, in rat BMSCs compared to traditional 2D monolayer culture over 3 weeks [114].

1.2.2 Pre-Conditioning with Oxygen Tension

Pre-conditioning with oxygen tension, also referred to as hypoxic pre-conditioning, involves culturing ASCs in an environment with reduced oxygen tensions compared to the atmospheric conditions that are commonly implemented in cell culture studies. In this context, the term ‘normoxic’ is used to refer to the atmospheric oxygen tension of ~20 % (factoring in the 5 % CO₂ used for buffering), while the term ‘hypoxic’ can refer to any oxygen tension lower than 20 %. Many studies have focused on culturing MSCs at 2 % oxygen tension, although lower oxygen tensions have also been investigated [75]. Reduced oxygen tension is used as a pre-conditioning strategy to mimic the low oxygen environment of injured or damaged tissues, where the cells will be delivered in clinical applications [115], [116]. Cells may experience oxygen tensions as low as 0.4 to 2.3 % when delivered to ischemic tissues, and for those cultured under atmospheric oxygen tension, this often leads to apoptosis [117].

Many different studies have investigated the effects of hypoxic pre-conditioning on MSC populations such as ASCs with the goal of increasing cell survival following
transplantation [89]–[91]. In one study, ischemia was stimulated using an *in vitro* oxygen-glucose deprivation model, where rat ASCs were pre-conditioned under hypoxic conditions of 2 % O$_2$ or <0.1 % O$_2$, or normoxic conditions for 24 hours before being exposed to both oxygen and glucose deprivation for 24 hours [118]. This study aimed to elucidate the effects of hypoxic pre-conditioning on cellular viability, apoptosis, and paracrine factor secretion within an ischemic environment. The hypoxic 2 % O$_2$ pre-conditioning resulted in significantly higher cell viability than the normoxic pre-conditioning group, as well as up-regulation of the pro-angiogenic genes VEGF-A and SDF-1 [118]. In another study, human BMSCs cultured under 2 % O$_2$ generated ~30-fold more cells than those grown under 20 % O$_2$ after 7 passages, with cell numbers showing significant differences by day 7 [90]. Another study with hASCs cultured under 2 % O$_2$ showed increased cell numbers starting from day 7 compared to those grown under 20 % O$_2$, as well as increased gene expression of the stem cell-associated markers reduced expression-1 (REX1), SRY (sex determining region Y)-box 2 (SOX2), octamer-binding transcription factor 4 (OCT4) and NANOG [89].

1.2.3 Pre-Conditioning with Dynamic Culture

Finally, to pre-condition cells with dynamic culture, shear forces and/or mechanical stimulation are applied to the cells with the aim of influencing their behavior [67]. Dynamic culture is often performed in bioreactor systems where the agitation rate, and the resulting shear forces, can be more easily controlled [119], [120]. Alternatively, if shear forces are examined using 2D culture models, shear stress inducing devices can be used such as piezoelectric devices and voltage controlled motors, to induce acoustic or vibrational simulation respectively [8].

Several studies have reported that shear stress can influence MSC behavior, including migration and angiogenic factor secretion [86], [88]. Yuan *et al.* showed that human BMSCs exposed to low levels of shear stress (~0.2 Pa) migrated to an artificial wound significantly faster than those cultured statically [86]. In probing the mechanisms of this response, western blot analysis showed increased levels of C-X-C chemokine receptor type 4 (CXCR4) expression in the BMSCs exposed to shear stress. Stromal cell-derived
factor 1 (SDF-1) is the ligand of CXCR4, and often acts as a homing factor for circulating stromal cells [41], [86]. In another study, hASCs cultured for 7 days on TCPS with exposure to intermittent fluid flow showed significantly enhanced secretion of VEGF, HGF, leptin, IL-8, and angiopoietin-2 compared to statically-cultured controls [88]. In addition, CdM generated by the mechanically stimulated hASCs significantly increased the migration of human umbilical vein endothelial cells (HUVECs) compared to CdM from the control cells, demonstrating a functional effect of the pro-angiogenic secretions [88].

1.3 Ex vivo Cell Expansion Strategies

As stated previously, the most common ex vivo expansion strategy for hASCs currently involves 2D planar culture in TCPS flasks. Known for their relative simplicity, TCPS flasks feature several limitations, including the large difference between rigid TCPS and the native 3D cell microenvironment in vivo, difficulty controlling culture parameters, such as oxygen tension, nutrients and pH, and increasing costs when substantial cell expansion is required [65], [69], [70]. Notably, 2D planar culture has been shown to reduce the proliferative capacity and differentiation potential of hASCs [121], [122]. As such, new ex vivo cell expansion strategies are needed to efficiently grow hASCs while providing control over culturing parameters to enable the integration of pre-conditioning strategies.

1.3.1 Bioreactor Systems

The purpose of using a bioreactor system for cell expansion is to obtain a large cell yield in a reliable manner while maintaining the fundamental characteristics of the cell population of interest [85], [123]. A variety of bioreactor designs have been investigated for the expansion of pro-regenerative cell types including MSCs, which can be broadly classified as stirred bioreactors, rotating wall bioreactors or perfusion bioreactors, and all feature their own advantages and disadvantages [124], [125].

Stirred bioreactors have been used extensively for regenerative cell expansion and differentiation, including hematopoietic stem cells (HSCs), NSCs, pluripotent stem cells
(PSCs), BMSCs and ASCs [126]. One of the mostly commonly-investigated forms of stirred bioreactor for mammalian cell culture is the spinner flask, consisting of a small vessel containing a stirring impeller [127]. Biomaterial substrates in the form of microcarriers can be suspended within these stirred bioreactors to support the growth of adherent cell populations, which will be discussed in more detail in Section 1.3.2. Culture parameters such as stirring rate, temperature, oxygen tension and pH can be easily controlled and adjusted to refine the culture conditions, ultimately influencing the cell fate [128]. There are two common configurations of the spinner flask, which differ in their mode of agitation. The first system has a hanging stir-bar assembly, while the second utilizes a vertical impeller [127]. The choice of configuration influences the shear forces generated within the system, which are also impacted by the stirring rate, fluid volume and microcarrier density [129], [130]. This is very important for hASC culture as they are sensitive to shear forces, which can impact the proliferative capacity and differentiation potential of the cells [130]. Stirred bioreactors allow for the exchange of both nutrients and gases, as well as provide a more homogenous culture environment in comparison to 2D planar culture [131], [132]. This has been shown to lead to increasing cell numbers in MSC expansion compared to expanding on TCPS, as well as help to better maintain their differentiation potential towards the adipogenic, osteogenic and chondrogenic lineages [133]–[136]. However, the major disadvantages associated with stirred bioreactors are the continuous fluid shear stress and collisions with both the vessel walls and other scaffolds, which can have damaging effects on the growing cells, such as cell death and limiting proliferation [137].

Rotating wall bioreactors consist of culture media, cells and scaffolds placed in concentric cylinders that are oriented horizontally and rotated. The competing gravitational and stirring forces keep the cells in suspension throughout the culture [138], [139]. Advantages of using rotating wall bioreactors include their ability to support high density cell culture and regulate cell growth and differentiation, while maintaining a low shear stress throughout the system [126]. However, limitations occur in scaling up these bioreactors, leading to issues producing the cell quantities needed for clinical applications [140]. Rotating wall bioreactors have been successfully used to expand regenerative cell
populations, such as human BMSCs, but to date have been primarily studied for applications in bone regeneration [141], [142].

Perfusion bioreactors involve the use of pumps, a culture media reservoir, a tubing circuit, and chambers holding the cells and scaffolds that can be perfused with media [137]. Common forms include hollow fiber or microfluidic chambers, where cells experience constant flow and media exchange [137], [143]. This design can provide a uniform culture environment with thorough perfusion and continuous addition of nutrients, which allows the cells to grow at high densities that are comparable to tissues [124], [144], [145]. However, the major disadvantage of perfusion bioreactors is the difficulty in retrieving the cells after the culture period, especially in hollow fiber perfusion bioreactors [146]. As well, with increases in cell density the media perfusion within the bioreactor can be negatively impacted [146].

1.3.2 Microcarrier Culture

Microcarriers were first developed in 1967 by A.L. van Wezel for vaccine production, where crosslinked dextran beads, also called Sephadex particles, were used for cultivating cells [147]. As previously mentioned, microcarriers are small spherical biomaterials that are designed to provide a matrix that supports cell attachment and proliferation within bioreactor systems [148]. The spherical design of microcarriers is advantageous as it provides a high surface area to volume ratio for cell growth [116]. Furthermore, microcarriers can be easily sampled throughout the culture period to monitor cell growth [149].

Many different commercially-available microcarriers exist and can be commonly used in bioreactor culture, including dextran-based microcarriers (Cytodex, GE healthcare), alginate-based microcarriers (GEM, Global Cell Solutions), and gelatin- or collagen-based microcarriers (CultispHERE, Percell) [150]. While synthetic microcarriers made of glass or polystyrene provide consistent, reproducible substrates, the use of microcarriers fabricated from decellularized tissues is of interest to harness the innate cell-instructive properties of the ECM, which will be discussed in more detail in Section 1.4.2 [151]. In addition, some commercially-available synthetic microcarriers are not bioresorbable,
requiring the cells to be harvested at the end of the culture period. In contrast, naturally-derived microcarriers could potentially be directly transplanted, thus overcoming the need for cell extraction, which can alter both cell viability and phenotype [70]. The use of microcarriers for cell transplantation may help to protect the cells during and post-injection, and could improve both cell viability and cell localization at the site of transplantation [152]–[154].

When using microcarriers within spinner flask bioreactors as a cell expansion platform, it is important to control and refine other parameters of the culture system to provide appropriate conditions for cell attachment, viability and growth [70]. Important parameters include the impeller geometry, stirring rate and cell seeding density [130], [155], [156]. Several studies have reported using 100-250 mL spinner flasks with magnetically-driven impellers having a paddle or bulb-shaped glass geometry, which influences the shear stress in the system [70], [130]. Recently, one study systematically explored how varying spinner flask parameters would effect placenta-derived MSC growth, including the impeller size, seeding density and microcarrier density using Cytodex-3 microcarriers comprised of a collagen coating over a dextran matrix [157]. Specifically, the study found that increasing the seeding density or microcarrier density had negative or neutral outcomes on cell expansion, while increasing the impeller surface area and including a static cell attachment period prior to agitation were favorable for cell expansion, thereby increasing the cell yield [157]. In another study, the effects of spinner flask parameters on cell attachment and expansion were investigated with BMSC on gelatin-based Cultispher-S microcarriers [158]. In particular, intermittent agitation at 100 rpm during the initial seeding phase resulted in improved cell attachment in comparison to continuous agitation [158].

1.4 The Extracellular Matrix (ECM)

As described briefly above, the ECM plays an intricate role in cell growth and development, providing not only structural support, but also mediating cell signaling and cell processes such as survival, adhesion, proliferation, migration and differentiation [159], [160]. As such, it is important to recognize the fundamental role the ECM plays in
modulating cell function both in vivo and when culturing cells in vitro [161]. The ECM is a highly organized protein-rich structure, that is a component of all tissues [159], [162]. The composition of the ECM is tissue-specific and heterogeneous in nature, but broadly consists of structural proteins including collagens and elastin, bioactive proteins including matricellular proteins, glycosaminoglycans (GAGs) and proteoglycans such as hyaluronan and chondroitin sulphate, and water [163]. The ECM also acts as a reservoir for signaling factors such as growth factors, cytokines and chemokines, which can be bound to proteoglycans within the ECM and released through cell-mediated mechanisms [164]. In turn, cells interact and respond to the ECM through synthesis, degradation and reassembly, constantly remodeling the environment, showing a dynamic relationship between the ECM and cells [165].

Cell adhesion to the ECM is regulated primarily by cell-surface adhesion receptors known as integrins, which function as a link between the cytoskeleton and the ECM [166], [167]. Integrins are heterodimers composed of one of 18 different alpha subunits and one of 8 different beta subunits, which can be assembled into 24 different combinations. The specific combination expressed by a cell can influence the ECM substrates that it will bind to, as well as dictate downstream signaling events that regulate cell behaviour within the ECM [168].

Collagens are the most abundant proteins within the human body, and they play important structural and bioactive roles within the ECM [169], guiding adhesion, migration, proliferation, and tissue development through integrin signaling [170]. Collagens form a right-handed triple helix of polypeptide chains comprised of three α-chain subunits stabilized by hydrogen bonds, which provide structural integrity and tensile strength to the ECM [171]. There are several main groups of collagens, which are grouped based on the structure they form: fibrillar (collagen type I, II, III, V, and XI), network-forming (collagen type IV, VIII, and X), fibril-associated collagens with interruptions in their triple helices (FACIT) (collagen type IX, XII, XIV, and XVI), short chain collagens (collagen type VIII and X), anchoring fibrils (collagen type VII), and other membrane-type collagens (collagen type VI) [172], [173]. In particular, fibrillar collagens are found in large quantities within all connective tissues, with type I collagen
being the most abundant [174], [175]. Elastin is the other main structural ECM protein, which provides elasticity to tissues, allowing the tissue to deform with mechanical stimulation and return to its original shape [176], [177].

Collagen is a commonly-used bioscaffold material due to its ease of processing, biodegradability, cell-interactive properties and relative abundance [178], [179]. In addition to having bioactive effects in its intact form, matrikines are fragments of ECM molecules that are generated by enzymatic activity, and are associated with cell recruitment and the modulation of adhesion and differentiation [180]. These degradation products generated by matrix metalloproteinases (MMPs) and ADAMs (a disintegrin and metalloproteinase) family members have been shown to modulate the immune response, as well as the recruitment and differentiation of stem/progenitor cells [108], [160]. For example, one study showed that collagen fragments were important for mediating the migration of human BMSCs in vivo [181].

1.4.1 Decellularized Tissue as an ECM Source

Decellularized tissue bioscaffolds offer several potential advantages over bioscaffolds produced from purified ECM components, such as higher levels of structural complexity, as well as more closely mimicking the complex composition of the native tissue source, which may be favorable for bioactivity [108], [182]. Further, the use of decellularized tissues provides an opportunity to match tissue-specific properties and functions with specific cell populations and applications [183]. The process of tissue decellularization aims to remove the cellular and nuclear components from tissues, while maintaining the complex biochemical composition and mechanical properties of the native ECM as much as possible [182]. Decellularization protocols involve unique methods that must be tailored to the physical and biochemical composition of the specific tissue of interest [184], [185]. Commonly, a combination of physical, chemical, and biological treatments are applied to fully decellularize the tissue [186]. The combination of these treatments, as well as the processing steps and preparation methods used, have a large impact on the final biochemical composition and mechanical properties of the decellularized tissues [184], [186].
Physical treatments for decellularization can include agitation, sonication, mechanical processing, and freeze/thaw cycles [182], [187]. The purpose of physical treatments is to disrupt the cell membrane, to aid in extraction of cellular and nuclear contents [188]. Specifically, freeze/thaw cycles allow for the lysing of cells through the formation of ice crystals which disrupt the cell membranes [189], [190]. Physical treatments must typically be followed by processes that can remove both the cellular and nuclear components, such as chemical treatments [191]. Agitation, mechanical processing and sonication can assist in extracting cellular components by increasing tissue exposure to the chemical treatments [182].

Chemical treatments can include detergents, acids, bases, alcohols, hypertonic or hypotonic solutions, and/or chelating agents [192]. The aim of chemical treatments is to solubilize the cellular and nuclear components, thereby separating them from the ECM [193]. Detergents are widely used in tissue decellularization as they solubilize cell membranes and dissociate deoxyribonucleic acid (DNA) from ECM proteins [194], [195]. However, the use of detergents leads to the loss of important proteins, growth factors and GAGs, which can significantly impact the structure and mechanical properties of the resultant decellularized tissues [196]. Both acids and bases are used to solubilize cellular components by changing the pH of the tissue [182], [197]. Unfortunately, their use may also lead to the extraction of valuable soluble factors from the ECM, as well as result in the degradation of proteins such as collagen, thereby altering the ECM structure and biomechanics [198]. Alcohols can extract and dissolve lipids from the tissues, but can have negative effects on the ECM proteins, including matrix stiffening [197], [199]. Finally, hypertonic and hypotonic solutions are often used with a chelating agent, which causes osmotic shock that leads to cell lysis and removal of cellular debris [200]. Overall, the use of hypertonic or hypotonic solutions are gentle compared to other chemical treatments [182].

Biological treatments include the use of enzymes such as nucleases, proteases, and lipases [182], [187]. Nucleases, such as deoxyribonuclease (DNase) and ribonuclease (RNase), degrade residual DNA and RNA after cell lysis, which decreases the risk of an immune response if the decellularized tissues are to be implanted [201], [202]. Proteases,
such as collagenase or trypsin, partially digest dense ECM proteins to allow for greater reagent penetration, and disrupt integrins to promote cell release from the ECM [203]. Generally, enzymes can have immunogenic effects and must be extensively washed out after digestion protocols [182], [197].

1.4.1.1 Decellularized Adipose Tissue (DAT)

Adipose tissue is an abundant ECM source found throughout the human body that is routinely discarded as surgical waste, and therefore represents a promising alternative to xenogenically-sourced ECM materials [10]. Both lipoaspirates and resected adipose tissue can be processed to generate decellularized adipose tissue (DAT) through a variety of decellularization protocols [200], [204]–[206]. In 2010, the Flynn lab pioneered a detergent-free adipose tissue decellularization protocol, which uses a combination of freeze/thaw cycles, enzymatic digestion with trypsin-ethylenediaminetetraacetic acid EDTA, RNase, and DNase, and lipid extraction with isopropanol to generate DAT bioscaffolds with mechanical properties that are similar to native human fat [207]. Other protocols in the literature often involve detergents, such as sodium dodecyl sulphate (SDS), to lyse cells [208], [209]. Detergent-based protocols have risks associated with them because residual detergent can be highly cytotoxic. Further, the use of detergents can lead to irreversible changes in the matrix structure and composition [210], [211]. Following decellularization, DAT has been shown to be composed of a diverse range of collagen types including collagen types I, III, IV, V, and VI, in addition to elastin, fibronectin, laminin, vitronectin and GAGs, along with growth factors and other proteins [212], [213]. For example, high throughput mass spectrometry techniques identified that the matricellular proteins dermatopontin, galectin-1,-7, and -9, osteopontin and osteocalcin were present in DAT [212].

1.4.2 Methods for Fabricating Bioscaffolds from Decellularized Tissues

In order to increase the utility of decellularized tissues, methods have been developed to process decellularized tissues into various forms such as microcarriers, coatings, hydrogels and porous foams [134], [214]–[216] to be able to tailor properties such as
bioscaffold architecture, stiffness, and porosity for a range of different applications [217]. Decellularized ECM must first be processed by mechanical and/or enzymatic digestion, prior to its incorporation into other scaffold forms [108], [215]. Initial steps involve fragmentation of the intact ECM and may include methods such as mincing and/or milling. Next, the ECM is homogenized into a solution or suspension, which can be done with or without the use of enzymatic digestion [148], [218]. Commonly, pepsin is used for enzymatic digestion to create ECM hydrogels, with the digestion time influencing the mechanical properties, biochemical composition, and gelation potential of the resultant hydrogels [219]. α-amylase has more recently been investigated as an alternative digestion enzyme due to its ability to preserve collagen fibrils during digestion. Specifically, α-amylase is believed to function through glycosidic cleavage of carbohydrate groups in the telopeptide regions of collagen in contrast to the non-specific proteolytic cleavage which occurs when utilizing pepsin [220]. The solution or suspension can be used on its own or combined with other ECM components as an injectable material [221]. Once in a suspension, the decellularized ECM can be fabricated into hydrogels, microcarriers and coatings, or used to create bioinks for 3D printing [148], [222]–[224].

For applications in bioreactor culture, the Flynn lab has previously established methods to fabricate microcarriers from α-amylase-digested DAT using the method of electrospraying [151]. Briefly, DAT suspensions were delivered via a syringe pump through a needle, where a high voltage of 15-20 kV was applied to disrupt the surface tension, causing small droplets to form. These DAT droplets were allowed to fall into liquid nitrogen, where they were collected on a grounded piece of aluminum foil. Following lyophilization and a controlled rehydration process, the DAT microcarriers that were generated were stable without chemical crosslinkers or other additives that may negatively impact ECM bioactivity [148]. Further, the stability of the DAT microcarriers is thought to be achieved through the formation of a complex network of interconnected collagen fibers [225]. These microcarriers were shown to support the attachment and growth of hASCs over 4 weeks in culture within a spinner flask system [151]. Furthermore, when compared to commercially-available Cultispher-S microcarriers, comprised of crosslinked gelatin, greater numbers of hASCs were recovered from the
DAT microcarriers after 2, 3, and 4 weeks of dynamic culture [151]. This study by Yu et al. also showed the hASCs maintained the ASC immunophenotype after 2 weeks of expansion on DAT microcarriers, although reduced expression levels of the cell adhesion receptors CD73, CD105 and CD29 were observed compared to controls statically cultured on TCPS, along with variable expression of CD146 and CD34. As well, the multilineage differentiation capacity of the hASCs towards the adipogenic, chondrogenic and osteogenic lineages was shown to be enhanced in comparison to cells expanded on both Cultispher-S microcarriers and TCPS [151].

1.5 Project Overview

1.5.1 Project Rationale

To date, culturing under hypoxia, on biomaterial substrates, and/or with mechanical stimulation have been explored as methods for hASC pre-conditioning, as introduced in the previous sections. The overall goal in these strategies is to enhance the survival and tune the paracrine secretion profile of the cells in order to more fully harness their capacity to stimulate angiogenesis and modulate inflammation for future clinical applications. In the current project, a spinner culture bioreactor system was applied and the key parameters investigated included the ECM composition within the microcarriers, the oxygen tension, and the effects of varying shear forces controlled through the stirring rate. More specifically, oxygen tensions of 2 % (“hypoxic”) and 20 % (“normoxic”; conventional culture conditions) were selected based on past studies in the Flynn lab indicating increased proliferation and angiogenic and immunomodulatory cytokine production under 2 % O$_2$ on larger 3-D DAT scaffolds cultured within a perfusion bioreactor [226], [227]. The stir rates selected included 20 rpm and 40 rpm, along with a static control, which are consistent with the range of stir rates seen in the literature and allow for probing the effects of varying shear stress on hASC proliferation and paracrine function [151], [157]. Previous studies have reported that an optimized agitation rate can increase hASC attachment to microcarriers increasing cell proliferation, but excessive stirring can have adverse effects on cell attachment and growth [157], [228], [229]. In terms of the ECM composition, custom microcarriers were fabricated from
commercially-available purified collagen for comparison to the DAT microcarriers across all culture conditions, to assess whether there were differences in hASC growth and phenotype between the differing ECM sources. Fundamentally, the goal of this study was to investigate the effects of microcarrier composition, oxygen tension and stirring rate on hASC growth, immunophenotype, and paracrine factor expression within spinner flask bioreactors, towards the goal of developing a novel system for hASC pre-conditioning for applications in soft tissue regeneration.

1.5.2 Hypothesis

The underlying hypothesis for this project was that hASC growth, immunophenotype and paracrine factor expression will be influenced by the microcarrier composition, oxygen tension, and shear stress applied within stirred culture bioreactor systems. More specifically, it was hypothesized that hASC expansion and pro-angiogenic gene expression would be augmented when the cells were cultured on the DAT microcarriers under 2 % O₂ with a stirring rate of 40 rpm.

1.5.3 Specific Aims

This Master’s thesis was divided into three aims:

Aim 1: To assess the effects of microcarrier composition, oxygen tension, and stirring rate on hASC growth over 14 days within a spinner culture bioreactor.

Aim 2: To characterize whether the hASC immunophenotype is affected by the varying bioreactor culture conditions.

Aim 3: To determine whether dynamic culture on the ECM-derived microcarriers alters the hASC gene expression levels of a range of pro-angiogenic and immunomodulatory secreted factors.
Chapter 2

2 Methods

2.1 Materials

All materials, chemicals and reagents were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Canada), unless otherwise stated.

2.2 Adipose Tissue Collection and Processing

Subcutaneous adipose tissue samples were collected with informed consent from female patients undergoing elective breast or abdominal reduction surgeries at the London Health Sciences Centre (London, ON) with Human Research Ethics Board approval from Western University (HSREB 105426). Patient information was collected and recorded including sex, age, height, weight, and donor site location. Samples were transported to the lab on ice in sterile phosphate buffered saline (PBS) with 2% bovine serum albumin (BSA).

2.3 Adipose Tissue Decellularization and Microcarrier Fabrication

To generate decellularized adipose tissue (DAT), freshly isolated subcutaneous adipose tissue samples were first transferred into hypotonic cell lysis buffer (10 mM Tris base and 5 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0) and frozen at -80 °C. Next, the tissues were processed using a published detergent-free decellularization protocol established by the Flynn lab, involving freeze-thaw cell lysis, enzymatic digestion with trypsin-EDTA and DNase/RNase, and lipid extraction with isopropanol [207].

Following decellularization, lyophilized DAT samples from 10 - 12 donors were pooled and finely minced [230]. Commercially-available purified collagen (COL) sourced from bovine flexor tendon was purchased (Cat. # 5162-1G) and similarly processed. Briefly, the lyophilized DAT or COL was cryomilled using a Retsch MM 400 mixer mill unit by
submerging milling chambers containing the minced extracellular matrix (ECM) and two
10 mm stainless steel milling balls into liquid nitrogen for 3 minutes before milling at
30 Hz for 3 minutes. The samples were again frozen and milled for a total of 2
cryomilling cycles. The resulting powder was then digested using α-amylase (1 % w/w)
in 0.22 M NaH$_2$PO$_4$ buffer (pH 5.4) for 72 hours under continuous agitation at 300 rpm.
Next, the suspensions were centrifuged at 1500 x g for 10 minutes and the digested ECM
pellet was washed 2 times for 10 minutes in 5 % NaCl, then once for 10 minutes in
deionized water (dH$_2$O) before being resuspended in 0.2 M acetic acid to achieve a
concentration of 50 mg ECM/mL based on the starting dry mass of milled ECM. The
suspensions were incubated overnight at 37 °C under agitation at 100 rpm, homogenized
(PowerGen Model 125 homogenizer, Fisher Scientific), and then stored at 4 °C until
further use.

Electrospraying was performed following previously-published methods [148], [216].
Before electrospraying, the ECM suspensions were warmed to 37 °C and homogenized
before being diluted to a concentration of 35 mg/mL using 0.2 M acetic acid, followed by
a second round of homogenization. In brief, the ECM suspensions were electrosprayed
directly into a Dewar flask containing liquid nitrogen using a 25 G winged infusion set,
with the needle tip positioned approximately 5 cm from the surface of the liquid nitrogen,
an applied voltage of 10.5 kV and an extrusion rate of 30 mL/hr, controlled with a

Following electrospraying, the frozen microcarriers were collected, transferred into
50 mL conical tubes, and immediately lyophilized overnight. Once dried, the
microcarriers were resuspended in absolute ethanol and stored at 4 °C until further use.
Prior to use, the microcarriers were rehydrated using a very gradual ethanol series to
avoid rapid swelling (96 %, 92 %, 88 %, 84 %, 80 %, 76 %, 72 %, 68 %, 64 %, 60 %,
56 %, 48 %, 40 %, 32 %, 24 %, 16 %, 8 %, 0 % ethanol in sterile phosphate-buffered
saline (PBS)). This process took place over the course of a day, allowing the
microcarriers to settle over 30 min before the next rehydration step. Upon complete
rehydration, the microcarriers were rinsed 3 times in sterile PBS to ensure all ethanol was
removed.
2.4 Microcarrier Characterization

2.4.1 Microcarrier Size Distribution

Rehydrated DAT and COL microcarriers were imaged using an EVOS XL Digital Inverted Brightfield and Phase Contrast Microscope (Life Technologies). Following imaging, the Feret’s diameter was measured using ImageJ to determine the microcarrier size distribution (n = 100 microcarriers/trial for both the DAT and COL microcarriers, N = 3 trials with different ECM batches).

2.4.2 Microcarrier Mechanical Testing

Compression testing was performed on the rehydrated DAT and COL microcarriers to determine the Young’s moduli using a CellScale MicroTester system. Briefly, individual rehydrated microcarriers (n = 6 microcarriers/trial, N = 4 trials with different ECM batches) were placed in a PBS bath at 37 °C and the cantilever was lowered until the platen contacted the top of the microcarrier. The microcarriers were then compressed to 50 % of their initial diameter during 3 pre-conditioning cycles at a strain rate of 0.01 s⁻¹, then data from the next 3 consecutive cycles were collected for each sample. The Young’s moduli were then calculated using nonlinear least squares curve fitting with a published extended mechanics model for large elastic deformations of spherical microparticles [231].

2.4.3 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to visualize the surface ultrastructure of both the DAT and COL microcarriers using published protocols [232]. Prior to imaging on a LEO 1530 microscope (Zeiss, Germany), the microcarriers were lyophilized and coated in osmium. Images were taken at a working distance of 6 mm and an accelerating voltage of 2 kV (Nanofabrication Facility, Western University).
2.4.4 Hydroxyproline and Dimethylmethylene Blue Assays

The hydroxyproline (OHP) and dimethylmethylene blue (DMMB) assays were performed to compare the relative hydroxyproline and sulphated glycosaminoglycan content respectively in the DAT and COL microcarriers. Lyophilized microcarrier samples (between 1 to 3 mg, exact mass recorded) were digested with 60 mU/mL of Proteinase K (Promega, Madison, WI, USA) in Tris-EDTA buffer (TE buffer) (Qiagen, Hilden, Germany) for 2 hours at 56 °C at 300 rpm, followed by a 10-minute incubation to inactivate the enzyme at 95 °C.

For the DMMB assay, 10 µL aliquots of the digested microcarrier samples were transferred into a 96 well plate in technical triplicates along with chondroitin sulphate standards of known concentrations. Next, 200 µL of a 1.6 % solution of DMMB in deionized water supplemented with 1 % ethanol and 0.2 % formic acid was added before the absorbance was measured at 525 nm using a CLARIOstar® spectrophotometer (BMG LABTECH Inc.). The sulphated glycosaminoglycan content was determined through comparison to the chondroitin sulphate standard curve and normalized to the dry weight of each respective sample.

For the OHP assay, 100 µL aliquots of the digested microcarrier samples were hydrolyzed with 100 µL of 12 N hydrochloric acid for 18 hours at 110 °C, then neutralized with 100 µL of 5.7 N sodium hydroxide. Next, 100 µL of dH2O was added and the samples were then centrifuged at 400 x g for 1 minute. Following this, the supernatant was collected and diluted (1:25 in dH2O) before 50 µL of diluted sample was added to a 96 well plate, along with hydroxyproline standards of known concentrations, in technical triplicates. Subsequently, 50 µL of 0.05 N chloramine-T was added with incubation at room temperature for 20 minutes, 50 µL of perchloric acid (3.15 N) was added with incubation at room temperature for 5 minutes, and 50 µL of Erlich’s reagent (200 mg/mL of 4-dimethylaminobenzaldehyde in 2-methoxyethanol) was added with incubation at 60 °C for 20 minutes. The well plates were then cooled at 4 °C for 5 minutes and the absorbance was measured at 560 nm using a CLARIOstar® spectrophotometer (BMG LABTECH Inc.). The total hydroxyproline content was then
determined through comparison to the hydroxyproline standard curve and normalized to the dry weight of each respective sample.

2.5 hASC Seeding and in vitro Cell Culture

Human adipose-derived stromal cells (hASCs) were isolated from fresh adipose tissue samples collected from elective lipo-reduction surgeries at the London Health Sciences Centre with Research Ethics Board approval from Western University (HSREB #105426). The cells were extracted and cultured following published methods and then frozen at passage 0 [207]. Following thawing, the hASCs were cultured in T75 flasks containing proliferation medium comprised of Dulbecco’s Modified Eagle’s medium (DMEM): Ham’s F12 (1:1) (Wisent Inc) supplemented with 10 % fetal bovine serum (FBS) (Wisent Inc) and 1 % penicillin-streptomycin (Life Technologies) prior to spinner flask seeding. Cells at passage 3 were used for all spinner culture studies.

To prepare the DAT and COL microcarriers for spinner flask culture, 5.2 g (wet weight in absolute ethanol) of microcarriers were weighed out for each CELLSPIN flask prior to rehydration following the methods described above. The microcarriers were rinsed once in proliferation medium and then resuspended in fresh proliferation medium and transferred into the CELLSPIN flasks. The microcarriers were allowed to equilibrate overnight at 37 °C and 95 % air/5 % CO\textsubscript{2}. The next day, the hASCs were trypsin-released and seeded onto the microcarriers at 250,000 hASCs/mg microcarriers, following a previously-established seeding regimen over 12 hours [155]. The microcarriers were then stirred at 25 rpm for the next 12 hours before being transitioned to their specific culture conditions (i.e. static, 20 rpm or 40 rpm; 2 % O\textsubscript{2} or 20 % O\textsubscript{2}) for up to 2 weeks of additional culture (Figure 2.1). The oxygen tension was controlled using a HypOxystation H35 system (HypOxygen) for 2 % O\textsubscript{2} trials and a standard cell culture incubator was used (37 °C, 95 % air/5 % CO\textsubscript{2}) for the 20 % O\textsubscript{2} trials. Half of the proliferation medium volume was changed every 2 - 3 days. All spinner culture studies were repeated with 3 different hASC donors (N = 3), with donor information provided in Supplementary Table A.1, Appendix A.
Figure 2.1: Experimental overview of the specific culturing conditions and corresponding assays for each of the aims. Aim 1 assays shown in light blue, Aim 2 in dark blue and Aim 3 in purple.

2.6 *In vitro* Assessment of Human ASC Growth on Microcarriers

2.6.1 hASC Visualization via the LIVE/DEAD® Assay

hASC growth on the DAT and COL microcarriers under the varying spinner culture conditions was qualitatively assessed through LIVE/DEAD® staining (Life Technologies) of the microcarriers on days 1, 3, 7 and 14 following the manufacturer’s instructions, with imaging performed on a Zeiss confocal scanning microscope (LSM 800 with Airyscan, Germany).

2.6.2 Quantification of Cell Growth on the DAT and COL microcarriers via the Picogreen® Assay

On days 1, 3, 7 and 14 of spinner culture, triplicate 1 mL samples of the hASC-seeded microcarriers were collected, lyophilized overnight, and then weighed. Unseeded microcarrier samples were included as controls. The samples were processed using the DNeasy Blood and Tissue Kit (Qiagen). In brief, the samples were enzymatically
digested using Proteinase K overnight at 56 °C under continuous agitation. Following digestion, the samples were diluted with 200 µL of absolute ethanol before being loaded into a DNeasy Mini spin column and centrifuged at 6000 x g. The samples were then washed and purified according to the manufacturer’s instructions before the double-stranded DNA (dsDNA) was eluted and collected. Next, the PicoGreen® assay (Thermo Fisher Scientific) was performed following the manufacturer’s instructions to quantify the dsDNA content in each sample using a standard curve with known dsDNA concentrations. The standard curve was made using an 8-point serial dilution of the λ-DNA provided with the kit, with values ranging from 1000 ng/mL to 0 ng/mL. The microcarrier samples were diluted 1:10 in TE buffer and 100 µL was combined with 100 µL of Quant-iT™ reagent within a 96 well plate. The samples and standards were run in technical triplicates and the fluorescence was read with a CLARIOstar® spectrophotometer (BMG LABTECH Inc.), using the manufacturer’s instructions. The dsDNA concentrations were then determined using the standard curve and normalized to the dry weight of each respective sample.

2.6.3 Assessment of Changes in Microcarrier Size and Mechanical Properties Following Cell Culture

At day 14, hASC-seeded DAT and COL microcarrier samples were collected for microcarrier size distribution characterization and mechanical testing following the methods described in Sections 2.4.1 and 2.4.2 respectively, to compare changes in the properties induced by hASC seeding and culture under the various conditions.

2.7 Flow Cytometry Analysis of hASC Immunophenotype

For the flow cytometry studies, hASCs were seeded onto DAT or COL microcarriers and cultured in vitro within the CELLSPIN flasks for 2 weeks following the methods described above. At 14 days, the microcarriers were collected into 15 mL conical tubes, washed with PBS, and the cells were extracted through digestion of the microcarriers with Liberase™ TL Research Grade. In brief, 300 µL of resuspended liberase was added to each tube (having a total collagenase concentration of 2.5 mg/ml), and the samples
were agitated at 100 rpm and 37 °C for 2 hours. Following digestion, an equal volume of complete medium was added, and the cell suspension was filtered through a 100 µm filter followed by a 70 µm filter, with both filters rinsed with 10 mL of PBS. The cells were then counted with a hemocytometer using trypan blue. For each cell donor, hASCs grown on tissue culture polystyrene (TCPS) were included as a baseline control, extracted using cell scraping, and counted. The isolated hASCs were centrifuged for 5 minutes at 300 x g and rinsed with PBS. The cells were then separated into single stain controls, fluorescence minus one (FMO) controls, and fully stained samples before being stained.

The cells were first stained with a LIVE/DEAD® fixable-aqua dead cell stain kit (Thermo Fisher Scientific). Additional cell surface markers of interest included CD90 (Thy-1) and CD29 (beta-1 integrin) as positive markers [20], CD34 and CD26 (dipeptidyl peptidase IV) as variable markers, and CD45 (hematopoietic) and CD31 (endothelial) as negative markers. Compensation beads were also used and both compensation beads and cells were stained (BioLegend, San Diego, CA) according to the manufacturer’s instructions for the surface markers of interest (Table 2.1). Following staining, the samples were fixed with 200 µL of 4 % paraformaldehyde (PFA) for 10 minutes before being rinsed with 1 mL of PBS supplemented with 5 % FBS. The cells were then centrifuged and resuspended in 300 µL of PBS supplemented with 5 % FBS and analyzed by flow cytometry the following day.

Table 2.1: Antibody conjugations and dilutions used for the flow cytometry study.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Conjugation</th>
<th>Dilution</th>
<th>Catalog number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD90</td>
<td>Brilliant Violet 605</td>
<td>1 : 400</td>
<td>328128</td>
</tr>
<tr>
<td>CD29</td>
<td>Alexa® Fluor 488</td>
<td>1 : 50</td>
<td>303016</td>
</tr>
<tr>
<td>CD45</td>
<td>PE-Cyanine7</td>
<td>1 : 100</td>
<td>368532</td>
</tr>
<tr>
<td>CD31</td>
<td>PE-Cyanine7</td>
<td>1 : 200</td>
<td>303118</td>
</tr>
<tr>
<td>CD34</td>
<td>PE-Dazzle™594</td>
<td>1 : 25</td>
<td>343534</td>
</tr>
<tr>
<td>CD26</td>
<td>APC</td>
<td>1 : 50</td>
<td>302710</td>
</tr>
</tbody>
</table>

The gating strategy for the panel was completed as follows; first the cell population was gated from the debris using the side scatter versus forward scatter plot before the single
cells were gated twice in the side scatter area versus side scatter height plot, and the forward scatter area versus forward scatter height plot. Following this, the live cells were gated using the LIVE/DEAD™ Fixable (LDF) Aqua plot, before the CD29 and CD90 positive populations were gated, with the CD31 and CD45 negative cells being gated last (Figure 2.2). Following this, the variable cell CD26 and CD34 positive populations were gated.

Figure 2.2: Representative flow cytometry plots showing gating strategy for hASC surface marker panel. Representative flow plots featuring hASCs grown on DAT microcarriers under 20 % O₂ and 20 rpm for two weeks showing gating strategy for flow analysis. Briefly, cells were gated from debris, before gating for the single cells. The FMO controls were utilized to gate the live cells from the dead cells, as well as to select the CD29⁺, CD90⁺ and CD45⁻/CD31⁻ (combined on the same channel) cell populations. Following this, the populations expressing the variable markers CD26 and CD34 were gated again using the FMO controls.
2.8 RT-qPCR analysis of Selective hASC Paracrine Secretion Secretome

For the RT-qPCR studies, baseline controls were collected from hASCs grown on tissue culture plastic at day 0 prior to microcarrier seeding by adding 10 mL of PureZOL™ (Bio-Rad) to the flasks then freezing at -80 °C until future use. For the spinner culture studies, triplicate 1 mL microcarrier samples were collected on Day 14 of culture. The culture media was removed and 1 mL of PureZOL™ was added before the samples were frozen at -80 °C until future use. Both microcarrier and baseline samples were thawed on ice. The microcarrier samples were sonicated with quick pulses using a Model 100 Sonic Dismembrator until the microcarriers were visibly broken down. The RNA was then extracted and purified using the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad), according to the manufacturer’s instructions. The amount of RNA and its purity were then quantified using a Nanodrop 1000 spectrophotometer. cDNA was then synthesized with an iScript™ cDNA Synthesis Kit using 250 ng of RNA in a 20 µL volume. No reverse transcriptase (RT) controls were also prepared for each sample.

Gene expression was analyzed by RT-qPCR using a BioRad CFX-384 system. The samples were run in technical triplicates, with 2 µL of sample and 8 µL of gene-specific TaqMan® Mastermix (Invitrogen), including the TaqMan® primers, precision blue, mastermix and nuclease-free water. No RT and no template controls were included on every plate, with the following cycling conditions: incubation at 50 °C for 2 min, activation at 95 °C for 20 s and 40 cycles of denaturing at 95 °C for 1 s and annealing/extension at 60 °C for 20 s. The gene expression panel included angiogenin (CAT # Hs02379000_s1), hepatocyte growth factor (HGF) (CAT # Hs00300159_m1), vascular endothelial growth factor (VEGF) (CAT # Hs00900055_m1), interleukin 6 (IL6) (CAT # Hs00174131_m1) and monocyte chemotactic protein-1 (MCP-1) (CAT # Hs00234140_m1) and ribosomal protein L 13a (RPL13A) (CAT # Hs04194366_g1) and importin 8 (IPO8) (CAT # Hs00183533_m1) as housekeeping genes. The data was then analyzed with the comparative delta delta C, method and normalized to the
geometric mean of the two stable housekeeping genes, utilizing the baseline samples for each ASC donor grown on TCPS as the calibrator.

2.9 Statistical Analyses

All numerical data are expressed as mean ± standard deviation (SD). All statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA). For the microcarrier characterization studies (Young’s modulus and size distribution of unseeded microcarriers, DMMB, OHP and PicoGreen® assays), unpaired Welch’s t-tests were performed to compare the DAT and COL microcarrier groups. For the PicoGreen® analyses in the in vitro hASC expansion studies, one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc multiple comparisons was performed to assess changes in the cell abundance over time for each individual culturing condition, and a three-way ANOVA with a Tukey’s post-hoc comparison of the means was performed to compare the cell abundance under all conditions at the final timepoint of 14 days. For the size distribution analysis of the hASC-seeded microcarriers after 14 days of culture, Welch’s t-tests were performed to compare the mean diameter of the seeded microcarriers at day 14 to the mean diameter of the corresponding microcarriers prior to seeding, and a three-way ANOVA with a Tukey’s post-hoc comparison of the means was performed to compare the mean diameter for all seeded groups after 14 days of culture. For the Young’s moduli analyses performed after 14 days of culture, Welch’s t-tests were performed to compare the Young’s modulus at day 14 to the modulus of the corresponding unseeded microcarriers. In addition, a three-way ANOVA with a Tukey’s post-hoc comparison of the means was performed to compare the modulus at day 14 between all culture conditions. Finally for flow cytometry and RT-qPCR analyses, Welch’s t-tests were performed to compare each condition relative to the baseline controls of hASCs cultured on TCPS prior to microcarrier seeding, and a three-way ANOVA with a Tukey’s post-hoc comparison of the means was performed to compare expression levels between all culturing conditions. In all statistical analyses, differences of p < 0.05 were considered statistically significant.
Chapter 3

3 Results

3.1 Characterization of the DAT and COL Microcarriers

Prior to initiating the cell culture studies, characterization of the decellularized adipose tissue (DAT) and collagen (COL) microcarriers fabricated via electrospraying was performed to confirm that they were structurally and biomechanically similar. Both microcarrier types appeared qualitatively spherical (Figure 3.1A) and were stable following the controlled rehydration process without chemical crosslinking or other additives. The DAT and COL microcarriers were similar in size, with the majority ranging between 1150 - 1450 µm in diameter when fully hydrated in phosphate-buffered saline (PBS) (Figure 3.1B). More specifically, the DAT microcarriers had an average diameter of 1321 ± 152 µm and the COL microcarriers had an average diameter of 1249 ± 148 µm, with no significant difference between the size distributions. Characterization of the Young’s moduli through compression testing with a CellScale MicroTester system under simulated physiological conditions (hydrated in PBS, 37 °C) showed no significant differences between the platforms, with the DAT microcarriers having a Young’s modulus of ~ 0.213 kPa ± 0.080 kPa and the COL microcarriers having a Young’s modulus of ~ 0.461 kPa ± 0.359 kPa (Figure 3.1C,D). While the COL microcarriers showed greater variability in their moduli, both microcarrier types were soft and compliant, and able to withstand repeated compression up to 50 % of their diameter over 3 cycles without any observable permanent deformation.
Figure 3.1: Soft and compliant microcarriers were fabricated from DAT and COL that were stable without chemical crosslinking. (A) Representative brightfield images of the rehydrated microcarriers fabricated with an extracellular matrix (ECM) concentration of 35 mg/mL. Scale bars = 1 mm. (B) Size distribution plots showing the Feret’s diameter of the DAT and COL microcarriers following rehydration in PBS, with a similar diameter and size distribution range for the two sources (pooled data from n = 100 microcarriers/test, N = 3). (C) Representative force versus deformation curves for individual microcarriers. The data from 3 cycles are shown with a strain rate of 0.01 s⁻¹. (D) Average Young’s moduli of both microcarrier types determined through compression testing on the CellScale MicroTester system (n = 6 microcarriers/ECM batch, N = 6).

The surface ultrastructure of the microcarriers was assessed through scanning electron microscopy (SEM), which revealed that both the DAT and COL microcarriers were comprised of a complex fibrous network with some sheet-like regions observed (Figure 3.2). Qualitatively, there were no obvious structural differences between the two groups.
Figure 3.2: Scanning electron microscopy (SEM) revealed that the DAT and COL microcarriers were structurally similar, with a complex fibrous ultrastructure. Both the COL and DAT microcarriers were shown to be comprised of a complex fibrous network including sheet-like regions. 100x scale bar = 100 µm, 500x scale bar = 10 µm.

Finally, the composition of both microcarrier types was investigated using biochemical assays. There was no significant difference in total collagen content between the DAT and COL microcarriers based on analysis of hydroxyproline (OHP) content, with the DAT microcarriers containing \(~61.91 \pm 4.85\ \text{µg/mg} \) (dry weight) and the COL microcarriers containing \(~56.20 \pm 14.23\ \text{µg/mg} \) (Figure 3.3A). Similarly, analysis of sulphated glycosaminoglycan (sGAG) content with the dimethylmethylene blue (DMMB) assay showed no significant differences between the groups, with \(~0.754 \pm 0.298\ \text{µg/mg} \) (dry weight) for the DAT microcarriers and \(0.321 \pm 0.145\ \text{µg/mg} \) (dry weight) for the COL microcarriers (Figure 3.3B). As expected, based on ECM sourcing, quantification using the Picogreen assay showed that the DAT microcarriers contained significantly higher double-stranded DNA (dsDNA) content than the microcarriers.
fabricated from the commercially-sourced purified collagen. However, both microcarrier types generally contained low levels of dsDNA, with the DAT microcarriers containing \(~20.8 \pm 3.1\) ng/mg (dry weight) and the COL microcarriers containing \(~7.7 \pm 1.6\) ng/mg (dry weight) (Figure 3.3C).

**Figure 3.3:** The DAT and COL microcarriers contained similar levels of collagen and sGAG, with the DAT microcarriers having higher dsDNA content. (A) OHP assay results showed no significant difference in total collagen content between the microcarrier groups. (B) DMMB assay results showed no significant difference between the sGAG content of the two microcarrier types. (C) Picogreen assay results showed significantly higher dsDNA content in the DAT microcarriers compared to the COL microcarriers (n = 3 samples/ECM batch, N = 3 - 4 different ECM batches), **p<0.01.

### 3.2 hASC Growth on DAT and COL Microcarriers Under Varying Culture Conditions within the Spinner Flask Bioreactors

Following microcarrier fabrication and characterization, the effects of microcarrier composition (DAT versus COL), oxygen tension (20 % versus 2 %), and stirring rate (static, 20 rpm, 40 rpm) on human adipose-derived stromal cell (hASC) growth were explored over 2 weeks in culture within CELLSPIN spinner flask bioreactors. At days 1, 3, 7, and 14 post-seeding, samples of the hASC-seeded microcarriers were stained with a LIVE/DEAD® assay and visualized by confocal microscopy to qualitatively monitor cell attachment and growth. Representative results for the day 1 and 14 timepoints for cell
donor C are shown in Figure 3.4. Results from all timepoints for all 3 cell donors are included in Supplementary Figure A.1 – A.6. In general, the imaging showed that both microcarrier types supported hASC attachment within the spinner flasks. Some qualitative differences were observed in the relative density of the cells on the microcarriers at day 14 between the groups. For 2 out of the 3 cell donors (donors B and C) under 2 % O₂, the cell density appeared qualitatively higher on the DAT microcarriers cultured at 20 rpm in comparison to the DAT microcarriers cultured statically or at 40 rpm. For all 3 cell donors under 20 % O₂, a qualitatively lower cell density was observed on the COL microcarriers cultured under static conditions in comparison to both dynamic conditions.
Figure 3.4: DAT and COL microcarriers qualitatively supported hASC attachment and growth within the spinner flask bioreactors. Representative confocal images under (A) 20% oxygen tension and (B) 2% oxygen tension showing calcein+ live (green) hASCs on the DAT and COL microcarriers (blue due to ECM autofluorescence) over 2 weeks in culture under static or dynamic conditions within the spinner flask bioreactors. No ethidium homodimer-1+ dead (red) cells were observed on the microcarriers. Scale bar = 100 µm.
To quantitatively probe cell growth on the microcarriers over the 14-day culture period, the dsDNA content associated with the hASCs on the microcarriers was measured via the Picogreen® Assay. Assessing the growth curves for each condition individually, a significant increase in the dsDNA content was observed from day 1 to day 14 for all groups cultured under 20 % O₂, as well as for the hASCs cultured on the DAT microcarriers under 2 % O₂ (Figure 3.5A). In comparing the dsDNA content measured at 14 days for all of the different conditions, there were no significant differences between the groups due to cell donor variability, which was particularly noticeable on the COL microcarriers cultured dynamically under 2 % O₂ (Figure 3.5B). However, for all three of the cell donors, the dsDNA content was lower in the static culture conditions for both the DAT and COL microcarriers under 20 % O₂ and 2 % O₂, with the exception of the DAT 40 rpm under 2 % O₂ condition, which also showed a lower cell abundance, consistent with the observations from the LIVE/DEAD® imaging.
Figure 3.5: Picogreen® analysis of hASC abundance on the DAT and COL microcarriers over 2 weeks in culture under varying stirring rates at either 20 % O₂ or 2 % O₂. A) Growth curves based on the dsDNA content associated with the hASCs, as determined by the Picogreen® assay. For the samples cultured under 20 % O₂ (shown in black), a significant increase in the cell content was observed over 2 weeks on both the DAT and COL microcarriers under all stirring conditions. For the samples cultured under 2 % O₂ (shown in purple), a significant increase in the cell content was observed over 2 weeks on the DAT microcarriers under all stirring conditions. Significant differences relative to day 1 are indicated for each condition on the plots; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. B) dsDNA content at day 14 determined by the Picogreen® assay, comparing the cell content on the microcarriers cultured under the varying conditions at the end of the culture period. (n = 3 samples/ASC donor, N = 3 ASC donors).
3.3 Characterization of Microcarrier Size and Mechanical Properties Following hASC Seeding and 2 Weeks of Culture Within the Spinner Flask Bioreactors

Additional characterization studies were performed to measure the changes in microcarrier diameter and compressive properties induced by hASC seeding and 2 weeks of culture within the spinner flask bioreactors. Macroscopically, both microcarrier types appeared to be more contracted when cultured dynamically as compared to statically under both 20 % and 2 % O₂ (Figure 3.6A). These results are generally consistent with the trend for increased cell content at day 14 in the dynamically-cultured groups as compared to the statically-cultured samples, with the exception of the DAT 40 rpm condition under 2 % O₂, which showed marked contraction despite having a lower relative cell content. In quantitatively analyzing the size distributions at day 14 under the varying culture conditions relative to baseline measurements prior to hASC seeding, there was a leftwards shift in the plots for all hASC-seeded groups compared to the unseeded microcarriers, indicative of contraction (Figure 3.6B). Main effects analysis showed significant effects of both microcarrier composition and agitation rate. In addition, there was a significant difference in the mean diameter of the seeded COL microcarriers cultured under 20 % O₂ at both 20 and 40 rpm compared to the baseline samples (Figure 3.6B).
Figure 3.6: Size distribution analysis showed that the microcarriers generally contracted following hASC seeding and 14 days of culture, with greater contraction macroscopically observed in the dynamic culture groups. (A) Representative brightfield images of the rehydrated seeded microcarriers for all growth conditions. Scale bar = 1 mm. (B) Size distribution plots showing the Feret’s diameter of the unseeded and seeded microcarriers for all growth conditions under 20 % (left) and 2 % O₂ (right) (n = 100 microcarriers/ASC donor, N = 3 donors).
Additionally, the Young’s moduli of the hASC-seeded microcarriers were analyzed through compression testing on the CellScale MicroTester system after 14 days of culture. When comparing the force compression plots for the seeded microcarriers (Figure 3.7A) to the unseeded microcarriers (Figure 3.1C), a higher maximum force was typically measured in the seeded groups, indicative of increased microcarrier stiffness, as more force had to be applied to reach an equivalent compression. Regardless, all microcarriers remained soft and compliant at the end of the culture period. While there was a general trend that the Young’s modulus of the microcarriers increased after 14 days of culture relative to the unseeded baseline samples, there were no significant differences between the groups due to cell donor variability (Figure 3.7B). Notably lower moduli values were measured for the samples seeded with hASC donor A and cultured under 2 % O₂, which also showed lower cell densities at 14 days under these conditions (Figure 3.5B).
Figure 3.7: Young’s moduli of the hASC-seeded microcarriers after 14 days of culture under the varying conditions. (A) Representative force versus deformation curves for individual microcarriers cultured at 20 rpm under 20 % O₂. The data from 3 cycles are shown with a strain rate of 0.01 s⁻¹. (B) Average Young’s moduli of the various seeded microcarriers under all growth conditions at 20 % O₂ and 2 % O₂ determined through compression testing on the CellScale MicroTester system, where the red dashed line represents the average Young’s modulus of the unseeded DAT microcarriers and the black dashed line represents the average Young’s modulus of the unseeded COL microcarriers (n = 6 microcarriers/ASC donor, N = 3 donors).

3.4 Effects of Dynamic Culture on the ECM-derived Microcarriers on the hASC Immunophenotype

Flow cytometry analysis was performed utilizing a panel of markers commonly used to define ASC populations [20]. More specifically, the percentage of cells expressing the positive markers CD90 and CD29, the variable markers CD26 and CD34, and the negative markers CD45 and CD31 (combined on one channel) were assessed (Figure 3.8). As expected, less than 2 % of the cells in all samples expressed CD45 or CD31.
Interestingly, the percentage of cells expressing CD90 was found to vary between the different culture conditions (Figure 3.8A,B). Based on main effects analysis, both agitation rate and oxygen tension had significant effects on CD90 expression. Relative to the baseline hASCs cultured on tissue culture polystyrene (TCP) prior to microcarrier seeding, there was reduced expression of CD90 in the hASCs that were cultured on the microcarriers under 2 % O₂ for all 3 cell donors studied. In addition, for 2 out of 3 of the cell donors (donors A and B), there was a lower percentage of CD90⁺ cells on both the DAT and COL microcarriers cultured statically under 20 % O₂ relative to the baseline samples. However, due to cell donor variability in the expression levels, the difference in the average percentage of CD90⁺ cells was only statistically significant for the hASCs cultured statically or at 20 rpm on the DAT microcarriers under 2 % O₂ relative to the baseline levels.

In contrast, no significant differences were observed in CD29 expression, with > 80 % of cells expressing CD29 under all of the different culture conditions (Figure 3.9A,B).
Figure 3.8: CD90 expression is impacted by culturing on the DAT microcarriers under 2 % O₂. (A) The percentage of cells expressing CD90 was significantly reduced in the samples cultured statically or at 20 rpm on the DAT microcarriers under 2 % O₂ for 2 weeks relative to the cells at baseline, (N = 3 donors), *p<0.05. The black dashed line represents the average expression in the baseline hASCs cultured on TCPS. (B) Representative flow plots showing CD90 expression for hASC donor A cultured under 20 % O₂.
Figure 3.9: There were no significant differences in the percentage of cells expressing CD29 under the varying culture conditions relative to the baseline samples. (A) The percentage of cells expressing CD29 was maintained under all conditions, showing over 80% of cells expressed CD29 (N = 3 donors). The black dashed line represents the average expression in the baseline hASCs cultured on TCPS. (B) Representative flow plots showing CD29 expression for hASC donor A cultured under 20% O₂.
Notably, CD26 expression was variable between the different hASC donors, with generally lower expression levels in the samples cultured under 2 % O₂ (Figure 3.10A,B). Main effects analysis indicated that microcarrier composition and oxygen tension had significant effects on CD26 expression.
Figure 3.10: CD26 expression showed variability under all growth conditions, with decreased expression in the hASCs cultured on the microcarriers under 2 % O₂ relative to the samples cultured under 20 % O₂. (A) The percentage of cells expressing CD26 showed variable expression, with main effects analysis showing significant effects of both oxygen tension and microcarrier composition (N = 3 donors). The black dashed line represents the average expression in the baseline hASCs cultured on TCPS. B) Representative flow plots showing CD26 expression for hASC donor A cultured under 20 % O₂.
The CD34 expression levels were also donor-dependent, with donor B showing no expression under any of the culture conditions or in the baseline samples. For the other two donors, there were significantly fewer CD34+ cells after 2 weeks of culture on both microcarrier types cultured dynamically under 2 % O₂, as well as in all DAT microcarrier groups and the COL microcarriers cultured at 20 rpm under 20 % O₂ relative to the baseline populations (Figure 3.11A,B).
Figure 3.11: The percentage of cells expressing CD34 is donor-dependent, but is generally reduced following culture on the microcarriers relative to the baseline levels. (A) No CD34+ cells were detected in the cell donor B samples. For the other two donors, the percentage of CD34 was significantly reduced in the majority of the conditions studied relative to the baseline levels for each donor grown on TCPS (N = 3 donors), *p<0.05. The black dashed line represents the average expression in the baseline hASCs cultured on TCPS. B) Representative flow plots showing CD34 expression for hASC donor A cultured under 20 % O₂.
3.5 Effects of Culturing on the ECM-derived Microcarriers on the hASC Gene Expression Levels of Pro-angiogenic and Immunomodulatory Paracrine Factors

RT-qPCR analysis was performed to assess whether culturing under the different conditions on the ECM-derived microcarriers altered the gene expression levels of a range of pro-angiogenic and immunomodulatory paracrine factors known to be secreted by hASCs (Figure 3.12). Main effects analysis showed that oxygen tension significantly influenced vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) expression, with higher levels generally observed in the samples cultured under 2 % O₂. In addition, main effects analysis indicated that the substrate and agitation rate significantly influenced monocyte chemotactic protein 1 (MCP-1) expression levels.

Interestingly, there was an overall trend for enhanced expression of all markers investigated in the cells cultured on the COL microcarriers under 20 rpm and 2 % O₂. This difference was statistically significant compared to the levels in the baseline samples of the hASCs prior to microcarrier seeding for angiogenin, VEGF, and HGF. In addition, there was significantly higher levels of MCP-1 expression detected in this group relative to all other culture conditions and the baseline samples.
Figure 3.12: The gene expression levels of a range of pro-angiogenic and immunomodulatory factors are increased in the hASCs cultured on the COL microcarriers at 20 rpm and 2% O2. Quantitative analysis of gene expression assessed by RT-qPCR following 2 weeks of culture on the DAT or COL microcarriers under different stirring rates and oxygen tensions. Data was analyzed with the delta delta C_t method and normalized to the geometric mean of the two stable housekeeper genes, IPO8 and RPL13A, using the baseline samples for each cell donor grown on TCPS as the calibrator. The black dashed line represents the average expression in the baseline hASCs cultured on TCPS. (n = 3 samples/ASC donor, N = 3 donors), $p<0.05$ in comparison to all other culture conditions including the baseline samples, $p<0.05$ in comparison to the baseline levels.
Chapter 4

4 Discussion

There is growing interest in the application of adipose-derived stromal cells (ASCs) within the field of regenerative medicine due to their secretion of paracrine factors, which can have a range of pro-regenerative effects such as promoting cell survival, modulating the immune response and stimulating angiogenesis [34]–[37]. However, there are critical barriers that need to be addressed before ASC-based therapies can be broadly translated to the clinic. Key challenges include: (i) the large number of cells needed for treatment doses compared to relatively low ASC yields [61], [62], (ii) the decline in the pro-regenerative functionality of human ASCs (hASCs) associated with long-term expansion on tissue culture polystyrene (TCPS) [67], and (iii) poor cell survival following implantation when delivered into harsh environments, such as poorly vascularized tissues or wounds [78], [79].

To help address these limitations and move closer to clinical translation, various pre-conditioning strategies are being investigated as a way to prime ASCs with the goal of enhancing the capacity of the cells to stimulate regeneration through paracrine-mediated mechanisms during in vitro expansion [233]. Many different pre-conditioning strategies have been explored, including culturing under reduced oxygen tensions [234], [235], the application of dynamic culture to provide mechanical stimulation [86], [87], [236], the use of cell-instructive biomaterials that can guide cell function [76], [237], and stimulation with soluble factors or cytokines [92], [93].

Previous work in the Flynn lab by Dr. Claire Yu explored the use of cell-instructive biomaterials and dynamic culture on hASC proliferation and multilineage differentiation capacity through the development of decellularized adipose tissue (DAT) microcarriers and their application within spinner flask bioreactors [151]. Dynamic culturing of the hASCs on the DAT microcarriers was shown to increase hASC proliferation relative to hASCs cultured on Cultispher-S microcarriers fabricated from cross-linked porcine gelatin, and augment their multilineage differentiation capacity towards the adipogenic,
osteogenic and chondrogenic lineages following expansion. In addition, when analyzing if the immunophenotype of the hASCs cultured on the DAT microcarriers within the spinner flask bioreactors was maintained compared to TCPS controls, flow cytometry analysis found preserved expression of CD90 and CD44, and reduced relative expression of CD73, CD105 and CD29 [151]. Notably, CD34 expression was lowest in the hASCs that had been expanded on the DAT microcarriers relative to the baseline hASCs prior to microcarrier seeding, along with hASCs expanded on the Cultispher-S microcarriers or serially-passaged on TCPS [151].

The effects of biomaterials, dynamic culture and reduced oxygen tensions on hASC phenotype and function have also been explored within the Flynn lab by Dr. Tim Han, who used a perfusion bioreactor to expand hASCs on DAT scaffolds [226], [227]. hASC proliferation was increased in the scaffolds cultured within the bioreactor under 2 % O₂ relative to controls cultured under 20 % O₂, as well as under static conditions, with a high cell density achieved on the DAT scaffolds [227]. Moreover, culturing on the DAT scaffolds within the perfusion bioreactor under 2 % O₂ was shown to promote the expression of inducible nitric oxide synthase (iNOS) in the hASCs, and increase the secretion levels of the immunomodulatory factors interleukin 10 (IL10), C-X-C motif chemokine ligand 10 (CXCL10) and hepatocyte growth factor (HGF), while reducing the secretion of pro-inflammatory interleukin 6 (IL6) [226]. Importantly, subsequent in vivo testing in a subcutaneous implant model in athymic nu/nu mice showed that culturing the scaffolds within the bioreactor under 2 % O₂ prior to implantation significantly enhanced blood vessel formation and adipose tissue regeneration within the DAT [226], [227].

While the results were promising, the perfusion bioreactor was complex and could not be easily scaled up to generate sufficient cell numbers or constructs sized for use in humans. In addition, a non-homogeneous distribution of hASCs was observed on the larger three-dimensional (3D) DAT scaffolds applied within the bioreactor, suggesting that media perfusion may have been limited into the central scaffold regions [226], [227]. In contrast, the spinner flask bioreactors also showed promising results but were much simpler in terms of their set up, allowing easier tuning of the culturing parameters and a more homogeneous distribution of cells on the microcarriers [148], [151], [225]. Based
on this previous experience, it was decided to focus the current study on investigating the effects of varying culture conditions within the spinner flask bioreactors on hASC growth and phenotype, towards the long-term goal of harnessing their pro-angiogenic capacity in strategies to promote vascular regeneration. Spinner flask bioreactors are commonly used in cell expansion and differentiation of hASCs [136], [155], [238], as well as bone marrow-derived mesenchymal stromal cells (BMSCs) [133], [158], [239], and represent a versatile platform for the development of dynamic pre-conditioning strategies.

The investigation of cell-instructive biomaterials for cell culturing has shown the importance of both matrix composition and stiffness in modulating cell phenotype and function [95], [108]. In the current study, the DAT and COL microcarriers were designed to have similar starting Young’s moduli to more specifically probe the effects of extracellular (ECM) composition within the microcarriers. As previously discussed, DAT substrates have been investigated for ASC culturing, with the goal of mimicking the composition of the native cellular microenvironment, showing the capacity to increase proliferation in vitro, while maintaining the ASCs multilineage differentiation capacity [134], [151], [214]. Similarly, collagen-based substrates have been incorporated in mesenchymal stromal cell (MSC) expansion strategies in a variety of forms, such as collagen coatings [157], [240], composite biomaterials containing collagen [241], [242], and collagen scaffolds [243], [244]. Collagen-based substrates have been shown to provide a supportive growth environment for MSCs, increasing MSC proliferation, while maintaining differentiation capabilities [245]–[248]. Studies in the past have compared the cellular response on decellularized tissue scaffolds to collagen-based scaffolds, showing increased hASC proliferation on decellularized porcine peritoneum and bladder scaffolds, potentially due to higher levels of growth factors in the matrix [249], as well as increased human fibroblast survival and angiogenic function on more compositionally complex DAT scaffolds as compared to collagen controls [250].

Within spinner flask bioreactors, a wide range of agitation rates can be found throughout the literature, with most studies ranging between 20 to 80 rpm for ASC culturing [251], [252]. It is well known that MSCs are affected by shear stress within the spinner flask environment, with low shear stresses leading to microcarrier clumping and high shear
stress leading to cell death [130], [253]. The paracrine functions of MSCs may also be impacted by shear stress. More specifically, studies have shown that shear stress can enhance the secretion of immunomodulatory and pro-angiogenic factors by hASCs. In particular, studies have revealed that shear stress stimulation can enhance protein expression levels of HGF, vascular endothelial growth factor (VEGF), interleukin 8 (IL8) and granulocyte-macrophage colony stimulating factor (GM-CSF) [88], [120]. Moreover, conditioned media generated by hASCs cultured under shear stress has been shown to suppress tumor necrosis factor-α (TNF-α) secretion by activated immune cells [87], [254]. However, limited research has been conducted to date investigating the effects of shear stress under different oxygen tensions on ASC/MSC expansion and paracrine factor expression.

Oxygen tensions below 20 % have frequently been studied as a pre-conditioning strategy, commonly in the range of 2 to 5 % O₂ [84], [85]. Reduced levels of oxygen have been shown to promote ASC proliferation and modulate the ASC phenotype [227], as well as promote the capacity of ASCs to mediate endothelial cell proliferation and tubule formation [42], [255]. Culturing ASCs under hypoxic conditions stabilizes hypoxia inducible factor (HIF)-1α, which in turn modulates the secretion of factors including VEGF [256]. VEGF specifically has been shown to promote both angiogenesis and wound healing, and is thought to be a key mediator of the paracrine effects of MSCs [257], [258].

In order to probe cell attachment and growth on the different microcarrier substrates under the varying culture conditions, confocal imaging utilizing the LIVE/DEAD® assay was performed in conjunction with the Picogreen® assay over the 14 days in culture. All 20 % O₂ culturing conditions were found to support ASC growth, with more variable results obtained in the samples cultured under 2 % O₂. Although it is generally thought that hypoxia enhances ASC growth [89]–[91], [259]–[261], conflicting reports exist, with some studies showing no effect or reductions in ASC cell numbers [262]–[265]. For example, Ranera et al. found decreased equine ASC expansion in samples cultured under 5 % O₂ in comparison to 20 % O₂ over 7 days [264], while Wang et al. saw reduced growth of hASCs cultured at 5 % O₂ compared to 20 % O₂ over 14 days [265]. An
additional study by Noronha et al., saw increased human umbilical cord derived MSC expansion at 20 % O\textsubscript{2} in comparison to 5 % O\textsubscript{2} over 5 days on collagen-coated microcarriers in a stirred-tank bioreactor [266]. This decrease in growth may be due to the effects of hypoxia alone, or potentially to an increased sensitivity to shear stress under hypoxia, which should be explored further in future work. Within this project, the hASCs cultured dynamically under 20 % O\textsubscript{2} showed a trend for increased growth compared to static conditions, which is consistent with literature reports on the positive effects of dynamic culturing. For example, increased cell expansion was reported when equine ASCs were cultured under fluid shear stress compared to static conditions over 14 days [267].

During the cell expansion process, cell-mediated matrix contraction can occur due to increased cell growth on soft biomaterials [268], [269]. Cell-mediated matrix contraction has been reported to occur with multiple cell types, including fibroblasts [270], endothelial cells [271], chondrocytes [272], osteoblasts [273], and BMSCs [269], [274]–[276]. Sumanasinghe et al. investigated the effects of human BMSC-mediated matrix contraction in 3D type I collagen gels, finding increased contraction with an increased seeding density, as well as increased contraction in proportion to increased cell expansion [274]. The main cause of matrix contraction has been shown to be the compaction and translocation of collagen fibrils due to the tractional forces induced by the cells, which in turn stiffens the surrounding ECM [277], [278]. Within the current project, increased matrix contraction and microcarrier mechanical properties were generally associated with increased cell growth, consistent with the literature on BMSCs [269], [274]–[276]. However, matrix contraction may also be induced by the cells becoming more contractile in nature, as mediated by the shear stress of the environment and spreading area of the cells [279], [280]. For example, Sonam et al. reported that human BMSCs showed a more contractile phenotype on planar TCPS following 48 hours of continuous fluid flow generating 1 Pa of shear stress in comparison to static culture controls [279]. A change in the contractile phenotype of the cells may explain the findings of enhanced contraction in the DAT 40 rpm condition cultured under 2 % O\textsubscript{2}, despite the lower levels of cell growth observed.
However, it is important to note that cell-mediated matrix contraction may not be advantageous, as it could reduce long-term cell growth by limiting cell infiltration into the interior of the microcarriers due to contraction of the porous structure and limited diffusion of nutrients into the interior of the scaffolds [281]. Ma et al. showed by increasing the mechanical properties of type I collagen scaffolds through chemical crosslinking, the scaffolds were able to resist cell-mediated contraction by human fibroblasts [281]. Further, non-crosslinked scaffolds showed a reduced cell density over 21 days of culture due to high contraction in the first week, while crosslinked scaffolds showed increases in cell density over the 21 days of culture, with an even distribution of cells both on the surface and in the interior of the scaffolds [281]. Future studies could potentially investigate the effects of crosslinking the DAT and COL microcarriers. However, cell infiltration into the microcarriers may not be desirable if the system is being applied for pre-conditioning, as it would be expected to alter the exposure of the cells to the shear stress stimulation and could result in greater heterogeneity in the cellular response.

In order to assess the effects of the different culturing conditions on the hASC immunophenotype, flow cytometry was performed after 14 days of culturing. Based on the immunophenotype outlined by the International Society for Cellular Therapy (ISCT) in conjunction with the International Federation for Adipose Therapeutics and Science (IFATS) for ASCs [20], the hASC populations were expected to be > 80 % positive for CD90 and CD29, < 2 % positive for CD45 and CD31, and have variable expression levels of CD26 and CD34. The baseline hASCs cultured on TCPS were shown to meet the ISCT criteria. However, following 14 days of culturing on the microcarriers, CD90+ expression was shown to decrease below 80 % for all hASCs cultured under 2 % O₂, as well as those cultured statically on the DAT and COL microcarriers under 20 % O₂ for 2 out of 3 of the cell donors. Decreases in CD90+ expression have been reported in the literature for hASCs cultured on alginate hydrogels in response to the 3D environment [282], [283]. Both Follin et al. and Karlsen et al. observed decreases in CD90+ cells after 7 and 10 days of culturing respectively in hASCs cultured on alginate hydrogels compared to TCPS controls [282], [283]. Additionally, Karlsen et al. further cultured the hASCs on the alginate hydrogels under 6 % O₂ and saw similar decreases in CD90+
expression [283]. It is important to note that the ISCT criteria for the hASC immunophenotype were established based on expression levels in plastic-adherent ASCs. As such, it is quite possible that the phenotype of the hASCs could differ with 3D culturing [282]. In contrast to the previous study that showed reduced CD29 expression levels in hASCs cultured dynamically on the DAT microcarriers, which was postulated to be due to the shear stress generated in the dynamic system [151], CD29 expression levels were maintained in the current study. Possible reasons for this difference could include variations in microcarrier size (i.e. mean diameter of 428 ± 41 µm compared to 1321 ± 152 µm in the current study), as well as timepoint of analysis (3 weeks as compared to 2 weeks in the current study).

There is recent interest in the investigation of ASC subpopulations, selected for their positive expression of variable surface markers such as CD26 and CD34, with the hopes that they may have increased angiogenic and immunomodulatory capabilities [20]. CD26 expression has been correlated with macrophage recruitment [284], [285], and is also associated with an adipocyte precursor phenotype [286]. Further, in a murine contact burn model, treatment with pullulan-collagen hydrogels seeded with a subpopulation of CD26^+CD55^+ ASCs led to accelerated healing, increased dermal appendage count and an improved scar quality in comparison to both hydrogels seeded with unsorted ASCs and unseeded hydrogels [287]. In this project, higher levels of CD26 expression were generally observed when the cells cultured under 20 % O_2. Loss of CD26 expression under hypoxic culture conditions has been reported for other cell types such as preadipocytes [288] and endometrial cells, which showed reduced CD26 expression linked to an enhanced migratory capacity when cultured under 6.5 % O_2 [289]. Further studies are warranted to investigate the functional effects of the reduced CD26 expression in hASCs. The other variable marker, CD34, was selected as it has been correlated with pro-angiogenic functionality [17], [290] and maintenance of clonogenicity [291]. In one study, a subpopulation of hASCs co-expressing CD34 and CD90 were shown to have higher proliferative and angiogenic capacity, promoting the extensive formation of new vessels when implanted subcutaneously within collagen scaffolds into immunocompromised athymic nude mice [292]. While donor variability in the CD34 expression levels is expected [293], CD34 expression has generally been shown to
decrease with increased passaging of ASCs [27], [28], [291], consistent with the results in the current study.

To further investigate the impact of the varying culture microenvironments on the hASCs and assess whether there were conditions that showed pre-conditioning effects, RT-qPCR was performed to examine the gene expression levels of a range of paracrine factors known to be secreted by hASCs. Specifically, the gene expression levels of monocyte chemotactic protein-1 (MCP-1), IL6, angiogenin, VEGF and HGF were characterized. MCP-1 has been shown to promote tissue repair and angiogenesis following ischemic injury [294], [295]. IL6 plays a role in angiogenesis and can suppress inflammatory immune responses [296]–[298]. Angiogenin stimulates angiogenesis by enhancing endothelial cell proliferation and tubule formation, as well as binding to endothelial cells to promote invasiveness [39], [40]. As previously discussed, VEGF is a key mediator of the paracrine effects of ASCs, promoting both angiogenesis and wound healing [258], [296]. Finally, HGF also promotes both angiogenesis and wound healing, including by stimulating fibroblast proliferation and migration [43], [296].

Interestingly, the gene expression levels for most of these factors were shown to be increased in the hASCs cultured on the COL microcarriers under 2 % O2 at 20 rpm. Consistent with the findings in the current study, other studies have reported that intermediate or low levels of shear stress can be beneficial for enhancing paracrine factor expression, as opposed to higher levels of shear stress [299], [300]. For example, when varying levels of shear stress were applied to human endothelial cells over 24 hours, low levels of shear stress (1.5 dynes/cm²) were found to enhance the gene expression levels of immunomodulatory genes including IL6, IL8, MCP-1 and platelet-derived growth factor-β (PDGF-β) as compared to cells cultured under higher levels of shear stress (15 dynes/cm²) and static controls [300].

Additionally, as previously discussed, hypoxia has been shown in many studies to increase VEGF expression, as well as other paracrine factors such as basic fibroblast growth factor (bFGF) [257], [258]. For example, a study by Hsiao et al., showed increases in VEGF, IL8 and angiogenin gene expression levels in hASCs cultured on
TCPS under $<$ 0.1 % O$_2$ over 72 hours [301]. Further studies have shown that hypoxic pre-conditioning of MSCs can enhance their capacity to promote functional recovery in ischemic tissues, although this may be attributed in part to the increased survival of these cells in vivo in addition to altered paracrine function [302], [303].

The enhanced pro-angiogenic and immunomodulatory gene expression levels on the COL microcarriers relative to the DAT microcarriers was unexpected, based on previous findings that have shown augmented pro-angiogenic gene expression in human fibroblasts cultured statically under 2 % O$_2$ on DAT scaffolds relative to collagen controls [250]. This difference may be related to synergistic effects of the shear stress stimulation applied in the current study, variations in the response due to differences in cell-cell and/or cell-ECM interactions between scaffold formats, or potentially due to differences in the response between cell types. Regardless, the current findings support that the application of shear stress stimulation by dynamic culturing on the COL microcarriers had a pre-conditioning effect on the hASCs, and that it may be possible to alter paracrine factor expression levels by tuning the stirring rate.

In the current study, notable cell donor variability was observed. In the literature, donor characteristics, such as age, co-morbidities, and BMI, have been shown to alter ASC function [304]–[307]. In particular, decreases in hASC proliferation have been noted in hASCs extracted from obese patients in comparison to lean patients [308]–[310]. Frazier et al. reported a decrease in colony forming potential with increasing BMI [309]. Further, hASCs from obese patients have been reported to have reduced pro-angiogenic and immunomodulatory capacities [311]–[313]. More specifically, a significant reduction in capillary-like structures produced by human umbilical vein endothelial cells (HUVECs) was found in the presence of conditioned medium from hASCs from obese donors as compared to lean donors [313]. Similarly, Zhu et al. found that obesity impaired the capacity of ASCs to promote a shift in macrophage phenotype from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype within both in vitro co-culture models and in vivo in a unilateral renal artery stenosis mouse model [311]. While only a limited number of donors were included in the current study due to the broad range of conditions that were explored, it is interesting to note that donor A had a much higher
BMI than the other two donors, potentially explaining the reduced cell expansion and paracrine factor gene expression levels observed under all conditions with that donor. In the future, it would be worthwhile to expand the studies within this thesis to include a larger of number of hASC donors to identify significant differences in the response and assess the effects of varying factors including age, sex, and BMI, which will be important to consider in the development and translation of cell-based therapies.
Chapter 5

5 Conclusion

5.1 Summary and Significance

The current study investigated the effects of varying microcarrier substrates and culture conditions on human adipose-derived stromal cells (hASCs) cultured within spinner flask bioreactors. More specifically, the growth and phenotype of hASCs cultured on custom-fabricated microcarriers comprised of decellularized adipose tissue (DAT) or commercially-sourced purified collagen (COL) was compared under varying stirring rates (0, 20, 40 rpm) and oxygen tensions (20 % and 2 % O₂). The overarching goal was to determine if conditions could be identified that would effectively pre-condition the hASCs for applications in vascular regeneration, by enhancing cell expansion and promoting a more pro-angiogenic phenotype.

In the first aim, the DAT and COL microcarriers were fabricated via electrospraying and then characterized, showing microcarrier stability without chemical crosslinking, as well as structural and biomechanical similarity between the microcarrier substrates. Following characterization, the effects of the varying culture conditions on hASC growth were explored over 2 weeks in culture within the spinner flask bioreactors. Through confocal imaging utilizing the LIVE/DEAD® assay in conjunction with quantification of the double stranded DNA (dsDNA) content associated with the hASCs on the microcarriers via the Picogreen® assay, hASC growth was assessed within the different environments. Overall, the qualitative and quantitative methods of analyzing cell growth aligned, showing all 20 % O₂ culturing conditions supported hASC growth, while more variable growth was observed in the samples cultured under 2 % O₂. Additionally, a trend for enhanced cell growth was observed in the hASCs cultured under dynamic conditions at 20 % O₂ compared to static conditions. In general, enhanced cell growth resulted in increased contraction of the microcarriers that were dynamically cultured at both 20 % and 2 % O₂. The mechanical properties of the hASC-seeded microcarriers were also assessed after 14 days in culture under the varying conditions, showing that the
microcarriers remained soft and compliant, with a trend that the Young’s moduli were increased following culturing relative to the corresponding unseeded baseline samples. Overall, the first aim suggested that hASC growth was better supported and more consistent in the samples cultured dynamically under 20 % O₂ on both microcarrier substrates.

In the second aim, the immunophenotype of the hASCs was assessed for possible changes in standard hASC markers, along with variable markers that may be indicative of a more pro-angiogenic or immunomodulatory cell population. The expression levels of the two positive hASC markers, CD29 and CD90, showed contrasting responses to the different culture conditions. More specifically, there were no significant differences in CD29 expression levels under all culture conditions in the current study, with > 80 % of the cells expressing CD29⁺. In contrast, CD90 expression levels were found to vary between the different culture conditions. Particularly, hASCs cultured under 2 % O₂, as well as hASCs cultured statically on the microcarriers under 20 % O₂ for 2 out of 3 of the cell donors studied, showed reduced percentages of CD90⁺ cells compared to baseline controls of the hASCs cultured on tissue culture polystyrene (TCP-S) prior to microcarrier seeding. As expected, less than 2 % of the cells in all samples expressed the negative markers CD31 and CD45. Finally, the expression levels of the variable markers CD26 and CD34 were assessed. CD26 expression levels varied between the 3 hASC donors in the current study, but generally lower expression levels were observed in the cells that were cultured under 2 % O₂. CD34 expression levels were also donor dependent, with one donor showing no expression under any conditions or in the baseline samples. For the other 2 hASC donors, decreased expression of CD34 was observed across all conditions relative to the baseline levels. Overall, the culture conditions were shown to influence the hASC immunophenotype to varying extents. Further studies are warranted to determine whether these phenotypic changes correlate with altered functionality, including in terms of the capacity of the cells to promote regeneration through paracrine-mediated mechanisms, such as stimulating angiogenesis and modulating the response of immune cell populations.
Finally, in the third aim, to probe the effects of the culture microenvironments on the pro-angiogenic phenotype of the hASCs, RT-qPCR was performed to assess if the different culture conditions impacted the gene expression levels of a range of pro-angiogenic and immunomodulatory paracrine factors in the hASCs. Interestingly, the gene expression levels of all markers investigated, more specifically monocyte chemotactic protein-1 (MCP-1), interleukin 6, angiogenin, vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), were enhanced in the cells cultured on the COL microcarriers at 20 rpm and 2 % O\textsubscript{2}. These findings support that stirred bioreactor systems can be applied to pre-condition hASCs, and that factors including microcarrier composition, stirring rate, and oxygen tension can be tuned to alter the cell phenotype.

Taken together, this study emphasizes the importance of the culture microenvironment in mediating the growth, immunophenotype and paracrine factor expression of hASCs. Stirred bioreactor systems incorporating custom-fabricated microcarriers comprised exclusively of extracellular matrix (ECM) were successfully applied to expand hASCs, comparing hASCs on the DAT and COL microcarriers for the first time. Notably, the effects of a diverse range of variables were explored within this system, expanding on previous work in the literature, where more limited conditions are typically studied. The findings suggest that there is an intricate relationship between oxygen tension and shear stress that can influence the cellular response, with varying outcomes depending on the specific microcarriers applied as a growth substrate. With further testing, it may be possible to tune the microenvironment within the bioreactor system to develop a robust \textit{in vitro} pre-conditioning strategy that can both expand the cells and enhance their pro-regenerative capacities \textit{in vivo}, towards their future clinical application in strategies to promote vascular regeneration.

### 5.2 Limitations and Future Directions

The wide range of conditions included in the current studies provide a basis for understanding the combined effects of microcarrier composition, shear stress stimulation, and oxygen tension on the expansion and pro-angiogenic phenotype of hASCs. Based on the findings of this thesis, future work should focus on exploring whether the observed
phenotypic changes correlate with altered functional effects to determine the potential benefits of bioreactor pre-conditioning of the hASCs through cell expansion on the ECM-derived microcarriers under dynamic conditions within the stirred bioreactor system.

Based on the findings from the first aim, it would be interesting to investigate if the microcarrier size impacted the results of the study. Notably, the microcarriers used in this thesis were larger than those studied in the past from the Flynn lab [148], [151], [225]. It would be interesting to repeat the study with microcarriers fabricated within a microfluidics device, capable of creating microcarriers with much smaller diameters of 100 – 300 µm [314], [315], as well as smaller microcarriers with diameters of approximately 350 – 550 µm fabricated with the electrospraying approach, to see if the size of the microcarriers applied alters hASC growth and/or the hASC phenotype observed under the various culture conditions. Further, stirring rates as high as 80 rpm have been studied in the literature for mammalian cells cultured on microcarriers within stirred bioreactor systems [252]. Therefore, the investigation of stirring rates higher than 40 rpm would be interesting to explore to probe the effects of higher shear stresses on hASC expansion, phenotype, and paracrine gene expression.

Additionally, based on the findings from the third aim, future studies should assess whether the observed differences in gene expression levels correlate with changes at the protein level by analyzing hASC protein secretion through Luminex assays or enzyme-linked immunosorbent assays (ELISA). Also, functional assays should be conducted to verify that changes in the hASC secretome correlate with biological effects in terms of the capacity of the hASCs to promote angiogenesis and modulate the immune response. In vitro assays to determine the effects of pre-conditioning on the pro-angiogenic capacity of the hASC secretome could include human microvascular endothelial cell (HMVEC) tubule formation and proliferation assays [316], as well as testing in a chick chorioallantoic membrane (CAM) assay [317]. In vitro assays to determine the effects of pre-conditioning on the immunomodulatory capacity of the hASC secretome could include T-cell immunosuppression assays [318] or macrophage polarization assays [319].

As discussed, high levels of donor variability were observed in all aims of this study.
Increasing the number of cell donors, and exploring a variety of donor factors such as age, sex and BMI, would be recommended to further investigate donor-dependent differences in the cellular responses, as one standardized pre-conditioning strategy may not be optimal for all cell donors.

Finally, the COL microcarrier 20 rpm 2 % O₂ condition should be further explored in future studies due to the significant increases observed in the gene expression levels of the paracrine markers, although cell growth was more variable between donors in comparison to the samples cultured under 20 % O₂. More specifically, an in vivo hindlimb ischemia model could be applied to determine whether the enhanced gene expression levels correlate with enhanced pro-angiogenic functionality, building from the in vitro assays suggested above to obtain essential pre-clinical data as a next-step towards clinical translation. Within this model, angiogenesis following femoral artery ligation would be studied through assessment of the restoration of hindlimb perfusion by laser Doppler perfusion imaging (LDPI) and immunostaining for vascular markers within the muscles at various endpoints [320], to assess whether bioreactor pre-conditioning on the COL microcarriers effectively enhances the therapeutic capacity of the hASCs.
References


J. M. Gimble, B. A. Bunnell, E. S. Chiu, and F. Guilak, “Concise review: Adipose-derived stromal vascular fraction cells and stem cells: Let’s not get lost in


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Reduced Hypertrophic Cartilage Formation,” *Bioengineering*, vol. 9, no. 6, pp. 1–16, 2022, doi: 10.3390/bioengineering9060232.


Appendix A: Supplemental Data

**Supplementary Table A.1: ASC donor information**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Sex</th>
<th>Age</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>BMI</th>
<th>Surgical Site</th>
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<td>63</td>
<td>87.0</td>
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<td>33.6</td>
<td>Breast</td>
</tr>
<tr>
<td>B</td>
<td>Female</td>
<td>37</td>
<td>86.1</td>
<td>175</td>
<td>28.1</td>
<td>Breast</td>
</tr>
<tr>
<td>C</td>
<td>Female</td>
<td>49</td>
<td>68.7</td>
<td>158</td>
<td>27.5</td>
<td>Breast</td>
</tr>
</tbody>
</table>
Supplementary Figure A.1: DAT microcarriers qualitatively supported hASC attachment and growth within the spinner flask bioreactors. Donor A confocal images under (A) 20 % oxygen tension and (B) 2 % oxygen tension showing calcein⁺ live (green) hASCs on the DAT and COL microcarriers (blue due to ECM autofluorescence) over 2 weeks in culture under static or dynamic conditions within the spinner flask bioreactors. No ethidium homodimer-1⁺ dead (red) cells were observed on the microcarriers. Scale bar = 100 µm.
Supplementary Figure A.2: COL microcarriers qualitatively supported hASC attachment and growth within the spinner flask bioreactors. Donor A confocal images under (A) 20 % oxygen tension and (B) 2 % oxygen tension showing calcein+ live (green) hASCs on the DAT and COL microcarriers (blue due to ECM autofluorescence) over 2 weeks in culture under static or dynamic conditions within the spinner flask bioreactors. No ethidium homodimer-1+ dead (red) cells were observed on the microcarriers. Scale bar = 100 µm.
Supplementary Figure A.3: DAT microcarriers qualitatively supported hASC attachment and growth within the spinner flask bioreactors. Donor B confocal images under (A) 20 % oxygen tension and (B) 2 % oxygen tension showing calcein\(^{\text{+}}\) live (green) hASCs on the DAT and COL microcarriers (blue due to ECM autofluorescence) over 2 weeks in culture under static or dynamic conditions within the spinner flask bioreactors. No ethidium homodimer-1\(^{\text{-}}\) dead (red) cells were observed on the microcarriers. Scale bar = 100 \(\mu\)m.
Supplementary Figure A.4: COL microcarriers qualitatively supported hASC attachment and growth within the spinner flask bioreactors. Donor B confocal images under (A) 20 % oxygen tension and (B) 2 % oxygen tension showing calcein$^+$ live (green) hASCs on the DAT and COL microcarriers (blue due to ECM autofluorescence) over 2 weeks in culture under static or dynamic conditions within the spinner flask bioreactors. No ethidium homodimer-1$^+$ dead (red) cells were observed on the microcarriers. Scale bar = 100 µm.
Supplementary Figure A.5: DAT microcarriers qualitatively supported hASC attachment and growth within the spinner flask bioreactors. Donor C confocal images under (A) 20 % oxygen tension and (B) 2 % oxygen tension showing calcein+ live (green) hASCs on the DAT and COL microcarriers (blue due to ECM autofluorescence) over 2 weeks in culture under static or dynamic conditions within the spinner flask bioreactors. No ethidium homodimer-1+ dead (red) cells were observed on the microcarriers. Scale bar = 100 µm.
Supplementary Figure A.6: COL microcarriers qualitatively supported hASC attachment and growth within the spinner flask bioreactors. Donor C confocal images under (A) 20 % oxygen tension and (B) 2 % oxygen tension showing calcein$^+$ live (green) hASCs on the DAT and COL microcarriers (blue due to ECM autofluorescence) over 2 weeks in culture under static or dynamic conditions within the spinner flask bioreactors. No ethidium homodimer-1$^+$ dead (red) cells were observed on the microcarriers. Scale bar = 100 µm.
Appendix B: Research Ethics Board Approval

Date: 21 July 2022
To: Dr. Lauren Flynn
Project ID: 105426
Review Reference: 2022-105426-68983
Study Title: Tissue Engineering with Adipose-derived Stem Cells
Application Type: Continuing Ethics Review (CER) Form
Review Type: Delegated
REB Meeting Date: 09/Aug/2022
Date Approval Issued: 21/Jul/2022 09:11
REB Approval Expiry Date: 13/Aug/2023

Dear Dr. Lauren Flynn,

The Western University Research Ethics Board has reviewed the application. This study, including all currently approved documents, has been re-approved until the expiry date noted above.

REB members involved in the research project do not participate in the review, discussion or decision.

Western University REB operates in compliance with, and is constituted in accordance with, the requirements of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The REB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000946.

Please do not hesitate to contact us if you have any questions.

Electronically signed by:

Patricia Sergeant, Ethics Officer

Reason: I am approving this document

Note: This correspondence includes an electronic signature (validation and approval via an online system that is compliant with all regulations).
Curriculum Vitae

Education
M.E.Sc. Biomedical Engineering
Western University, London, ON, Canada
Start date: September 2020
B.Eng. Biological Engineering
University of Guelph, Guelph, ON, Canada
September 2015 – April 2020

Coursework
BME 9550B: Principles of Communication and Knowledge for Biomedical Engineers (Grade Received: 87)
MSK 9100B: Musculoskeletal Health Research B – Fundamental Concepts in Clinical and Health Services Research (Grade Received: 91)
BME 9508A: Fundamentals of Biomedical Engineering (Grade Received: 90)
MSK 9000A: Musculoskeletal Health Research A – Biomedical and Bioengineering Concepts (Grade Received: 94)

Awards
(M.E.Sc.) 2022 Till & McCulloch Meeting Travel Award, $2000 (October 2022)
(M.E.Sc.) Ontario Graduate Student Scholarship, $15,000 (Sept. 2021 – Aug. 2022)
(M.E.Sc.) Transdisciplinary Bone & Joint Training Award, $10,000 (Sept. 2020 – Aug. 2021)
(B.Eng.) University of Guelph Dean’s List (Sept. 2015 – Apr. 2020)
(B.Eng.) Linamar Entrance scholarship, $2500 (Sept. 2015)
(B.Eng.) Earl B. MacNaughton Entrance Scholarship, $2000 (Sept. 2015)
(B.Eng.) University of Guelph Entrance scholarship, $3000 (Sept. 2015)

Presentations
Tosh M and Flynn L. Investigation of dynamic culture on matrix-derived microcarriers as a strategy to modulate the pro-regenerative phenotype of human adipose-derived stromal cells. 2022 Till & McCulloch Meeting. Vancouver, British Columbia, Canada. October 2022

Volunteer Positions
Let’s Talk Science / Stem Cells Talks Volunteer (May 17, 2022)
Engineers without Borders / Gender Equity Pen Pal Program Volunteer (Sept. 2020 – Apr. 2022)
Let’s Talk Science / Stem Cells Talks Volunteer (May 4, 2021)

Teaching Assistantships