Scaffold Design Considerations for Soft Tissue Regeneration

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Graduate Program in Biomedical Engineering

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Abstract

Tissue engineering has emerged as a promising strategy for the replacement of degenerating or damaged tissues in vivo. Also known as regenerative medicine, integral to this therapeutic strategy is biomimetic scaffolds and the biomaterial structural components used to form them. In this study, three different biomaterial scaffolds for tissue engineering applications were fabricated: three-dimensional reverse embedded collagen scaffolds, polymer fusion printed polycaprolactone (PCL) scaffolds, and electrospun gelatin scaffolds. Three-dimensional collagen and PCL scaffolds promoted human adipose-derived stem/stromal cell (ASC) spreading, proliferation, and fibronectin deposition in vitro. Secondly, this study investigated the efficacy of exogenous galectin-3 delivery as a therapeutic in skin healing, given that galectin-3 has been implicated in several wound healing processes. Gelatin polymer blended with recombinant galectin-3 was electrospun into a protein delivery scaffold and employed in a murine model of cutaneous wound healing. Treatment of wounds with the galectin-3/gelatin scaffolds, or with topical galectin-3, did not enhance wound closure, re-epithelialization, or influence macrophage phenotypes in vivo.

Keywords

Wound Healing, Chronic Wound, Inflammation, Biomaterials, Tissue Engineering, Three-Dimensional Printing, Electrospinning, Collagen, Polycaprolactone, Gelatin, Galectin-3
Summary for Lay Audience

Following injury, the body’s natural healing mechanisms mount a defense against invading pathogens and repair skin to close the wound. Factors such as disease and advanced age may reduce the body’s ability to repair, resulting in a non-healing chronic wound. Chronic wounds pose a severe threat, causing pain, impaired limb function, prolonged infection, and may require hospitalization and limb amputation. Thus, research and design of biomaterials and tissue engineered scaffolds attempts to initiate healing and eventually restore tissue function. In this study, we use scaffold fabrication methods such as three-dimensional printing and polymer electrospinning to design materials that mimic the natural microenvironment and stimulate wound healing cell responses.
Co-Authorship Statement

This thesis was written by M.M. Di Gregorio with input, suggestions, and revisions from Dr. D.W. Hamilton. Experiments were designed by Dr. D.W. Hamilton. All studies were conducted by M.M. Di Gregorio.
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Thank you to all members of the Hamilton lab. It has been a pleasure to meet and work alongside an amazing group of scientists; I am thrilled to see what the future holds for each of you. JT and Georgia taught me animal studies. Adam taught me the fundamentals of electrospinning and was an excellent resource when I had questions. Sarah helped edit this thesis; Sanduni and Alex offered unlimited positivity and friendship. Thank you, Dr. John de Bruyn, for teaching me rheological testing. Thank you also to Dr. Rizkalla and Khalid for your collaboration and introducing me to polymer fusion 3D printing.

Thank you to my family especially; Dad, Oma and Nonno, for your endless encouragement and support. Sonja, my sister, who’s work ethic is inspirational. Thank you, Hunter, for always being there for me, and for all the laughs.
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<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>AMM</td>
<td>Amniotic mesenchymal stem cell</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ADSC</td>
<td>Adipose-derived stem cell</td>
</tr>
<tr>
<td>AD-MSC</td>
<td>Adipose-derived mesenchymal stem cell</td>
</tr>
<tr>
<td>AMSC</td>
<td>Adipose MSC</td>
</tr>
<tr>
<td>ASC</td>
<td>Adipose-derived stem/stromal cell</td>
</tr>
<tr>
<td>Asc 2-P</td>
<td>Ascorbic acid 2-phosphate</td>
</tr>
<tr>
<td>AM</td>
<td>Additive manufacturing</td>
</tr>
<tr>
<td>BGP</td>
<td>β-glycerophosphate, glycerol 2-phosphate</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone marrow derived macrophage</td>
</tr>
<tr>
<td>BO</td>
<td>Bio-Oss</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>Computer-aided design</td>
</tr>
<tr>
<td>CCN2</td>
<td>Connective tissue growth factor 2</td>
</tr>
<tr>
<td>CKI</td>
<td>Cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>CSR</td>
<td>Controlled shear rate</td>
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<tr>
<td>CXCL</td>
<td>Chemokine ligand</td>
</tr>
<tr>
<td>c-Abl</td>
<td>Abelson tyrosine kinase</td>
</tr>
<tr>
<td>DCB</td>
<td>Decellularized bone</td>
</tr>
<tr>
<td>DFU</td>
<td>Diabetic foot ulcer</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FDA</td>
<td>Federal Drug Association</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FRESH</td>
<td>Freeform reversible embedding of suspended hydrogel</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glycogen synthase kinase-3 beta</td>
</tr>
<tr>
<td>G’</td>
<td>Storage modulus</td>
</tr>
<tr>
<td>G”</td>
<td>Loss modulus</td>
</tr>
<tr>
<td>HBOT</td>
<td>Hyperbaric oxygen therapy</td>
</tr>
<tr>
<td>HF</td>
<td>Hair follicle</td>
</tr>
<tr>
<td>HFSC</td>
<td>Hair follicle stem cell</td>
</tr>
<tr>
<td>IBMX</td>
<td>Isobutylmethylxanthine</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IFE</td>
<td>Interfollicular epidermis</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M1</td>
<td>Classical activated macrophage</td>
</tr>
<tr>
<td>M2/a</td>
<td>Alternative activated macrophage</td>
</tr>
<tr>
<td>M2b</td>
<td>Type 2 macrophage</td>
</tr>
<tr>
<td>M2c</td>
<td>Deactivated macrophage</td>
</tr>
<tr>
<td>M2d</td>
<td>M2-like macrophage</td>
</tr>
<tr>
<td>MCR</td>
<td>Modular compact rheometer</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MRWD</td>
<td>Moisture-retentive wound dressing</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NPWT</td>
<td>Negative-pressure wound therapy</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered solution</td>
</tr>
<tr>
<td>PC</td>
<td>Progenitor cell</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly(ε-caprolactone)</td>
</tr>
<tr>
<td>PCLDMA</td>
<td>Polycaprolactone dimethylacrylate</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
</tbody>
</table>
PEGDA Poly(ethylene glycol) diacrylate
PEO Poly ethylene oxide
PI Propidium Iodide
PLA Polylactic acid
PLGA Poly(lactic-co-glycolic)acid
PPARγ Peroxisome proliferator-activated receptor gamma
PSA Prostate-specific antigen
PU Pressure ulcer
PVA Polyvinyl alcohol
RM Regenerative medicine
RNA Ribonucleic acid
ROI Reactive oxygen intermediate
RPM Revolutions per minute
RUNX2 Runt-related transcription factor 2
SC Stem cell
SG Sebaceous gland
SMA Smooth muscle actin
SMC Smooth muscle cell
STL Stereolithography
SVF Stromal vascular fraction
T3 Triiodothyronine
TCP Tricalcium phosphate
TCPS Tissue culture polystyrene
TBS Tris-buffered saline
TE Tissue engineering
TGF Transforming growth factor
TIMP Tissue inhibitor of matrix metalloproteinase
TNF Tumor necrosis factor
TRM Tissue resident memory
UV Ultraviolet
VEGF Vascular endothelial growth factor
<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>VLU</td>
<td>Venous leg ulcer</td>
</tr>
<tr>
<td>V/V</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Viscosity</td>
</tr>
<tr>
<td>$</td>
<td>\eta^*</td>
</tr>
<tr>
<td>$\tau$</td>
<td>Shear stress</td>
</tr>
</tbody>
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1 Introduction
1.1 Cutaneous Wound Healing

1.1.1 Anatomy and Physiology of Skin

Skin is part of the integumentary system and represents the largest organ of the human body. Skin is multifunction: it acts as a selectively permeable barrier to protect the body from harmful pathogens in the external environment; thermoregulation is achieved through blood vessel control and perspiration via sweat glands; cutaneous sensory receptors, including mechanoreceptors, nociceptors (pain) and thermoreceptors, mediate the body’s interactions with the external environment. The epidermis, dermis, and subcutaneous hypodermis of skin are comprised of a variety of cell types with specific biological roles (Table 1).

The epidermis is subdivided into five layers: the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum. The epidermis is the outermost layer of skin and provides a cell-dense barrier between the body and the external environment. Keratin, the main structural protein found in the epidermis, is water insoluble and mechanically protects epithelial cells from damage or stress while preventing the invasion of foreign bodies. This water-resistant barrier is synthesized by keratinocytes, which are the dominant cell type within the epidermis, accounting for 95% of the cells located in this layer. Cornification, or keratinization, is the process by which proliferating keratinocytes of the basal layer undergo terminal differentiation into highly specialized corneocytes. In this process, keratinocytes migrate superficially, become flat, polyhedral, anucleated, and lose 55% of their water volume. Other cell types present in the epidermis include melanocytes, which produce melanosomes containing melanin. This protein provides skin with pigmentation and protects skin from ultraviolet radiation that can cause sunburn injury to skin, damage to nucleic DNA, and accelerated aging of skin. Merkel cells, also known as Merkel-Ranvier cells or tactile epithelial cells, are oval-shaped mechanoreceptors essential for light touch sensation in hairless skin of vertebrates. Langerhans cells also occupy the epidermis and act as antigen-presenting cells.

The dermal layer confers pliability, elasticity, and tensile strength to skin. These mechanical properties are achieved through a diverse arrangement of connective tissues, cell types, and structural components. The extracellular matrix (ECM) is a three-dimensional network consisting of extracellular fibrous proteins such as reticulin, elastin, fibronectin, laminin.
and collagen, and hydrated gels of glycosaminoglycans (GAGs) linked to proteoglycans. The ECM occupies the interstitial space, providing structural support and biochemical cues to cells, while also acting as a compression buffer. The most abundant ECM protein is collagen (types I and III), which is synthesized by dermal fibroblasts and conveys structural integrity to the skin by resisting stress. In addition to fibroblasts, various leukocyte populations (including neutrophils and macrophages) can enter the dermis through the vascular networks in response to different stimuli. Blood vessels, lined with squamous endothelial cells, can become permeable allowing leukocytes and clotting proteins access to connective tissue. On the outside of blood vessels, pericytes wrap around capillaries and confer structural integrity to the vessel wall. Other components of the dermal layer include epidermally derived appendages such as hair follicles, nails, and sebaceous, sweat and mammary glands; also, dermal dendrocytes, mast cells, histiocytes, blood vessels, nerves, and lymphatics. The dermis is subdivided into the upper papillary dermis and lower reticular dermis, with distinctions in the structure and organization of collagen and elastin fibers between these sublayers. The dermal vasculature provides nutrition to the skin and assists the body in thermoregulation. Dilation and constriction of blood vessels leads to heat dissipation or conservation, respectively. Thermoregulation is also controlled by arrector pili muscles (attached to hair follicles) and sweat glands. Piloerection, the erection or bristling of hairs, traps air close to the skin for an additional layer of insulation. Endothermic evaporation of sweat cools the surface of the skin.

The subcutaneous hypodermis consists primarily of fatty adipocytes but also contains fibrous septa of loose connective tissue, nerves, and blood vessels. Hypodermal fat lobules insulate and cushion the body, provide buoyancy, and store energy. In addition to adipocytes, fibroblasts and macrophages are also found in the hypodermis. Together, the epidermis, dermis, and hypodermis allow skin to maintain a physical barrier to the external environment; if this barrier is disrupted via injury to the skin, a spatiotemporally coordinated process is initialized in order to restore barrier function, maintain internal homeostasis and guard sterility.

Skin cells are continually shed and replenished through desquamation, the shedding of the outermost layer of the stratum corneum. In this process, individual corneocytes are shed following degradation of cell-cell junctions known as corneodesmosomes. The rate of corneodesmosome degradation is highly regulated. The self-perpetuating skin barrier is
maintained by permanently residing stem cells that sustain principal differentiated epidermal lineages, the interfollicular epidermis (IFE), sebaceous gland (SG), hair follicle (HF), and Merkel cell mechanoreceptors. Through radiation dose-survival studies, it was proposed that stem cells comprise about 2-7% of basal layer cells while another study of murine basal layer cells suggested a larger stem cell population, 10-12% of cells in the basal layer. Maintenance of skin homeostasis is dependent on the ability of stem cells to replenish the turnover of apoptosing epithelial lineages.

Epithelial stem cells (SCs) reside in a specific microenvironment called the niche, where stem cell behaviour is influenced by cell-cell communication, cell-ECM interactions, and growth factors. Cutaneous mesenchymal stem/stromal cells (MSCs) include dermal papilla cells (DPC) involved in hair follicle cycling, and the dermal sheath cells (DSC), which are capable of differentiating into wound healing fibroblasts that repair the dermis. Human FH-derived DPCs and DSCs have been shown to differentiate into adipogenic and osteogenic lineages in vitro, suggesting multi-lineage potential of these MSCs. Hair follicle stem cells (HFSCs) reside in the follicle bulge and have been shown to give rise to IFE, HF, and SG lineages after transplantation. Under normal physiological conditions, however, lineage tracing in mice has shown that HFSCs only contribute to HF regeneration and not the SG, IFE, or infundibulum.

In the IFE, a single basal layer of proliferative cells replenishes suprabasal terminally differentiated cells. This basal layer consists of a heterogeneous proliferative population of quiescent, long-lived IFE SCs and short-lived, differentiation-fated progenitor cells (PC). Between the bulge and SG is the isthmus, where another pool of resident SCs maintain the isthmus, SG, SG ducts, and infundibulum. Epithelial SCs are normally confined to compartmental niches but can be activated and recruited to different regions during wound repair where these SCs contribute to regeneration of wounded skin.
Table 1. A summary of cutaneous cell types within the epidermis, dermis, and hypodermis.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epidermis</strong></td>
<td></td>
</tr>
<tr>
<td>Keratinocyte</td>
<td>Express involucrin and keratin; form barrier to external environment&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>Merkel Cell</td>
<td>Mechano-sensitive; Merkel cell-neurite complexes&lt;sup&gt;14&lt;/sup&gt;</td>
</tr>
<tr>
<td>Melanocyte</td>
<td>Produce melanin pigment for skin colour&lt;sup&gt;12&lt;/sup&gt;</td>
</tr>
<tr>
<td>Langerhans Cell</td>
<td>Capture microbial antigens to become antigen-presenting cells&lt;sup&gt;15&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tissue-resident memory (TRM) (αβ) T Cell</td>
<td>Rapid recognition of previously encountered pathogens&lt;sup&gt;60&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epidermal-Resident (γδ) T Cell</td>
<td>Balance keratinocyte differentiation and proliferation with the destruction of infected or malignant cells&lt;sup&gt;61&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Dermis</strong></td>
<td></td>
</tr>
<tr>
<td>Fibroblast</td>
<td>Secrete ECM, mainly collagen types I and III&lt;sup&gt;62&lt;/sup&gt;</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>Phagocytosis and intracellular degradation, release of granules, and formation of neutrophil extracellular traps&lt;sup&gt;63&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
| Macrophage                                    | Classical (M1) phagocytose foreign bodies, release pro-inflammatory cytokines<sup>64</sup>  
                          | Alternative (M2) induce proliferation and collagen production, release anti-inflammatory cytokines<sup>64</sup> |
| Endothelial Cell                              | Line blood vessels (arteries, veins, capillaries and sinusoids)<sup>18</sup> |
| Pericyte                                       | Contractile cells present at intervals along capillaries; attribute structural integrity and constrict during ischemia<sup>19</sup> |
| Dendrocyte                                    | Antigen presentation<sup>65</sup>                                        |
| Mast Cell                                     | Regulate vasodilation, vascular homeostasis, innate and adaptive immune responses, angiogenesis, and venom detoxification<sup>66</sup> |
| Histiocyte                                    | Connective tissue resident; phagocytosis and antigen presentation<sup>67</sup> |
| **Hypodermis**                                |                                                                          |
| Adipocyte                                      | Store energy and cushion body<sup>68</sup>                              |
| Mechanoreceptors*                             | Type 1 sense quivering and touch<sup>13</sup>                          
                          | Type 2 sense vibration and pressure<sup>13</sup>                       |
| Thermoreceptors*                              | Cold type sense temperatures < 30°C<sup>13</sup>                       
                          | Heat type sense temperatures 32-48°C<sup>13</sup>                      |
| Nocireceptors*                                | Mechano sense significant pressure, inflammatory mediators, ischemia mediators<sup>13</sup>  
                          | Polymodal sense inflammatory mediators<sup>13</sup>                   |
| Pruriceptors*                                 | Sense histamine and inflammatory mediators<sup>13</sup>               |

*Cutaneous sensory endings of the peripheral nervous system innervating the skin derive from the dorsal root ganglia and the trigeminal ganglia. Nerves in the skin form epidermal plexus from which some fibres cross the dermo-epidermal junction.<sup>13</sup>
1.1.2 Acute Wound Healing

Following injury, normal wound healing is achieved via four overlapping phases: hemostasis, inflammation, proliferation, and remodeling (Table 2). Thrombin, the principal enzyme involved in hemostasis, is produced primarily in the liver and circulates systemically in the blood plasma. This serine protease cleaves fibrinogen to create a polymerized fibrin matrix that captures circulating platelets to form a hemostatic plug and establish hemostasis. Within the first hour post-injury, inflammatory cytokines such as interleukin (IL) -1α, IL-1β, IL-6, IL-8, tumor necrosis factor (TNF) -α, platelet derived growth factor (PDGF), and transforming growth factor (TGF) -β, are released by aggregated platelets to recruit early inflammatory cells and initiate healing. When injury first occurs, vasoconstriction is induced locally to reduce blood loss. However, this is subsequently reversed to allow changes in blood flow, an increase in permeability of blood vessels, and the infiltration of fluid, proteins, and leukocytes from the circulatory system into the site of tissue damage.

Recruited leukocytes produce a chemotactic gradient that stimulates the migration of neutrophils, monocytes, smooth muscle cells (SMCs), and fibroblasts. Neutrophils, which comprise 60-70% of the leukocyte population, are part of the innate immune response and one of the first cell types to respond to injury. Neutrophils are granulocytes and lack immune memory; they function by releasing reactive oxygen species to produce toxic metabolites (hydrogen peroxide) that kill invading bacteria and certain fungal species, secreting serine proteases and matrix metalloproteinases (MMPs) to debride necrotic tissue, and phagocytosing dead bacteria and wound debris. Neutrophils cannot renew their lysosomes and are short-lived, resulting in the formation of a white exudate called pus within the wound bed. Monocytes responding to paracrine signals, including TGF-β, differentiate into macrophages capable of phagocytosing debris and exhausted neutrophils. Macrophages that are classically activated (M1 polarization), typically by interferon (IFN) -γ or lipopolysaccharide (LPS) in vitro, produce pro-inflammatory cytokines, phagocytize microbes, express inducible nitric oxide synthase (iNOS), and produce nitric oxide (NO) or reactive oxygen intermediates (ROI) to protect against bacteria and viruses. Alternatively activated (M2) macrophages, typically activated by exposure to certain cytokines such as IL-4, IL-10, or IL-13, produce either polyamines to induce proliferation or proline to induce collagen production. These M2 macrophages secrete arginase I and anti-inflammatory cytokines, and consequently are associated with resolution of the inflammatory phase, pro-
regenerative wound healing and tissue repair. Following exhaustion of inflammatory cells, exocytotic release of a second wave of signaling molecules, including TGF-α, TGF-β, fibroblast growth factor (FGF) 2/basic FGF (bFGF), PDGF, and vascular endothelial growth factor (VEGF), recruits keratinocytes, fibroblasts, endothelial cells, and pericytes to initiate the overlap of late-stage inflammation with the proliferative stage of healing.71

During fibroplasia, fibroblasts proliferate and migrate into the wound bed, depositing collagen-rich ECM to slowly replace the fibrin clot with a permanent matrix.72 This ECM improves structural integrity of the damaged site and provides a supportive scaffold for neovascular growth (angiogenesis) and re-epithelialization.73 Through cadherin-11-mediated adhesion between macrophages and fibroblastic cells, TGF-β-producing macrophages and TGF-β-activating myofibroblasts are held in close proximity, facilitating efficient myofibroblast activation and stimulating fibrosis.74 Persistent fibroplasia leads to the accumulation of a dense fibrotic scar tissue.72 As ECM is deposited, re-epithelializing keratinocytes upregulate anti-fibrotic urokinase-type plasminogen activator, MMP-1 and MMP-3, and downregulate pro-fibrotic connective tissue growth factor 2 (CCN2), collagen I and II, fibronectin, plasminogen activator inhibitor-1, α-smooth muscle actin (α-SMA), and tissue inhibitor of matrix metalloproteinase (TIMP)-2/3.75 Maturation of the granulation tissue, composed of the newly deposited collagen network and migrating endothelial cells and macrophages, is enhanced by angiogenesis and re-epithelialization.76

Angiogenesis, vascular growth from pre-existing vasculature via endothelial cell migration, proliferation and vessel formation, re-establishes normoxia and nutrient supply to tissues.71,77 Post-injury hypoxic conditions promote the release of inflammatory mediators to increase vascular permeability and dilation, facilitating endothelial cell migration into the wounded tissue.78,79 Angiogenic factors, particularly VEGF-A and FGF-2/bFGF, promote endothelial cell proliferation and the formation of new capillary tubules in the developing granulation tissue to restore vascularization.80–83 To prevent excessive scar formation, wound progression through fibroplasia and angiogenesis must be tightly regulated. Proteolytic ECM remodeling decreases ECM density, decreases cell-ECM interactions (by cleaving matrix components such as collagen, fibronectin and laminin84), and releases matrix-bound angiogenic factors to stimulate endothelial cell migration.85
During re-epithelialization, epithelial cell proliferation and migration is initiated. Fibroblasts and macrophages release epidermal growth factor (EGF), TGF-α and FGF, initiating re-epithelialization. Hemidesmosome links between the epidermis and the basement membrane separate, releasing cell-ECM linkages. Keratinocyte migration over the granulation tissue separates the eschar from viable tissue. Behind the migrating epithelial tongue, keratinocytes proliferate and mature, ultimately restoring the barrier function. Macrophages secrete TGF-β that signals fibroblasts to migrate into the granulation tissue and produce new ECM components. Macrophage-activated myofibroblasts, highly contractile cells, anchor to the ECM and contract the wound, pulling opposing edges together. Myofibroblast contractility is dependent on a positive feedback loop initiated by endogenous TGF-β and tensile forces, which increases the density of α-SMA enrichment in stress fibers, increases force production and tension development, and consequently upregulates TGF-β. Re-modeling of granulation tissue occurs as fibroblasts upregulate expression of collagen I, and MMPs degrade the temporary collagen III matrix. In the final stages of remodeling, the scar tissue consists of mainly an acellular matrix of parallel collagen I fiber bundles. The repaired tissue which has the composition of a scar does successfully restore barrier function, but possesses approximately 80% of the mechanical elasticity of unwounded skin. However, in certain instances, skin can remain compromised and demonstrate a reduced ability to heal.
Table 2. Spatiotemporal coordination of normal wound healing.

<table>
<thead>
<tr>
<th>Post-Injury Period</th>
<th>Within Minutes</th>
<th>Within Hours</th>
<th>~3 Days</th>
<th>~1 Week</th>
<th>Weeks</th>
<th>Years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound Healing Stage</td>
<td>Hemostasis</td>
<td>Inflammation</td>
<td>Proliferation</td>
<td>Remodeling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active Cell Types</td>
<td>-Platelets</td>
<td>-Neutrophils -M1 macrophages -Mast cells</td>
<td>-Fibroblasts -M2 macrophages -Endothelial cells -Keratinocytes</td>
<td>-Myofibroblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major Events</td>
<td>-Vasoconstriction -Hemostatic plug formation -Chemotactic recruitment of inflammatory cells</td>
<td>-Inflammatory cell migration into the wound bed -Defense against foreign infection</td>
<td>-Granulation tissue -Fibroplasia -Randomly oriented ECM -Angiogenesis -Re-epithelialization</td>
<td>-Parallel collagen ECM -Contraction of wound bed -Scar management</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.1.3 Chronic Wounds

Reduced ability to heal is often the result of a variety of morbidities including vascular insufficiency, diabetes, malnutrition, patient age, pressure, infection, and edema.\textsuperscript{70} A persistent tissue injury that fails to proceed through the reparative process after 3 months is classified as a chronic (non-healing) wound.\textsuperscript{92} Such wounds become a significant burden to the patient, causing pain, impaired limb function, sepsis, hospitalization and the need for amputation. Worldwide, chronic wounds account for approximately 4\% of the global healthcare expenditure.\textsuperscript{93–96} Within Canada, chronic wounds pose a significant burden to the Canadian healthcare system, costing on average $10,000 to treat a single wound.\textsuperscript{97} The most common chronic wound classifications are venous leg ulcers (VLUs), arterial ulcers, diabetic foot ulcers (DFUs) and pressure ulcers (PUs).

The largest class by occurrence is VLUs, caused by chronic venous insufficiency as a result of trauma, aging, obesity, pregnancy, phlebitis, deep vein thrombosis, congestive heart failure, and a history of ulcers.\textsuperscript{70,89} Arterial ulcers, formed following arterial disease and inadequate blood supply to the skin, are most commonly the manifestation of systemic atherosclerosis.\textsuperscript{98,99} DFUs affect approximately 10\% of diabetics annually.\textsuperscript{89} Hyperglycemia-induced impaired nerve function reduces sensation, causing foot deformity, limited joint mobility, and structurally compromised peripheries.\textsuperscript{98} DFUs most frequently appear on the sole of the foot where peripheral neuropathy of the foot increases the risk of ulceration from repeated mechanical stress.\textsuperscript{100} Finally, PUs are localized areas of necrotic tissue formed as a result of extended periods of soft tissue compression, causing localized hypoxia and ischemic-reperfusion injury. PUs, also called bedsores or decubitus ulcers, are associated with impaired mobility, decreased level of consciousness, diabetes mellitus, peripheral vascular disease, malnutrition, and fecal/urinary incontinence.\textsuperscript{101} With increasing prevalence of obesity, diabetes and atherosclerosis, as well as an aging population, the impact of chronic wounds on the healthcare system will continue to rise.

At the molecular level, chronic wounds are characterized by dysfunctional proteolytic activity and prolonged inflammation, bacterial infection, cellular senescence and constrained cell proliferation.\textsuperscript{102} Enrichment of proteases and reactive oxygen species leads to a deficient ECM structure, damages cell membranes leading to premature senescence, and interferes with the transcription of pro-inflammatory signaling molecules, up-regulating various MMPs and down-regulating antagonistic TIMPs.\textsuperscript{103,104} Prolonged inflammation and greater than normal
populations of neutrophils and macrophages are hallmarks of chronic wounds. Abundance of these inflammatory cells cause accumulation of proteolytic factors such as MMP-9, neutrophil elastase, and proteinase-3 within chronic wounds, indicating a disturbed balance between proteases and their inhibitors. This imbalance can lead to excessive breakdown of matrix, ultimately hindering re-epithelialization and angiogenesis.

During prolonged inflammation, neutrophils and macrophages release pro-inflammatory cytokines such as IL-1β and TNF-α which advances further MMP and TIMP imbalance. Under normal conditions, pro-fibrotic macrophages release soluble factors (such as TGF-β, PDGF-β and galectin-3) to counteract the degradation of extracellular matrix by MMPs. TGF-β, PDGF-β and galectin-3 increase the deposition of matrix and the release of TIMPs by myofibroblasts. Chronic wounds however represent a function of significantly increased neutrophil and pro-inflammatory macrophage populations. Thus, the switch from inflammation to proliferation, requiring downregulation of anti-inflammatory factors and initiation of fibroplasia, neoangiogenesis, and remodeling, is severely compromised in chronic wounds. In particular, the expression of IL-1β and TNF-α is upregulated during pathological wound healing. These cytokines signal macrophages to release MMPs, and also suppress the synthesis of ECM proteins and TIMPs. A structurally immature ECM reduces or inhibits cellular migration, prolonging the wound healing process. This characteristic unbalanced proteolytic activity results in extreme degradation of ECM components, protease inhibitors, growth factors, and other wound repair mediators, creating a harsh microenvironment with abnormal biochemical and physical cues. Wound healing cell types rely on biochemical and physical signals in order for normal cellular activity to take place, which again delays the final stages of wound healing. Bacterial colonization of the affected tissue is an additional challenge resulting from compromised barrier function. This continuous bacterial presence contributes to a feedforward mechanism which elevates the proinflammatory response, preventing inflammatory resolution even further. Substantial bacterial presence generates a biofilm, a slippery buildup of bacteria, which impedes wound closure and blocks topical application of antibiotic treatments.

Angiogenesis normally ensures adequate nutrition and oxygenation during tissue healing. However, in the chronic wound microenvironment, antiangiogenic factors, for example myeloperoxidase, are upregulated while angiogenic stimulators, such as extracellular superoxide dismutase and VEGF, are diminished. Proteolytic degradation of angiogenic factors and
pathological angiogenesis are possible mechanisms that lead to the development of inadequate capillary growth witnessed in chronic venous leg ulcers.\textsuperscript{113–116}

1.1.4 Clinical Treatment of Chronic Wounds

Medical intervention for a chronic wound includes debriding (cleaning) the affected region, removal of necrotic tissue, management of localized and systemic infection, application of bandages, mechanical off-loading to reduce shear forces on the skin and injury recurrence, and restoration of blood flow to the wounded tissue.\textsuperscript{117} Furthermore, the application of advanced multidisciplinary wound care strategies, such as combining pressure off-loading and revascularization with biomedical engineering approaches, has under certain circumstances significantly improved chronic wound healing outcomes.\textsuperscript{118–120}

Debridement involves the removal of unwanted calluses, necrotic tissue, foreign debris, and pathogens from the wound in order to minimize infection and expose underlying healthy tissue to the wound edge.\textsuperscript{90} Surgical debridement is the preferred method and involves excising tissue with scissors or a scalpel. However, this procedure can be painful and may damage viable tissue depending on the surgeon’s ability to distinguish regions of affected tissues from unaffected. Thus, a more accurate means of targeting only the nonviable tissue is by autolytic debridement, which degrades nonviable tissue via the catabolic action of endogenous enzymes. DuoDerm, a clinically available hydrocolloid dressing with autolytic properties, reduced pericapillary fibrin cuffs that can cause venous disease and chronic venous ulcers.\textsuperscript{121} Similarly, enzymatic debridement involves exogenous enzymes added exogenous to topical ointments to degrade and remove necrotic material from the wound bed.\textsuperscript{122} Collagenase, the sole enzymatic debriding agent approved by the Federal Drug Association (FDA), degrades bioactive fragments within the wound bed in order to increase endothelial cell and keratinocyte migration.\textsuperscript{123} Lastly, biosurgical debridement involves the usage of medical grade maggots (\textit{Lucilia sericata}, \textit{Phaenicia sericata}, \textit{Lucilia cuprina}) that ingest necrotic tissue.\textsuperscript{122,124–126}

Infection management includes the use of cleansing agents such as water, saline, or 0.5\% acetic acid, and topical antimicrobials such as low-concentration povidone iodine, cadexomer iodine gel beads, metronidazole gel, silver, and medical grade manuka honey, to reduce bacterial growth.\textsuperscript{122} Unchecked bacterial invasion can be detrimental as infection spreads into adjacent
tissues, leading to necrotizing infection, gangrene, or deep abscesses with the need for amputation.\textsuperscript{127}

Covering a wound with dressings provides protection from infection, maintains moisture, absorbs exudate, and promotes tissue regeneration.\textsuperscript{89,122} Specifically, moisture-retentive wound dressings (MRWDs) limit moisture vapor transmission from the wound to less than 35 g/m\textsuperscript{2}/hr, which promotes keratinocyte migration and wound healing.\textsuperscript{128–130} MRWDs can be fabricated from films, foams, hydrocolloids, alginates, and hydrogels.

MRWDs differ in their efficacy, advantages and disadvantages (Table 3). Films are comprised of thin, elastic sheets of polyurethane applied mainly to the site of acute surgical wounds.\textsuperscript{90,122} While films are gas permeable, transparent for visual inspection of wound progression, and exclude bacteria from entering the wound, they offer limited liquid drainage and can also damage tissue when removed. MRWD foams are moisture absorbent, comfortable to wear, and drape over tissue, but may require application of additional dressings to facilitate occlusion or drainage.\textsuperscript{90,122,131} Hydrocolloid dressings combine a foam or polyurethane film dressing with a hydrocolloid matrix that, when applied to wound exudate, forms a gel that promotes autolytic debridement and granulation tissue formation.\textsuperscript{90,122,131} However, hydrocolloids should not be employed for highly exudative or infected wounds as they may cause skin maceration.\textsuperscript{132,133} Alginate dressings are composed of sodium and calcium salts of alginic acid. Alginates have haemostatic properties, thus are highly absorbent and ideal for highly exudative wounds, but not for dry wounds.\textsuperscript{90,122,131} Lastly, hydrogels are macromolecular polymer gels that absorb wound exudate and stimulate autolytic debridement.\textsuperscript{90,122} Hydrogels may be applied to a range of wounds from mildly to highly exudative, but maceration can occur.\textsuperscript{131} These MRWDs are largely passive dressings, and the ideal clinical treatment of chronic wounds would also possess an ability to actively stimulate biological healing responses.

Bioengineered skin substitutes, in addition to retaining moisture, stimulate healing and are of interest in tissue engineering and regenerative medicine. Epidermis substitutes, including Epitel®, Epidex®, Myskin®, Bioseed®, and Cellspray®, are predominantly autogenic keratinocyte expansions formulated into delivery dressings or as suspensions that can be topically ejected or sprayed onto the wound site.\textsuperscript{134} Dermal substitutes are 3D biomaterial matrices that match the native ECM in terms of structural, elastic, and mechanical properties. Acellular dermal substitutes, such as Oasis® and Alloderm®, are nonimmunogenic,
mechanically robust, and can be used off the shelf; however, these substitutes depend on host cell infiltration to initiate angiogenesis and remodeling.\textsuperscript{134} In contrast, collagen-GAG cellular matrices such as Dermagraft\textsuperscript{®} and OrCel\textsuperscript{®} are pre-seeded with neonatal human foreskin fibroblasts and/or keratinocytes and have been shown to reduce wound contractility to allow re-epithelialization to restore a more natural tissue barrier.\textsuperscript{135,136} Bilayer skin substitutes contain both epidermal and dermal components. Apligraf\textsuperscript{®} is an FDA-approved skin substitute consisting of an epidermal neonatal human foreskin keratinocyte layer and an underlying dermal bovine type I collagen layer seeded with neonatal human fetal fibroblasts.\textsuperscript{90,122} Apligraf\textsuperscript{®} dressings already contain within them the cells responsible for the production and delivery of cytokines, growth factors and ECM components to the wound bed, however these bilayer substitutes are expensive and have a limited shelf life of 5-10 days.\textsuperscript{137,138}

Healing outcomes can also be influenced by employing adjuvant therapies in conjunction with standard medical practice. Negative-pressure wound therapy (NPWT) or vacuum-assisted closure therapy applies sub-atmospheric pressure to a local area of tissue damage through a specialized pump.\textsuperscript{139} NPWT reduces tissue edema, improves circulation, promotes granulation tissue formation and inhibits bacterial growth.\textsuperscript{140} Hyperbaric oxygen therapy (HBOT) involves briefly inhaling 100\% oxygen while inside a pressurized chamber.\textsuperscript{141} Heightened blood and tissue oxygenation by HBOT improves oxygen delivery to hypoxic tissues and vasoconstricts surrounding healthy tissue, alleviating edema and permitting innate biological combating of infection and ischemia to ensue.\textsuperscript{142} Topical addition of growth factor formulations have also been used in chronic wound healing. For instance, a gel composition of recombinant PDGF (Regranex\textsuperscript{®}/Becaplermin) has been employed to mediate cell division, migration and proliferation. Daily Becaplermin application significantly aided wound closure in PUs and in DFUs.\textsuperscript{143,144} However, there is increased risk of cancer mortality associated with excessive use (three or more dispensaries) of Becaplermin.\textsuperscript{145} Notably, a significant limitation of topical growth factors is their rapid proteolytic degradation.\textsuperscript{146}

Current clinical strategies, including wound debridement, moisture-retentive dressings, and adjuvant therapies, have various disadvantages and do little to resolve a chronic wound. In Canada, 3.5 million individuals live with diabetes and DFUs will affect one quarter of these patients.\textsuperscript{95,147} DFUs are the most common cause of non-traumatic lower limb amputations, with 20\% of DFU patients requiring amputation.\textsuperscript{89} Furthermore, these amputations are associated with
a high of mortality rate; in Canada, 30% of patients with diabetes die within one year of amputation and 69% of patients within five years.\textsuperscript{96,148} Despite the multitude of wound healing products available, chronic wounds continue to be a significant burden on patients and the healthcare system. In recent years, attention has focused on development of biologically active and tissue mimetic technologies.
Table 3. Examples of clinically available moisture-retentive wound dressings.\textsuperscript{90,122,149,150}

<table>
<thead>
<tr>
<th>Category</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Examples</th>
</tr>
</thead>
</table>
| Films          | - Suitable for flat, shallow wounds with low to medium exudates  
                 - Promote moist environment  
                 - Adhere to healthy skin but not to wound  
                 - Allow visual checks  
                 - May be left in place several days  
                 - Useful as secondary dressing | - Provide no cushioning  
                 - Not for infected or heavily exuding wounds | Tegaderm (3M Healthcare), Polyskin II (Kendall Healthcare), Bioclusive (Johnson & Johnson Medical), Blisterfilm (The Kendall Co), Omniderm (Omikron Scientific Ltd), Proclude (ConvaTec), Mefilm (Mölnlycke Health Care), Carrafilm (Carrington Lab), and Transeal (DeRoyal) |
| Foams          | - Flat, shallow wounds (control of exudate depending on type of foam)  
                 - Provide a degree of cushioning | - Need secondary dressing  
                 - Need to be replaced after 2 to 3 days | Polymem (Ferris Corp), Allevyn (Smith & Nephew United), Biopatch (Johnson & Johnson Medical), Curafilm (The Kendall Co), Flexzan (Dow Hickam), Hydrasorb (Tyco/Kendall Co), Lyofoam (ConvaTec), and Mepilex (Mölnlycke Health Care) |
| Hydrocolloids  | - Useful for flat, shallow wounds with low to medium exudate  
                 - Absorbent  
                 - Conformable  
                 - Suitable for heel, elbow, sacrum | - May cause maceration  
                 - Need secondary dressing | Duoderm (ConvaTec), NuDerm (Johnson & Johnson Medical), Comfeel (Coloplast Sween, Inc), Hydrocol (Dow Hickam), Cutinova (Smith & Nephew), Replicare (Smith & Nephew United), and Tegasorb (3M) |
| Alginates      | - Useful in cavities and for undermining wounds  
                 - Highly absorbent | - Need secondary dressing  
                 - Need to be changed daily | Algiderm (Bard), Algisite (Smith & Nephew), Algisorb (Calgon-Vestal), Algosteril (Johnson & Johnson Medical), Kaltostat (ConvaTec), Curasorb (The Kendall Co), Sorbsan (Dow |
| Hydrogels | -Supply moisture to wounds with low to medium exudate  
-Suitable for sloughy or necrotic wounds  
-Useful in flat wounds and cavities  
-May be left in place several days | -Need secondary dressing  
-May cause maceration | Hickam), Melgisorb (Mölnlycke Health Care), SeaSorb (Coloplast), and Kalginate (DeRoyal)  
Vigilon (CR Bard), Nu-gel (Johnson & Johnson Medical), Tegagel (3M), FlexiGel (Smith & Nephew), Curagel (The Kendall Co), Clearsite (Conmed Corp), Curafil (The Kendall Co), Curasol (The Kendall Co), Carrasyn (Carrington Laboratories), Elasto-Gel (SW Technologies), Hypergel (Scott Health Care), Normgel (SCA Hygiene Products), 2nd Skin (Spenco Medical, Ltd), and Transigel (Smith & Nephew) |
1.2 Tissue Engineering and Regenerative Medicine

Tissue engineering is an area of research that is focused on the combination of natural and synthetic scaffolds, cells, and biologically active molecules in an attempt to form biologically functional tissues. Similarly, regenerative medicine describes the use of engineered biological materials in conjunction with the body’s innate proliferative mechanisms to expand cell populations and reconstruct tissues in vivo. Focuses of tissue engineering research include the development of bioengineered materials, skin substitutes, biomolecule delivery systems, and stem cell therapies.

1.2.1 Biomaterial Scaffold Design Requirements

Biomaterials can be considered natural or synthetic materials that are designed to interact with biological tissues for augmenting or replacing a native tissue. Tissue engineering scaffolds have the potential to mimic the native extracellular matrix at the nanoscale level, which is important for matrix production, neoangiogenesis and cellular ingrowth, leading to regeneration of tissues. Biomaterials must be biocompatible, nonimmunogenic, and if desirable, biodegradable; that is, a biomaterial should perform safely within the biological environment and any degradation products should be nontoxic. Scaffold structures should ideally mimic native ECM and display a fiber diameter of 50 to 500 nm, 90% porosity, and average pore size of 100 μm to facilitate mass transfer and cell infiltration. Various fabrication methods for construction of three-dimensional biomimetic scaffolds have been investigated, including electrospinning, phase-separation, freeze drying, and self-assembly. However, the challenge of fabricating complex and functional tissues still exists. Current biomaterial fabrication methods are limited in the ability to control hierarchical architecture of scaffolds, formed by nanofibers and nanopores. The insufficient vascularization systems of biological substitutes results in limited diffusion properties of these biomimetic scaffolds. As a consequence, biomaterial scaffolds contain a necrotic center where oxygen transport, nutrient deposit, and waste removal are limited, and viable cells do not penetrate. As such, a scaffold manufacturing technique with controlled material deposition is desirable in order to create complex architectures with optimized porosity.
1.2.2 Three-Dimensional Bioprinting

Additive manufacturing (AM), also known as three-dimensional (3D) printing, is a mechanized fabrication method that builds objects by repeatedly depositing material layer-by-layer. Adjacent layers fuse together during the process until the final object has been achieved. This technique first requires the creation of a 3D digital model that is generated using computer-aided design (CAD) software. Since building takes place layer-by-layer, the 3D model must be sliced into horizontal planes in order to generate a G-code, or programming language that will control the printer’s extrusion path.¹⁵⁸ A basic 3D printer typically includes a movable print bed that can displace in the vertical (Z) direction and supports the print object, an ejector nozzle that moves in the horizontal (X, Y) plane, and the print material, which can be a wide range of materials, from plastics and metals to soft materials. G-code is sent to the printer and the object can be 3D printed.¹⁵⁸,¹⁵⁹ Advantages of 3D printing include the availability of low-cost hardware that is easily customizable, free, open-source software, and limited waste of print materials.¹⁶⁰

In 3D bioprinting, the print material is often referred to as a “bioink.” Material properties to consider when choosing a bioink include the desired print resolution, ability for the printed device to be easily sterilized, shear strength and ability to recover scaffold shape, and the material stiffness. As is required for all biomaterials, bioinks need to display biological compatibility with cells and the body such that the biomaterial can adequately function in the biological environment while minimizing any foreign-body response mounted by the host immune system.¹⁶¹ Furthermore, optimized biomaterials should also exhibit the ability to be remodeled, biodegraded and bioadsorbed by cells, producing nontoxic degradation products.¹⁶¹ Commercially available bioinks include native structural extracellular matrix proteins such as collagen, gelatin, hyaluronic acid, and calcium phosphate.¹⁶²

Three-dimensional printing of soft materials, such as photocurable resins, polymer powders, or thermoplastic monofilaments, creates the additional challenge of apparent ink viscosity, which can cause the print material to flow out of its extruded shape.¹⁶³ One approach to 3D printing of soft materials is to eject the ink layer-by-layer into a secondary yield-stress support bath, embedding the print object in a granular medium. Embedding scaffold structures into a temporary, sacrificial support material is advantageous in that this technique allows for the printing of soft natural and synthetic materials that exhibit low viscosities prior to self-polymerization and gelation, allowing for the production of structures with complex architectures
and porous regions. Freeform reversible embedding of suspended hydrogels (FRESH), is a type of 3D printing that utilizes a support bath with reversible gelation properties, such that the bath can be liquified in order to liberate the 3D printed object post-printing.\textsuperscript{164–166}

1.2.3 Collagen-Based Biomaterials

Twenty-nine collagen types have been identified which account for 33\% of the protein in humans and 66\% of the dry weight of skin.\textsuperscript{167} Collagen types I-III, V, and XI have fibrillar quaternary structures. Collagen molecules are comprised of three polypeptide chains aligned in a parallel manner and coiled in a left-handed polyproline II-type (PPII) helix.\textsuperscript{168} These chains arrange themselves further into a right-handed triple helix that is stabilized by interstrand hydrogen bonds and intrastrand non-covalent interactions.\textsuperscript{169} Animal collagens arranged in triple helices, known as tropocollagen, form macroscopic fibers and extracellular matrix networks in tissue, bone, and basement membrane. Ninety percent of the total collagen content in human skin is type I collagen, and this type is most frequently employed for biomedical applications.\textsuperscript{170}

Biomaterial scaffolds fabricated using collagen have been applied extensively as a major component in dermal skin substitutes due to collagen’s high biocompatibility, biodegradability, and molecular composition.\textsuperscript{171} Collagen extraction for biomedical application is commonly from bovine skin and tendons; porcine skin, intestine, or bladder mucosa; and rat tail sources.\textsuperscript{172} The quality and properties of extracted collagen depends on the source species and the tissue from which it was harvested. One risk associated with collagen derived from animal sources is allergic reaction and pathogen transmission.\textsuperscript{173,174} To overcome this complication, recombinant collagen can be produced via heterologous expression in mammalian, insect, yeast, or bacterial cells.\textsuperscript{175–177} Biorecognition of collagen is necessary since may cell-surface portions interact and bind to collagen. Cell-collagen communications are mediated by four different kinds of proteins: Pro-Hyp-Gly-recognizing receptors (for example, glycoprotein VI); integrin family receptors; integrin-type receptors that recognize cryptic motifs within collagen; and receptors with affinity for non-collagenous domains.\textsuperscript{178,179} Proteins containing Arg-Gly-Asp or similar integrin-recognition sequences, such as decorin and laminin, can bind to both collagen and integrins, promoting cell adhesion and proliferation.\textsuperscript{180}

Collagen types I-III are hydrolyzed by collagenases MMP-1, MMP-2, MMP-8, MMP-13, and MMP-14. Intrinsic biodegradability by endogenous collagenases makes exogenous collagen
ideal for use in biomedical applications. Exogenous degradation products of collagen types I-III have been shown to induce chemotaxis of human fibroblasts and is thought to promote regeneration of tissue structure and functionality.\textsuperscript{181,182} Collagen can be cross-linked via chemical cross-linking agents including formaldehyde, glutaraldehyde, carbodiimides, polyepoxy compounds, acyl azides, and hexamethylene diisocyanate;\textsuperscript{167} physical cross-linking methods using ultraviolet light or dehydrothermal treatment;\textsuperscript{183} and enzymatic cross-linking such as via tissue transglutaminase.\textsuperscript{184} Although chemical cross-linking can enhance biomaterial stability, residual electrophilic reagents and compounds produced upon degradation \textit{in vivo} can be cytotoxic. An advantage of cross-linking with enzymes is this method is benign and generates no cytotoxic byproducts.

Despite limitations such as immunogenicity of xenogeneic sources and high costs, the use of natural biological materials is of interest in biomaterial fabrication. For instance, collagen-based biomaterials have been demonstrated for a variety of applications, including neural stem cell scaffolds,\textsuperscript{185} cartilage,\textsuperscript{186,187} osteochondral,\textsuperscript{188} and skin.\textsuperscript{189} Tissue substitutes comprised of natural biomaterials have garnered interest for applications in transdermal and topical formulation discovery, dermal toxicity studies, and autologous grafts for wound healing. Collagen-based biomaterials can be classified as either decellularized collagen matrices or more refined scaffolds based on the extent of their purification. Decellularized collagen matrices maintain the natural tissue properties and ECM structure; cellular matter is removed from collagen matrix by physical methods such as snap freezing or high pressure, chemical treatment with acid or alkali treatment, chelation with EDTA, or treatment with detergents or solutions of high osmolarity, and trypsin enzymatic digestion to produce the biomaterial.\textsuperscript{190} In contrast, more refined scaffolds are fabricated via collagen protein extraction, purification, and polymerization. Natural collagen can dissolve in aqueous solutions depending on the extant cross-linking. Refined scaffolds require collagen to first be dissolved in aqueous solution; the most common solvent systems include a neutral NaCl solution, dilute acetic acid, or a solution of proteolytic enzymes.\textsuperscript{167}

1.2.4 Polycaprolactone-Based Biomaterials

In certain cases, the use of natural materials is not possible and thus synthetic materials have been proposed. Advantages of synthetic materials include high reproducibility, availability,
consistent quality, and tunable material properties.\textsuperscript{191} One such example of a widely employed synthetic material is polycaprolactone (poly(\(\epsilon\)-caprolactone), PCL), a biocompatible polyester. PCL is a semi-crystalline, aliphatic polymer, having highly ordered molecular structure.\textsuperscript{192} The structure comprises of a repeating unit of one ester group and five methylene groups. Its melting temperature is above body temperature, thus within the body the semi-crystalline structure of PCL results in high toughness.\textsuperscript{193} Advantages of PCL include its strength and durability, which are tunable, and its ease of fabrication while allowing for precise control of product architecture.\textsuperscript{194}

PCL is degraded in the body under physiological conditions via hydrolytic mechanisms. Due to its high molecular weight, the polyester has a slow degradation rate of approximately two years in the biological environment.\textsuperscript{195–197} PCL is biocompatible and non-toxic, while its durability means that PCL has less chance to induce immunological effects.\textsuperscript{198} Physical properties of PCL are easily manipulated by compounding the polymer with secondary constituents. By using a copolymer of PCL with dl-lactide, a more flexible material with a faster degradation rate than the homopolymer can be achieved.\textsuperscript{195} Moreover, the high degree of permeability has made PCL an important candidate for the development of drug delivery systems and in bone tissue regeneration.\textsuperscript{199–203}

PCL has been established as an important biomaterial. It has been approved by the Food and Drug Administration (FDA) for several medical applications, including suture materials and subdermal contraceptive implants.\textsuperscript{192,204,205} In dentistry, PCL has been employed as a root canal-filling material; PCL-filled roots demonstrated proper seals to protect against the aqueous environment.\textsuperscript{206} In wound healing, PCL was employed as wound-dressing material and delivery system for chemical antiseptic; PCL fibers were shown to exhibit desirable tensile properties following compounding with chlorhexidine diacetate while the antiseptic conferred antimicrobial properties, even at concentrations as low as 1\% (w/w).\textsuperscript{207} Furthermore, PCL composites have been widely studied for applications in tissue engineering scaffolds that regenerate bone, ligament, cartilage, skin, nerve and vascular tissues.\textsuperscript{204}

1.2.5 Electrospinning

In electrospinning, a polymer solution is ejected through a needle using an applied force from a syringe pump. An electric potential is applied to the needle through which the polymer
solution passes such that as each droplet accumulates at the tip of the needle, it experiences electrostatic repulsion between the surface charges of the droplet and Columbic force exerted by the applied electric field.\textsuperscript{208} As charge accumulates on the surface of the droplet, a Taylor cone is formed. Once the electrostatic charge exceeds the surface tension of the polymer solution, a polymer jet is expelled and travels towards a grounded mandrel.\textsuperscript{208} The solvent evaporates, leaving a porous, nonwoven fiber mat deposited on the collector. Electrospinning allows for customization of scaffold morphology by varying parameters known to affect fiber shape and size, for instance the concentration and resultant viscosity of the polymer solution, solvent identity, distance from needle to collecting surface, applied voltage, flow rate, temperature, and humidity.\textsuperscript{209,210}

Electrospun nanofibrous scaffolds have been proposed as a novel alternate strategy to conventional wound dressings for management of chronic skin wounds.\textsuperscript{211,212} Electrospinning polymer solutions can also be supplemented with bioactive agents to improve scaffold biocompatibility, biodegradability, biorecognition, sterilizability, and mechanical properties.\textsuperscript{213} Due to the high surface-area-to-volume ratio, nanoscale diameter, and highly porous structure of electrospun fibers, even distribution and controlled release of bioactive molecules is possible.\textsuperscript{214} Immediate burst release with subsequent prolonged release of bioactive factors via passive diffusion occurs during degradation of polymer fibers.\textsuperscript{215} Antimicrobial peptides, cytokines, and growth factors, are absent in chronic wounds, but could be delivered exogenously using a biomolecule delivery system.\textsuperscript{216} For instance, electrospun polyvinyl alcohol (PVA)-silk nanofibers have previously been supplemented with EGF, bFGF, and the antimicrobial peptide LL-37. Biological gradients of these bioactive factors were established and tested using full-thickness excisional wounds on the dorsal surface of diabetic rabbits, demonstrating this combination of factors accelerated wound healing, lowered MMP expression, regulated ECM secretion, and reduced biofilm or bacterial colonization.\textsuperscript{217}

Several limitations of electrospun scaffolds have been identified however, including 1) the structure which is densely packed nanofibers, resulting in small pore sizes, 2) potential toxicity of residual solvents or cross-linking agents, and 3) difficulty with industrial upscaling.\textsuperscript{218–221} Small pore sizes and densely packed fibers result from the fabrication process where overlying layers compress underlying layers during electrospinning. As a result, cellular infiltration and ingrowth is inhibited, which in turn reduces vascularization and tissue
regeneration.\textsuperscript{222} Pore size can be increased however, by changing the polymer solution flow rate, increasing the concentration of the polymer solution to produce thicker fibers, or selective and controlled heating of the electrospinning environment to facilitate solvent evaporation.\textsuperscript{214,223,224} However, while thicker fibers can counteract the limitation of pore size, increasing fiber diameter inhibits cellular adhesion and migration.\textsuperscript{225} Moreover, the use of organic solvents or chemical cross-linking agents is a common concern associated with blended electrospinning due to cytotoxicity.\textsuperscript{220} Safe crosslinking options include enzymatic crosslinking, electrostatic crosslinking, or hydrogen bonding with sugars or polyphenols.\textsuperscript{226} However, these alternative cross-linking methods are associated with more complicated fabrication procedures and excessive degradation of bioactive factors loaded into the scaffold for slow release.\textsuperscript{226} Lastly, the soluble nature of most biomolecules often results in rapid release from the nanofibrous scaffolds, as fast as 70\% released within 30 minutes, followed by degradation or aggregation due to the instability of these proteins against proteolysis, acidity, and heat in the wound microenvironment.\textsuperscript{227–229} Despite these challenges, electrospinning is a viable biomaterials technique due to the great potential for customization of scaffold morphology and material composition.

Electrospun nanofibrous scaffolds can be produced using natural and synthetic polymers.\textsuperscript{230} Natural polymers such as collagen, gelatin, silk, fibrinogen and chitin are biocompatible, biodegradable and abundant in the natural environment. However, natural polymers exhibit fragile structural properties, complicated processability, vulnerability to enzymatic degradation and potential immunogenicity. Weak mechanical properties and rapid degradation of natural polymers are overcome using cross-linking to improve scaffold stability. Chemical crosslinkers such as glutaraldehyde, formaldehyde, polyether oxide, hexamethylene diisocyanate and polyurethane, acyl azide and carbodiimides, and glycerol, and physical crosslinkers including drying, heating, and UV/gamma radiation, covalently bond amino acids on adjacent scaffold structures.

Gelatin is a natural polymer alternative to collagen for use in electrospun biomaterial scaffolds.\textsuperscript{231} Gelatin, a derivative of collagen, is obtained by the denaturation of collagen in either acidic or basic processing. Type A gelatin is produced by acid pre-treatment of animal samples while type B gelatin is produced by alkaline pre-treatment.\textsuperscript{232} Gelatin maintains biocompatibility and biodegradability at much lower production costs than collagen.\textsuperscript{232}
Moreover, gelatin scaffolds have been shown to stimulate cellular adhesion, migration and proliferation, and accelerate wound healing.233–235

1.2.6 Stem Cell Therapy in Wound Healing

Characteristics of stem cells (SCs) include the ability to self-renew, maintain long-term viability, and multipotent differentiation.236 SC therapies for the treatment of chronic wounds involves delivering adult stem and progenitor cells to the site of injury. Autologous SC transplant uses a person’s own SCs while an allogeneic transplant uses stem cells from a donor. In wound healing, exogenous progenitor and stem cells have been employed to improve healing and scarring outcomes through SC differentiation and secretory activities. For instance, transplanted human amniotic mesenchymal stem cells (AMMs) into a diabetic murine excisional skin wound showed that AMMs promoted wound healing and increased re-epithelialization and cellularity; AMMs demonstrated engraftment and expression of keratinocyte-specific proteins in vivo, while the secretome was rich in angiogenic factors IGF-1, EGF and IL-8.237 A clinical study of autologous bone marrow (BM)-derived MSC intramuscular injection into the affected limb of 24 patients with non-healing ulcers demonstrated enhanced wound healing and improved clinical parameters such as painless walking.238 In a study of 41 type 2 diabetic patients with critical limb ischemia and foot ulcers, ulcer healing, limb perfusion, and painless walking were improved in groups injected intramuscularly with BM-MSCs compared to those that received BM-derived mononuclear cells or normal saline.239 Thus far, SC therapies for wound healing applications are limited to animal models and small clinical trials, but promising results warrant further investigation into the effective delivery of autologous and allogeneic stem and progenitor cells.240

Adipose-derived stem/stromal cells (ASCs) are MSCs derived from fat tissue and have garnered interest in SC therapy. ASCs have been shown to proliferate rapidly and differentiate in vitro toward the osteogenic, adipogenic, myogenic, and chondrogenic lineages.236,241,242 Moreover, ASCs are resistant to mechanical damage243 and easily harvested from the body by mechanical liposuction, manual aspiration (Coleman technique), or direct surgical excision.244 The stromal vascular fraction (SVF) derived from adipose tissue is heterogeneous, comprised of MSCs, pre-adipocytes, endothelial cells, pericytes, T cells, and alternative M2 macrophages.245 MSCs can be purified from the SVF by plastic adherence and sorting based on expression of cell
surface markers including CD44, CD90, CD105, CD166, and Stro-1. Notably, ASC phenotype is controversial and under speculation; the International Federation for Adipose Therapeutics and Science and the International Society for Cellular Therapy include ASC positivity for CD13, CD36, CD73, and CD10, and negativity for CD45, CD31 and CD106. Despite the need for a distinct set of quality control criteria that can define the standard ASC phenotype, ASCs are a promising focus for cell therapy.

In wound healing studies, ACSs injected locally into excisional wounds in diabetic rats with compromised healing abilities resulted in rescue of wound healing rates in diabetic rats almost equivalent to those of wild type rats. Moreover, ASCs appear to promote wound healing under ischemic conditions. Ischemia of rabbit ears was created by ligating two of three main arteries of the ear and subsequently wounding the ear; ASC therapy enhanced wound granulation in rabbit ears under ischemia. Radiation models of chronic wounds in rats and mice have demonstrated that ASCs administered directly into the irradiated region, intramuscularly into the irradiated limbs, or intravenously resulted in enhanced wound repair. ASCs within the subcutaneous adipose tissue niche are in close proximity to cutaneous wounds and are implemented in the wound healing process. ASCs exhibit great migration potential and may infiltrate the wound to foster wound repair. For instance, ASCs transplanted into the subretinal space of injured rat retinas migrated into the retinal pigmented epithelium after 4 weeks.

ASCs are capable of affecting other cells through the release of hormones, cytokines, growth factors, and micro RNAs. Extracellular vesicles released by ASCs transport gene regulatory information that in turn affects angiogenesis, adipogenesis, and other cell pathways in recipient cells. Hormones released from ASCs have been shown to affect cancer cell proliferation. In diabetic rats, ASCs injected subcutaneously into full-thickness skin wounds stimulated angiogenesis and enhanced tissue regeneration after 8 weeks; ASCs amassed in the subdermal layer of the wound boundary and amplified angiogenesis via expression of von Willebrand factor and VEGF. Through co-culturing it was demonstrated that ASCs have an anti-inflammatory effect on monocyte-derived dendritic cells in vitro. Moreover, proliferation of lymphocytes was diminished following treatment with conditioned media from ASCs. In vivo, ASC-conditioned media applied topically to rodent wounds increased capillary density and wound closure kinetics. Together, these results implicate the ASC secretome in wound
healing processes, including tissue regeneration, angiogenesis, and inflammatory modulation, and are of significant interest for wound healing therapeutic strategies.

1.3 Galectin-3 as a Therapeutic for Chronic Wounds

1.3.1 Galectin-3 Overview

Galectin-3 is 250 amino acids in length, and is encoded by a single gene, LGALS3, located on chromosome 14. Galectin-3 is defined as a matricellular protein and is a member of the beta-galactoside-binding protein family. It has been shown to play important roles in cell-cell adhesion, cell-matrix interactions, macrophage activation, angiogenesis, metastasis, and cell apoptosis. Implicated in several inflammatory and immunomodulatory processes, galectin-3 is of interest for applications in chronic wound treatment.

1.3.2 Roles in Inflammation

Galectin-3 can interact with a variety of inflammatory cell types including neutrophils, monocytes, and macrophages. Neutrophils, considered to be the first responders at the start of inflammation, eliminate foreign particles following injury. Treatment of neutrophils in vitro with recombinant human galectin-3 suggested that the matricellular protein was capable of activating neutrophils through its carbohydrate recognition domain. Similarly, in another study, galectin-3 increased exudate neutrophil activity corresponding to increased surface-bound protein, while activity of peripheral neutrophils was unaltered. In addition to increasing neutrophil activity, galectin-3 has also been shown to facilitate neutrophil adhesion to laminin in vitro and has been implicated in the recruitment of neutrophils during in vivo murine cutaneous infection.

Inflammation is also mediated by migrating monocytes that differentiate into macrophages. Galectin-3 affects monocyte migration in vitro, stimulating chemotaxis at high concentrations and chemokinesis at lower concentrations. A similar migratory effect from galectin-3 is also observed in macrophages. Monocyte and macrophage migration is increased in the presence of fibronectin, indicating that galectin-3 may mediate linkage of these cells to the ECM protein. Macrophages are known to clear exhausted neutrophils from the wound by phagocytosis. Galectin-3 may influence this process given that addition of exogenous galectin-3 increases apoptotic neutrophil uptake in macrophages in vitro. Moreover, it has been suggested that galectin-3 acts as an opsonin, physically linking phagocytic macrophages to neutrophils,
ensuring close proximity for easy ingestion of neutrophils.\textsuperscript{270} Regarding macrophage phenotype and activation phases, IFN-\(\gamma\) and LPS or TNF-\(\alpha\) signals monocytes to undergo classical activation into M1-polarized macrophages, which are associated with inflammatory roles. M1 macrophages produce inducible nitric oxide synthase (iNOS) as well as pro-inflammatory cytokines. In mice, markers of M1 macrophages include iNOS, chemokine ligand 9 (CXCL9), CXCL 10, and CXCL11. Monocytes can also undergo alternative activation through stimulation with IL-4 or IL-13 into M2-polarized macrophages. M2 macrophages are associated with tissue remodeling and secrete arginase I and anti-inflammatory cytokines. M2 markers in mice include arginase I, Mrc I, Fizz I, Ym1, and Ym 2.\textsuperscript{88,267,271} A study investigating the effect of galectin-3 on macrophage activation in bone marrow-derived macrophages \textit{in vitro} and in resident lung and recruited peritoneal macrophages \textit{in vivo} demonstrated that macrophages derived from galectin-3 deficient mice exhibited diminished IL-4/IL-13-induced M2 macrophage polarization, suggesting that galectin-3 is involved in the regulation of alternative macrophage activation.\textsuperscript{272}

1.3.3 Roles in Angiogenesis

Galectin-3 has been shown to induce angiogenesis. Capillary tube formation of human umbilical cord endothelial cells grown on a matrigel was stimulated with galectin-3 supplementation \textit{in vitro}. \textit{In vivo}, a galectin-3-loaded matrigel was able to induce angiogenesis in nude mice.\textsuperscript{273} Galectin-3 may modulate VEGF and FGF-2-mediated angiogenesis by activating focal adhesion kinase-mediated signaling pathways which control endothelial cell migration.\textsuperscript{274} The protein has also been implemented in angiogenesis and endothelial cell migration through integrin-linked kinase signaling.\textsuperscript{275} Galectin-3 binds vascular endothelial growth factor receptor 2 (VEGFR2), promoting its phosphorylation and preventing its internalization, thus increasing angiogenic processes of human umbilical cord endothelial cells \textit{in vitro}.\textsuperscript{276} Similarly, galectin-3, together with galectin-1, can activate and prevent the internalization of VEGFR1, to again enhance angiogenesis.\textsuperscript{276} However, despite these findings, a study of murine cutaneous wound repair from our research group demonstrated that galectin-3 deficient mice exhibited no difference in vascular density or expression of angiogenic markers relative to wild-type mice.\textsuperscript{277}
1.3.4 Roles in Re-Epithelialization

Surface expression of galectin-3 in type I and II alveolar epithelial cells was described in a model of irradiation-induced lung inflammation and repair.\textsuperscript{278} In corneal healing, re-epithelialization was reduced in galectin-3 deficient mice compared to wild-type counterparts.\textsuperscript{279} While galectin-3 did not alter the rate of epithelial cell proliferation, the protein may have influenced epithelial cell migration as elevated levels of galectin-3 were detected in the migrating epithelial tongue following injury.\textsuperscript{279} This influence was also seen in human corneal epithelial cells where galectin-3 promoted cell scattering, lamellipodia formation, and motility.\textsuperscript{280} Notably, in murine corneal healing the addition of exogenous galectin-3 increased re-epithelialization in wild type (WT) mice, but not galectin-3 deficient mice.\textsuperscript{279} Epithelial wounds in monkey corneal explants also exhibited enhanced re-epithelization following human galectin-3 exogenous treatment.\textsuperscript{281}

Cutaneous wound healing models have demonstrated that keratinocytes from galectin-3 knockout mice exhibit a migratory defect, and that re-epithelialization, but not wound closure itself, is delayed in galectin-3 deficient mice.\textsuperscript{277,282} Overall, galectin-3 has been implicated in numerous wound healing processes including inflammation and contributing to re-epithelialization. As a result, topical delivery of this protein during the wound healing process is of interest to augment repair in challenged wound healing environments.

1.4 Hypothesis and Objectives

1.4.1 Rationale

Pathologies such as cutaneous chronic wounds are a challenge to treat clinically and new treatment strategies are desperately needed. Excessive inflammation, a deficient ECM structure and composition, cell senescence, and imbalance of signaling molecules contribute to a non-healing wound bed that persists beyond three months, leading to pain, impaired limb function, bacterial infection, and hospitalization. High mortality rates following lower limb amputation, necessitated by chronic wounds, indicate a severe need for a bioengineered scaffold that can resolve inflammation and serve as a temporary support for fibroplasia, neoangiogenesis, and re-epithelialization.

A three-dimensional scaffold more closely mimics the structure and function of native ECM and can be used in the delivery of bioactive molecules. The scaffold provides a site for cell
biorecognition and adhesion, necessary for cell infiltration and proliferation. Electrospinning and 3D printing are two means of biomimetic scaffold production. Electrospun scaffolds are highly nanofibrous and randomly organized, while 3D printed scaffolds allow for controlled material architecture and void pores.

Three-dimensional printing has gained interest in biomaterial engineering as an accessible means of scaffold fabrication with the opportunity for customized scaffold shape and porosity. Thus, our laboratory is interested in establishing a protocol for reproducible printing of soft materials which can be investigated for use in tissue engineering applications.

Electrospinning nanofibrous scaffolds is well-established in our laboratory. We have previously demonstrated that electrospun gelatin/galectin-3 scaffolds are biocompatible in vitro. The conditions under which galectin-3 might attenuate prolonged inflammation by modulating alternative M2 macrophage polarization remains to be elucidated.

1.4.2 Hypothesis

I hypothesize that scaffold design considerations will depend on their intended application. Firstly, I predict that 3D printed soft collagen scaffolds and rigid PCL scaffolds will exhibit desired architecture, porosity, and biocompatibility; these engineered scaffolds will support human adipose-derived stem/stromal cell (ASC) bioactivity in vitro. Secondly, I predict that local delivery of human recombinant galectin-3, either topically or using a gelatin scaffold, in a murine model of wound healing will cause the enrichment of pro-regenerative, arginase I-positive cells within the wound in vivo.

1.4.3 Objectives

The aims of this study were to investigate 3D printed and electrospun scaffolds for tissue engineering. Electrospun scaffolds are composed of nanofibers that are randomly organized, while 3D printed scaffolds allow for controlled deposition of print materials to ensure desired material architecture and void pores.

**Aim 1: Three-Dimensional Bioprinting for Tissue Engineering**

- Establish a protocol for soft collagen scaffold printing
- Polycaprolactone scaffold polymer fusion printing
- *In vitro* proof-of-concept scaffold performance and ASC phenotype analysis
Aim 2: Effects of Exogenous Galectin-3 in a Murine Wound Healing Model

- Electrospin galectin-3-loaded gelatin scaffolds
- Evaluate effects of exogenous galectin-3 \textit{in vivo}
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Design and Validation of 3D Printed Scaffolds for Adipose Derived Stromal Cell Tissue Engineering

2.1 Introduction

Skin, the largest organ of the human body, is responsible for providing barrier protection as well as receiving sensation input from the external environment, and finally is responsible for thermal regulation of the body. Structurally, skin is comprised of three major layers, the epidermis, dermis and subcutaneous hypodermis. Interestingly, the skin surface is not smooth but is laced with multiple networks of fine grooves called sulci cutis, which can be either deep or shallow. The slightly elevated areas that are surrounded by shallower areas of sulci cutis are called crista cutis. Sweat pores fed by the sweat glands open to the crista cutis. The orientation of the sulci cutis, which differs depending on body location, is called the dermal ridge pattern. For instance, fingerprints and patterns on the palms of your hand and soles of your feet are formed by the sulci cutis. In addition, at the extracellular matrix level, skin contains significant topographical features; such biological topography provides important physical cues for oriented migration (contact guidance), cell orientation, spreading, contractility, migration and signaling.

Skin contains numerous stem and progenitor cell populations, which are self-renewing as skin constantly replaces itself following desquamation, the shedding of the outermost layer of skin. Non-pathologic desquamation of the skin occurs approximately every two weeks, when keratinocytes are individually shed unnoticeably. Humans keratinocytes turn over from stem cells to desquamation every 40-56 days, whereas in mice the estimated turnover time is much faster, 8-10 days. The continual regeneration of skin is maintained by permanently residing stem cells that sustain principal differentiated epidermal lineages, the interfollicular epidermis (IFE), sebaceous gland (SG), hair follicle (HF), and Merkel cell mechanoreceptors. Through radiation dose-survival studies, it was suggested that stem cells comprise about 2-7% of basal layer cells while another study of murine basal layer cells suggested a larger stem cell population, 10-12% of cells in the basal layer. Maintenance of skin homeostasis is dependent on the ability of stem cells to replenish the turnover of mature epithelial lineages.

Stem cell-based therapies employs the regenerative nature of stem and progenitor cells to treat disease or pathological conditions. In regenerative medicine, stem cells can be removed
from their natural environment, expanded to increase the population, and finally, implanted into a pathological tissue environment with or without a delivery scaffold. How stem and progenitor cells interact with their native microenvironment (stem cell niche), to establish and maintain their properties is crucial to tissue engineering applications particularly when the microenvironment is pathological in nature.\textsuperscript{11} Of interest in tissue engineering is the cell population of adipose-derived stem/stromal cells (ASCs). ASCs have been shown to proliferate rapidly and differentiate \textit{in vitro} towards adipogenic, osteogenic, chondrogenic and myogenic lineages.\textsuperscript{12,13} ACSs are found in the stromal vascular fraction along with pre-adipocytes, endothelial cells, pericytes, T cells, and alternative M2 macrophages.\textsuperscript{14} Following harvesting from the body by mechanical liposuction, manual aspiration, or surgical excision, enzymatic procedures, culture expansion with plastic adherence, and sorting using immunomagnetic beads coated with specific antibodies can be employed to isolate ASCs from contaminating cell types.\textsuperscript{15} ASCs have been implicated in tissue regeneration processes though their differentiation potential and secretion of bioactive molecules which signal and influence bioactivity of surrounding cells.\textsuperscript{16–21}

A biomaterial for stem cell culture and delivery should provide biomimetic physical and chemical cues. Collagen type I composes 90\% of the total collagen content in the skin, conferring compressive and tensile strength. Current approaches to the assembly of three-dimensional (3D) biomaterials use additive manufacturing (3D printing) to deposit materials layer by layer for controlled structure and architecture. Printing of biological hydrogels include syringe-based extrusion,\textsuperscript{22,23} printing with fibrin,\textsuperscript{24,25} gelatin,\textsuperscript{26} and protein mixtures obtained from decellularized tissues.\textsuperscript{27} However, 3D printed biological hydrogels and proteins must gel \textit{in situ} in order to prevent their collapse or shape deformation; moreover the structural integrity of printed scaffolds remains to be studied \textit{in vivo}.\textsuperscript{28–30} Direct printing into a secondary hydrogel that acts as a temporary support bath is of interest to preserve 3D printed architectures of soft biomaterials.\textsuperscript{31,32} In this study, 3D printed scaffolds are employed to assess \textit{in vitro} ASC bioactivity and phenotype.

2.2 Materials and Methods

2.2.1 Preparation of Gelatin Support Slurry

A gelatin microparticle support slurry was adapted from Hinton \textit{et al.}\textsuperscript{31} The preparation protocol is summarized in Figure 2.1. A 100 mL volume of 4\% (w/v) type A 275 bloom porcine
gelatin powder (Advanced Biomatrix, San Diego, CA, USA) hydrate in 1X phosphate buffered solution (PBS) pre-heated to 45°C was prepared in a glass 500 mL mason jar (Ball Inc., Broomfield, CO, USA). The solution was mixed until gelatin had fully dissolved and then stored overnight at 4°C to produce a semi-rigid gelatin colloid. The following day, 100 mL of 1X PBS chilled to 4°C was transferred to the jar and a rubber spatula was used to gently dislodge the gelatin. The jar was overflowed with 4°C 1X PBS and a rubber sealing ring, Osterizer® Ice Crusher Blade (Sunbeam Products, Inc., Boca Raton, FL, USA), and threaded bottom cap were twisted onto the jar rim. The sealed jar was placed at -20°C until ice crystals began to form between the colloid and fluid layers, approximately 45 min. Immediately following removal from the -20°C freezer, the jar was inverted onto an Osterizer® Heritage Blend 400 (Sunbeam Products, Inc., Boca Raton, FL, USA) consumer-grade blender and the gelatin was blended to mechanically disrupt the gel for 60, 90, or 120 s. The gelatin slurry was placed on ice and aliquoted into 50 mL conical centrifuge tubes, also placed in ice. The slurry was centrifuged at 4000xg and 4°C for 5 min to separate the supernatant, composed of excess PBS and soluble gelatin, from the gelatin microparticle layer. The supernatant was poured off and 10 mL of 4°C 1X PBS was added to each conical centrifuge tube; all tubes were kept on ice to prevent the gelatin from melting. The slurry was vortexed briefly to resuspend the gelatin, and centrifugation followed by resuspension in fresh 1X PBS was repeated three more times. Once the supernatant had been completely cleared from the gelatin microparticle layer, 10 mL of fresh 4°C 1X PBS was added to each tube, tubes were vortexed, and stored in a 4°C fridge. To prepare the gelatin support slurry for use, it was recovered from the fridge, vortexed, then centrifuged at 4000xg and 4°C for 5 min. The supernatant was poured off and the slurry was scooped into a sterile 60 x 15 mm round polystyrene dish (Corning Inc. Life Sciences, Durham, NC, USA) using a metal spatula to prevent air bubble formation within the support slurry. The slurry surface was smoothed using a metal spatula and two light-duty tissues (VWR International, LLC, Radnor, PA, USA) were placed on top of the slurry to absorb residual liquid.

2.2.2 Gelatin Support Slurry Rheology

Gelatin slurries were prepared as described above and rheological properties of slurries blended for 60, 90, or 120 s (N=2, n=3) were measured. Approximately 5 cc of slurry was loaded
onto a Modular Compact Rheometer (MCR) 302 (Anton Paar GmbH, Graz, Austria) equipped with a 50 mm parallel plate measuring system equipped with a moisture trap to prevent samples from drying out. Samples were pre-sheared at 10 Hz for 5 min then subject to a 10 min wait period. Viscoelasticity was analyzed by frequency sweep from 0.1 to 100 rad/s at 1 mm separation and 3% strain. A second wait period was observed for 10 min. Viscosity was analyzed by controlled shear rate (CSR) flow testing from 0.01 to 30 Hz. The MCR 302 was held at 23°C for the duration of all rheological measurements as to accurately reflect the ambient temperature during 3D printing.

2.2.3 Gelatin Support Slurry Microparticle Morphology

To study gelatin microparticle morphology, support slurries were blended for 60, 90, or 120 s (N=2). Gelatin slurries were recovered from 4°C, vortexed, and diluted 1:4 in 4°C Immuno-Mount mounting medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 2.5% (v/v) blue dye (McCormick & Co., Inc., London, ON, Canada). Samples were mounted on a coverslip and imaged using a Nikon SMZ 1500 stereomicroscope (Nikon Instruments Inc, Melville, NY, USA) and CoolSNAP cf camera (Teledyne Photometrics, Tucson, AZ, USA). ImageJ software (National Institutes of Health, Bethesda, MD, USA) was employed to convert each image to 8-bit greyscale and adjust brightness, contrast, and thresholding. Particles were counted and analyzed, and the effect of blend time on gelatin microparticle area, perimeter, Feret diameter, and circularity was measured (n=250).

2.2.4 Scaffold Computer-Aided Design

Collagen scaffolds were designed using SolidWorks modeling program (Dassault Systèmes, Vélizy-Villacoublay, France) to generate a 10 x 10 x 0.5 mm rectangular prism stereolithography (STL) file. The 10 x 10 x 0.5 mm STL was uploaded in quadruplicate to Slic3r (open source GNU AGPL license) for Gcode generation. STLs were sliced using either a rectilinear or hexagonal/honeycomb infill pattern at 20% infill density.

Polycaprolactone (PCL) scaffolds were designed using Simplify3D (Cincinnati, Ohio, USA) slicing software. A 50 x 50 x 2 mm rectangular prism stereolithography (STL) file was sliced with a 50% density, rectilinear infill pattern.
2.2.5 Printing of Porous Collagen Scaffolds

A r3bEL mini bioprinter (SE3D, Santa Clara, CA, USA) was plugged into a 12V DC power adaptor and controlled using Pronterface free open-source software (Printrun software licensed under the GNU General Public License, version 3). Porous collagen scaffolds were printed using freeform reversible embedding as described by Hinton et al.\textsuperscript{31} Lifeink® 200 collagen bioink (Advanced Biomatrix) consisting of 35 mg/mL pH neutral type I collagen purified from bovine hide with physiological salt concentration, was loaded into a 5 mL syringe and centrifuged at 100xg and 4°C for 1 min. Any air collected at the top of the syringe was expelled, and the syringe was fitted with a stainless steel 30G blunt luer stub to serve as the printing nozzle. The syringe was manually primed until collagen ink filled the nozzle, then mounted into the syringe pump extruder of the bioprinter. A 60 x 15 mm round polystyrene dish containing gelatin microparticle support slurry prepared as described in section 2.2.1 was positioned on the bioprinter bed and secured with vinyl-coated laboratory tape (VWR) to prevent slippage during movement of the print bed. The nozzle tip was positioned at the center of the support bath in x and y dimensions, and approximately 2 mm from the bottom of the bath in the z (lateral) direction. G-code instructions were immediately initiated to avoid clogging the nozzle.

All printing was completed at ambient temperatures (~23°C) over a period of 30 min and unused collagen ink was stored in the sealed syringe at 4°C immediately after printing. The polystyrene dish containing porous collagen scaffolds embedded within the gelatin support slurry was incubated at 37°C for 2 hr to melt the gelatin. Liquified gelatin was aspirated, and liberated collagen scaffolds were washed three times with PBS.

2.2.6 Printing of Porous Polycaprolactone Scaffolds

Low temperature polycaprolactone (PCL) 3D Filament (eSUN eMate, Shenzhen Esun Industrial Co., Ltd., Shenzhen, China) was fed into a F400-S (Fusion 3, Greensboro, NC, USA) operated using Duet Web Control (Creative Commons CC BY-SA 3.0 license). A 0.4 mm extrusion nozzle was heated to 90°C and the print bed to 30°C before execution of Gcode. Upon completion of printing, 6 mm diameter circular samples were cut using a biopsy punch (Integra Miltex, York, PA, USA) and functionalized using argon plasma treatment.
2.2.7 3D Printed Scaffold Morphology

Collagen and polycaprolactone scaffolds were prepared as described in sections 2.2.5 and 2.2.6 then imaged by digital camera and Nikon SMZ 1500 stereomicroscope to demonstrate scaffold architectures.

2.2.8 Collagen Scaffold Hydrophilicity

3D printed porous collagen scaffolds (N=4) were imaged using a Nikon SMZ 1500 stereomicroscope following printing, then incubated at 37°C in PBS for 14 days. Scaffolds were imaged again, and inner strut dimensions (n=45) were analyzed using ImageJ software to quantify the degree of swelling.

2.2.9 Cell Isolation and Culture

Human Research Ethics Board approval for this study was obtained from Western University (Appendix G). Subcutaneous adipose tissue samples were collected with informed consent a 67-year-old female undergoing lower limb amputation procedures at the London Health Sciences Centre (London, ON, Canada). Tissue samples were transported to the lab on ice in sterile, cation-free Dulbecco’s phosphate buffered saline (DPBS; Sigma) supplemented with 20 mg/mL bovine serum albumin (BSA) and processed within 2 hr for adipose-derived stem/stromal cell (ASC) isolation. Adipose tissue was washed with PBS by shaking, then treated with a digestion solution consisting of 0.1% (w/v) collagenase type 1, 1% (w/v) CaCl$_2$, and 1% (w/v) BSA. Fat tissue was minced and incubated for 1 hr at 37°C. The sample was centrifuged for 5 min at 420xg and the liquid supernatant was aspirated off. The cellular pellet was resuspended in proliferation medium comprised of DMEM supplemented with 10% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific), and 3% (v/v) antibiotic-antimycotic (Life Technologies Inc., Burlington, ON, Canada) and cultured on tissue culture polystyrene (TCPS; Corning, New York) at 37°C (20% O$_2$ and 5% CO$_2$). After 24 hr, the media was replaced and adherent cells were expanded further. The culture medium was replaced every 3 days and cells were cryopreserved in freezing medium comprised of 80% FBS, 10% Dulbecco’s Modified Eagle Medium (DMEM) and 10% dimethyl sulfoxide (DMSO) at passage one. ASCs were thawed and propagated on TCPS with 3rd passage cells used for all studies.
2.2.10 \textit{In Vitro} Proliferation Assay

Collagen scaffolds in two architectures, rectilinear and hexagonal, and rectilinear PCL scaffolds were placed in a 24-well culture plate and ultraviolet (UV) treated for 2 hr. P3 ASCs were seeded onto each scaffold at 80,000 cells per well and cultured in proliferation medium comprised of DMEM supplemented with 10\% (v/v) FBS and 3\% (v/v) antibiotic-antimycotic (37°C, 20\% O₂ and 5\% CO₂). Cells were also seeded onto TCPS as a control. After 24 hr culturing on scaffolds, cells were stained using the iFluor 488 EdU proliferation assay (ab219801, Abcam). Cells were incubated with 40 μM EdU for 2 hr then fixed in 4\% paraformaldehyde for 15 min at room temperature. Samples underwent two washes with PBS containing 3\% BSA before permeabilization for 20 min. Samples were washed twice and incubated with a reaction mixture composed of 1:420 iFluor 488 azide, 20 mg/mL sodium ascorbate and 4 mM copper sulfate in 1X Tris-buffered saline (TBS). The reaction mixture was prepared fresh and used within 15 min of preparation. Samples were rocked to ensure even distribution of the reaction mixture, then incubated at room temperature for 30 min protected from light. Samples were washed once with PBS containing 3\% BSA, then again in fresh PBS. Samples were then incubated with Hoechst 33342 (Trihydrochloride Trihydrate; Thermo Fisher Scientific) or propidium iodide (PI) at a dilution of 1:1000 for 1 hr at room temperature and subsequently washed with PBS three times. TCPS controls were imaged using an Axio Observer Z1 fluorescence microscope (Carl Zeiss) and 491/520 nm excitation/emission. Collagen and PCL scaffolds were imaged using z-stack acquisition on an LSM 800 confocal microscope (Zeiss). Images for each sample (n=10) were taken at 10X magnification and used to calculate the fraction of EdU-positive cells.

2.2.11 \textit{In Vitro} Cytoskeleton Morphology

Collagen scaffolds in two architectures, rectilinear and hexagonal, and rectilinear PCL scaffolds were placed in a 24-well culture plate and UV treated for 2 hr. P3 ASCs were seeded onto each scaffold at 80,000 cells per well and cultured in proliferation medium comprised of DMEM supplemented with 10\% (v/v) FBS and 3\% (v/v) antibiotic-antimycotic (37°C, 20\% O₂ and 5\% CO₂). Cells were also seeded onto TCPS as a control. TGF-β1 (R&D Systems, Minneapolis, MN, USA) or bovine serum albumin (BSA) were added to the proliferation media at 10 ng/mL. After either 3 days or 14 days of culturing on scaffolds, cells were washed three
times with PBS, then fixed for 10 min in 4% paraformaldehyde at room temperature. Samples
underwent three PBS washes then were permeabilized using 0.1% Triton X-100 for 5 min.
Washes were repeated and cells were incubated with 10% horse serum for 30 min to block non-
specific antibodies. Samples were incubated overnight at 4°C with a vinculin primary antibody
(MAB3574, Millipore) at 1:1000 in 1% horse serum. The next day, samples were washed and
incubated with 1:1000 Alexa Fluor 488 (Abcam), 1:1000 Hoechst 33342, and 1X rhodamine-
conjugated phalloidin (ab235138, Abcam) for 1 hr at room temperature, protected from light.
Samples were washed with PBS three times to remove unbound antibodies. TCPS controls were
imaged using an Axio Observer Z1 fluorescence microscope (Carl Zeiss) and collagen and PCL
scaffolds were imaged using z-stack acquisition on an LSM 800 confocal microscope (Zeiss).
Images for each sample (n=10) were taken at 10X magnification.

2.2.12 In Vitro Fibronectin Detection

Collagen scaffolds in two architectures, rectilinear and hexagonal, and rectilinear PCL
scaffolds were placed in a 24-well culture plate and UV treated for 2 hr (N=3). P3 ASCs were
seeded onto each scaffold at 80,000 cells per well and cultured in proliferation medium
comprised of DMEM supplemented with 10% (v/v) FBS and 3% (v/v) antibiotic-antimycotic
(37°C, 20% O2 and 5% CO2). Cells were also seeded onto TCPS as a control. Transforming
growth factor beta 1 (TGF-β1) or bovine serum albumin (BSA) were added to the proliferation
media at 10 ng/mL. After either 3 or 14 days of culturing on scaffolds, samples were treated
using the immunofluorescent protocol described in section 2.2.11. A fibronectin primary
antibody (sc 8422, Santa Cruz Biotechnology) was employed at 1:1000; the next day, samples
were incubated with 1:1000 Alexa Fluor 488 (Abcam), 1:1000 Hoechst 33342, and 1X
rhodamine-conjugated phalloidin. TCPS controls were imaged using an Axio Observer Z1
fluorescence microscope (Carl Zeiss) and collagen and PCL scaffolds were imaged using z-stack
acquisition on an LSM 800 confocal microscope (Zeiss). Images for each sample (n=10) were
taken at 10X magnification.

2.2.13 In Vitro Phenotype Study

Collagen scaffolds in two architectures, rectilinear and hexagonal, and rectilinear PCL
scaffolds were placed in a 24-well culture plate and UV treated for 2 hr (N=3). P3 ASCs were
seeded onto each scaffold at 50,000 cells per well and cultured in either proliferation medium, osteogenic differentiation medium, or adipogenic differentiation medium. Cells were also seeded onto TCPS as a control. The proliferation medium was comprised of DMEM supplemented with 10% (v/v) FBS and 3% (v/v) antibiotic-antimycotic (37°C, 20% O₂ and 5% CO₂). Osteogenic differentiation medium, used to induce osteogenic lineage, was comprised of proliferative medium supplemented with 10 mM β-glycerophosphate and 50 μg/mL ascorbate 2-phosphate. Adipogenic differentiation medium was comprised of serum-free DMEM media supplemented with 3% antibiotic-antimycotic, 33 μM biotin (vitamin B₇), 17 μM pantothenic acid (vitamin B₅), 66 nM insulin, 1 nM triiodothyronine (T3), and 10 μg/mL transferrin. For the first 3 days of culture, 1μg/mL of troglitazone and 0.25 mM isobutylmethylxanthine (IBMX) were also included in the adipogenic media. After 21 days of culturing on scaffolds, samples were treated using the immunofluorescent protocol described previously. Primary detection antibodies for runt-related transcription factor 2 (RUNX2; EPR14334, abcam) and peroxisome proliferator-activated receptor gamma (PPARγ; A3409A, R&D Systems) were employed at 1:1000 and 1:400, respectively. Samples were subsequently incubated with Alexa Fluor 488 goat α mouse, Alexa Fluor 647 donkey α rabbit (Abcam), and Hoechst 33342, each at 1:1000 dilution. TCPS controls were imaged using an Axio Observer Z1 fluorescence microscope (Carl Zeiss) and collagen and PCL scaffolds were imaged using z-stack acquisition on an LSM 800 confocal microscope (Zeiss). Images for each sample (n=10) were taken at 10X magnification.
Figure 2.1. Preparation of the Sacrificial Support Slurry. A gelatin microparticle slurry was employed as a secondary, thermo-reversible support medium for 3D printing of collagen.
1. Gelatin solidified overnight at 4°C

2. Gelatin blended for 120 s

3. Soluble gelatin (red), PBS washing solution (green), and gelatin slurry (blue) after centrifugation

4. PBS washing solution (green) and gelatin slurry (blue) after final wash

5. Slurry product before smoothing

6. Final product ready for printing
2.3 Results

2.3.1 Preparation of 3D Printed Scaffolds

To manufacture collagen scaffolds for human ASC culturing, collagen bioink was printed by positive-displacement ejection from a syringe. Collagen bioink printed directly onto TCPS resulted in material flowing out of the deposited architecture (Figure 2.1). Moreover, following gelation, printed scaffolds adhered to the TCPS surface and could not be lifted off without further collapse of the scaffold shape. Collagen bioink was too viscous to direct print, thus reverse embedded printing was considered. Suspension of printed materials within a secondary, sacrificial material in order to preserve the desired print architecture and allow for easy scaffold liberation.

A gelatin microparticle slurry was adapted with modification from Hinton et al.\textsuperscript{31,32} and employed as a secondary, thermo-reversible support medium for 3D printing of collagen. Gelatin in PBS was mechanically blended for 60, 90, or 120 s to fabricate a temperature-sensitive microparticle slurry (Figure 2.2). Following centrifugation of blended particles, a white raft of soluble gelatin was apparent in all samples. Soluble gelatin was removed from all samples by repeated washes with cold PBS. Longer blend durations resulted in notably greater soluble gelatin content, indicative of gelatin melting during the longer period off ice. At 4°C the slurry remained a solid-like gel, however incubation at 37°C completely melted the gelatin to a liquid. Thus, materials embedded within the gelatin slurry could be easily removed by melting the gelatin. Gelatin slurries blended for 60, 90, or 120 s were subject to rheological testing to study deformation and flow (Figure 2.3). Independent of blend duration, all samples behave as a Bingham plastic fluid and displayed comparable viscoelasticity and viscosity (p>0.05). At low stress, the slurry behaved as a solid, while at high stress it flowed as a viscous fluid. For all samples, the measured storage moduli (G’) was greater than the loss moduli (G” or E”), indicating that the gelatin slurry displayed more dominant gel-like structure than fluid-like. Supplementary rheological data is shown in Appendix A.

The effect of blend duration on gelatin microparticle morphology was investigated by blending gelatin samples for 60, 90, or 120 s, then observing suspended particles under a stereomicroscope (Figure 2.4). The mean particle area for slurries blended for 60, 90, or 120 s were 0.30 ± 0.12 mm\textsuperscript{2}, 0.14 ± 0.03 mm\textsuperscript{2}, and 0.04 ± 0.01 mm\textsuperscript{2}, respectively. Significant differences in the particle area (p<0.05), perimeter (p<0.01) and Feret diameter (p<0.01) were
observed between 60 s blended samples and 120 s blended samples. No significant differences involved the 90 s blended samples. All samples generated particles of comparable circularity, with reported index values of 0.74 ± 0.03, 0.72 ± 0.02, and 0.71 ± 0.03 for 60, 90, or 120 s blends (where 1.0 circularity index represents a perfect circle). Given that smaller particles would displace with higher resolution, 120 s blending was employed for all future embedded printing experiments.

For embedded printing of collagen bioinks, a 10 x 10 x 0.5 mm rectilinear prism STL was uploaded in quadruplicate to Slic3r for G-code generation (Figure 2.5). STLs were sliced using either a rectilinear or hexagonal (honeycomb) infill pattern at 20% infill density. A summary of early embedded printing attempts can be found in Appendix C. Rectilinear pores measured approximately 1 mm in diameter and exhibited interior angles of 90°. Hexagonal pores also measured approximately 1 mm in diameter and exhibited interior angles of 120°. The degree of scaffold swelling was assessed after two-week incubation in PBS at 37°C (Figure 2.6). Both infill patterns exhibited hydrophilicity, however while the hexagonal scaffold swelling was minimal enough to be considered not significant (p>0.05), rectilinear swelling was significant (p<0.001). This difference was likely a result of hexagonal struts being larger than their rectilinear counterparts, thus already containing free regions for water molecule infiltration, rather than being a difference in bioink hydrophilicity, since both architectures were printed using the same high concentration collagen type I bioink.

Polycaprolactone (PCL) scaffolds were printed using low temperature (90°C) polymer fusion printing. A 50 x 50 x 2 mm Rectangular prism CAD was sliced using rectilinear 50% density infill pattern from which 6 mm diameter circular samples were cut. The printed PCL construct displaced a rectilinear architecture with 90° interior angles where struts met, and struts approximately 0.4 mm in thickness.
Figure 2.2. Justification for Reverse Embedded Printing. Collagen bioink printed directly onto TCPS resulted in collapse of the scaffold architecture.
Figure 2.3. Rheological Properties of the Gelatin Support Slurry. Gelatin slurries prepared by blending for 60, 90, or 120 s were subject to viscoelasticity and viscosity testing. A) Storage (G’), loss (G”) and complex viscosity (|η*|) as a function of sweeping oscillatory angular frequency. B) Shear stress (τ) as a function of controlled shear rate (rotational speed) C) Viscosity (η) as a function of rotational shear rate. N=2, n=3, Two Way ANOVA, p>0.05. All data is represented as mean.
Figure 2.4. Effect of Blend Duration on Gelatin Microparticle Morphology. A)
Representative stereomicroscopic images of gelatin particles. B) Particle area, perimeter, Feret Diameter and circularity as a function of blend time. N=2, n=250, Two Way ANOVA with Tukey test, **p<0.01, *p<0.05. All data is represented as mean ± SEM.
A

60 s  90 s  120 s

B

Particle Area (mm²)

Particle Perimeter (mm)

Feret Diameter (mm)

Circularity Index

Gelatin Blend Time (s)
**Figure 2.5. Collagen Scaffold Computer-Aided Design.** A 10 x 10 x 0.5 mm rectilinear prism STL was uploaded in quadruplicate to Slic3r for G-code generation. STLs were sliced using either a rectilinear or hexagonal infill pattern at 20% infill density.
Figure 2.6. Porous Collagen Scaffold Morphology. A) Scaffolds were successfully embedded within a gelatin support slurry and subsequently liberated. B) Scaffold swelling was assessed 2 weeks post-printing. N=4, Student’s test, ***p<0.001, ns=not significant at p>0.05. All data is represented as mean ± SEM.
Figure 2.7. Polycaprolactone (PCL) Scaffold Morphology. A 50 x 50 x 2 mm Rectangular prism CAD was sliced using rectilinear 50% density infill pattern from which 6 mm diameter circular samples were cut. A) Digital photograph of the printed PCL construct. B) Stereomicroscopic image of the printed PCL showing scaffold architecture.
2.3.2 In Vitro Validation of 3D Printed Scaffolds as a Support for ASC Cell Growth

Three-dimensional printed scaffolds were assessed using human adipose-derived stem/stromal cells (ASCs) isolated from subcutaneous adipose tissue obtained from a female aged 67. In all studies, PCL rectilinear and collagen rectilinear and hexagonal 3D printed scaffolds were used.

Collagen and PCL scaffolds were first assessed using the EdU proliferation assay (Figure 2.8). Hoechst was used to counterstain DNA on TCPS and collagen scaffolds, while propidium iodide (PI) was used for PCL samples since this material auto fluoresces at 450 nm (Appendix D). ASCs seeded onto PCL scaffolds were over 90% EdU positive, and this difference was statistically significant when compared to collagen scaffolds of either architecture previously described, rectilinear or hexagonal (p<0.01). While approximately 10% of cells seeded onto rectilinear or hexagonal collagen scaffolds were proliferating, it was significantly less than the control TCPS group (p<0.01). ASC proliferation on PCL scaffolds compared to those cultured on TCPS was not significant (p>0.05), likely as a result of low cell numbers present on PCL scaffolds themselves. Together, these results confirm that human ASCs are capable of proliferation, especially on a PCL scaffold.

ASC cytoskeletal morphology was assessed three days post-cell seeding on scaffolds and with media supplemented with transforming growth factor beta 1 (TGF-β1) or bovine serum albumin (BSA) as a control (Figure 2.9). TGF-β1 is a secreted cytokine that influences many cellular functions, including the control of cell growth, cell proliferation, cell differentiation, and apoptosis. The actin cytoskeleton was visualized as well as vinculin, a membrane-cytoskeletal linker protein. Qualitatively, TGF-β1 increased cell density and spreading on all culturing surfaces, 3D scaffolds or 2D TCPS. Cells were seen to align along rectilinear or hexagonal geometries, and frequently were found stretched inside of pores, anchored to opposing struts on either side. Even in BSA control groups, cells plated on rigid PCL scaffolds exhibited robust cell spreading, an extensive actin network, and expression of vinculin; this contrasted with ASCs seeded onto soft collagen protein scaffolds, where cells appeared less spread.

ASC cytoskeletal morphology was also assessed 14 days post-seeding (Figure 2.10). Interestingly, ASCs cultured for 14 days on scaffolds exhibit a noticeable increase in cell density and spreading after 2 weeks, indicating a delayed cellular response and slower proliferative response. These results demonstrate that ASC activity can be stimulated using TFG-β1 for rapid
(3 days) tissue regeneration while scaffolds alone are capable of maintaining viable ASCs for prolonged growth periods (2 weeks).

As an important component of tissue regeneration is the synthesis of new ECM, the presence of fibronectin was assessed three days post-cell seeding on scaffolds and with media supplemented with transforming growth factor beta 1 (TGF-β1) or bovine serum albumin (BSA) as a control (Figure 2.11). Fibronectin is a high-molecular weight (~440 kDa) glycoprotein of the ECM that cells attach to through membrane-spanning receptor proteins, integrins. Fibronectin also binds to other extracellular matrix proteins such as collagen and fibrin.

Consistent with previous findings, TGF-β1 was sufficient to stimulate fibronectin production on all 3D scaffolds and the 2D TCPS. Between the BSA groups, printed collagen scaffolds appeared to increase fibronectin protein production in comparison with PCL scaffolds or 2D TCPS surfaces.

The presence of fibronectin was also evaluated 14 days post-seeding (Figure 2.12). By day 14, the presence of fibronectin was comparable to day 3 TGF-β1 supplemented groups. The increase in fibronectin detected at day 14 suggests deposition of new fibronectin matrix by the ASCs. Fibronectin content appeared relatively uniform between PCL and collagen scaffolds by week 2. Taken together, these results have shown that ASCs can be stimulated with additional bioactive molecules and reinforces the strategy of using a biomaterial as a delivery system as well as a support structure for cells.

ASC phenotype was studied using adipogenic and osteogenic induction media types, as well as a proliferative media control. After 3-week culturing on PCL or collagen scaffolds, PPARγ and RUNX2 expression was visualized by immunofluorescence (Figure 2.13). PPARγ is a nuclear receptor mainly present in adipose tissue; it regulates fatty acid storage and stimulates lipid uptake. In contrast, RUNX2 is a transcription factor associated with osteoblast differentiation. Most ASCs appeared positive for PPARγ, which was not affected by culturing in either osteogenic induction media or standard proliferative media. Varying the 3D scaffolding architecture did not appear to influence ASC phenotype, however this might be a caveat of ASCs derived from pathological lower limbs.
Figure 2.8. ASC Proliferative Potential. A) Hoechst was used to stain DNA on TCPS and collagen scaffolds, while propidium iodide (PI) was used for PCL samples. EdU-positive cells are indicative of cells that were in the process of cell division. B) The fraction of EdU-positive cells was quantified for TCPS, PCL rectilinear, and collagen rectilinear and hexagonal 3D printed scaffolds. N=3, n=10, Two Way ANOVA with Tukey test, **p<0.01. All data is represented as mean ± SEM.
A

Collagen Hexagonal Scaffold  
Collagen Rectilinear Scaffold  
PCL Rectilinear Scaffold  
TCPS

Fraction of EdU-Positive Cells

B

**

EdU-Positive  
Hoechst/PI
Figure 2.9. ASC Cytoskeletal Morphology. ASCs cultured on scaffolds or TCPS for 3 days with media supplemented with either A) 10 ng/mL BSA or B) 10 ng/mL TGF-β1.
Collagen Rectilinear Scaffold

TCPS

PCL Rectilinear Scaffold

Collagen Rectilinear Scaffold

Collagen Hexagonal Scaffold

Hoechst

Vinculin

F-Actin
B

TCPS

PCL Rectilinear Scaffold

Collagen Rectilinear Scaffold

Collagen Hexagonal Scaffold

Hoechst
Vinculin
F-Actin
Figure 2.10. ASC Cytoskeletal Morphology after 14 Days. ASCs cultured on scaffolds or TCPS for 14 days with media supplemented with 10 ng/mL BSA.
TCPS

Collagen Rectilinear Scaffold

PCL Rectilinear Scaffold

Collagen Hexagonal Scaffold

Hoechst
Vinculin
F-Actin
Figure 2.11. Fibronectin Detection After 3 Days. ASCs cultured on scaffolds or TCPS for 3 days with media supplemented with either A) 10 ng/mL BSA or B) 10 ng/mL TGF- β1.
A

TCPS

PCL Rectilinear Scaffold

Collagen Rectilinear Scaffold

Collagen Hexagonal Scaffold

Hoechst

Fibronectin
TCPS

Collagen Rectilinear Scaffold

PCL Rectilinear Scaffold

Collagen Hexagonal Scaffold

Hoechst

Fibronectin
Figure 2.12. Fibronectin Detection After 14 Days. ASCs cultured on scaffolds or TCPS for 14 days with media supplemented with 10 ng/mL BSA.
TCPS

PCL Rectilinear Scaffold

Collagen Rectilinear Scaffold

Collagen Hexagonal Scaffold

Hoechst
Fibronectin
Figure 2.13. ASC Phenotype Analysis. ASC phenotype after culturing for 3 weeks in osteogenic induction media, adipogenic induction media, or proliferative media control.
Proliferative Media

Osteogenic Media

Adipogenic Media

PCL Rectilinear Scaffold

Collagen Rectilinear Scaffold

Collagen Hexagonal Scaffold

Hoechst

PPARγ

RUNX2
2.4 Discussion and Conclusion

In this proof-of-concept tissue engineering study, soft collagen bioink scaffolds were reproducibly printed using a gelatin microparticle support bath. The gelatin support slurry was shear-sensitive and behaved similarly to a Bingham plastic fluid. Gelatin microparticle size was found to be dependent on the preparation method. Longer durations of mechanical blending resulted in smaller gelatin microparticles that displaced during embedded printing with higher resolution. Embedded printing has become a promising technique for the additive manufacture of viscous, soft materials such as elastomers, gels, and hydrogels.\textsuperscript{28,29} Specifically, the printing of biological hydrogels, composed of polysaccharides and proteins, are difficult to print because the biological materials must gel \textit{in situ} after extrusion from the print nozzle. To be suited for bioprinting, a material must have appropriate mechanical fluid properties. If print materials are not fluid enough, or if they gel quickly, a blockage will form inside the small-diameter print nozzle and affect printing results. Fluid-like materials require surrounding support from a sacrificial material such that the printed object does not collapse or deform due to material viscosity and excess weight deposited layer-by-layer. Embedded printing of biological materials has been employed previously, such as in the fabrication of a sodium alginate coronary arterial tree with a perfusable, hollow lumen\textsuperscript{31} as well as the printing of perfusable polydimethylsiloxane (PDMS) tubes within a hydrophilic Carbopol gel support.\textsuperscript{32} Embedded printing of biologically relevant materials has the potential to precisely control microstructure and anisotropy during layer-by-layer assembly of 3D constructs.

Embedded printed collagen type I scaffolds and fusion printed PCL scaffolds were printed with reproducible quality and fidelity. Both material scaffolds were sterilizable, making them suitable for \textit{in vitro} biological applications. Collagen and PCL scaffolds supported ASC proliferation, with the majority of ASCs seeded onto PCL scaffolds undergoing cell division. PCL scaffolds were quite rigid; the material has a documented Young’s (elastic) modulus in the range of 340 to 365 MPa.\textsuperscript{35} Matrix stiffness is known to regulate cell behavior (mechanosensation) and migration (durotaxis); stiff matrix environments have been shown to promote proliferative behavior in a variety of cell types, including hepatocytes, stellate cells, and fibroblasts.\textsuperscript{36} Our findings are consistent in this regard, with ASCs demonstrating a robust proliferative response on PCL scaffolds compared to soft collagen protein scaffolds. Importantly,
cell proliferation for tissue engineering applications should be regulated to prevent pathological cell growth and tumor formation.\textsuperscript{37}

Cell response to the 3D printed scaffolds was evaluated by studying ASC cytoskeletal morphology and fibronectin deposition. On rectilinear PCL scaffolds, cells exhibited a high degree of spreading, a mature F-actin network, and interaction with the ECM via vinculin. However, fibronectin synthesis on PCL scaffolds appeared reduced compared to collagen scaffolds after 3 days of culturing. ASCs responded positively to TGF-\(\beta\)1 treatment; independent of scaffold material or infill architecture, including this bioactive molecule in culture improved cell density, spreading, and fibronectin synthesis. This is expected, given that TGF-\(\beta\)1 protein regulates cell proliferation, differentiation and growth, and can modulate expression and activation of other growth factors including interferon gamma and tumor necrosis factor alpha.\textsuperscript{38}

This study has demonstrated that ASC \textit{in vitro} activity can be stimulated using TFG- \(\beta\)1 for rapid (3 days) tissue regeneration while scaffolds alone are capable of maintaining viable ASCs for prolonged periods (2 weeks).

In this study, ASC phenotype appeared unaffected by scaffold material (PCL or collagen) or architecture (rectilinear or hexagonal). This may have been a caveat of using ASCs isolated from adipose tissue obtained from lower limb amputation. ASCs in this study were largely PPAR\(\gamma\)-positive, suggesting an adipogenic phenotype. Even when seeded onto TCPS, the majority of ASCs were PPAR\(\gamma\)-positive, with minimal RUNX2 expression detected (Results not published; ASC cultures reached hyperconfluency rapidly on TCPS, with culture decline and apoptosis occurring well before the 3-week experimental end point. In contrast, ASC differentiation on PCL and collagen scaffolds took approximately 3 weeks to obtain expression of PPAR\(\gamma\) and RUNX2). Three-dimensional geometry has been shown to successfully influence stem cell lineage. For example, mesenchymal stem cells (MSCs) seeded onto 3D printed poly(propylene fumarate) scaffolds demonstrated increased expression of early osteogenic markers when cultured onto cylindrical pores, while cubic pores influenced gene expression for MSCs undergoing adipogenesis and chondrogenesis.\textsuperscript{39} Scaffold geometry of 3D-printed gelatin constructs, containing either 90° square shaped pores or 60° triangular pores, significantly influenced the differentiation and function of seeded hepatocytes.\textsuperscript{40} In addition to pore geometry, pore size also influences cell behavior. PCL scaffolds printed into square, triangular, and rhomboidal pore geometries of varied pore diameters from 550 to 750 \(\mu\)m were shown to
influence human MSC behaviors such as adhesion, viability and proliferation.\textsuperscript{41} Similarly, MSCs seeded onto either PCL or 300PEOT55PBT45 (PolyActive300/55/45) printed scaffolds presenting an axial gradient of pore size and total porosity displayed improved osteogenic differentiation.\textsuperscript{42}

In conclusion, 3D printing of biomaterials allows for rapid prototyping and precise control of construct geometry, microstructure, and anisotropy during layer-by-layer assembly. Relevant biomaterials will ideally be biocompatible, supporting cell adhesion, expansion, and viability. Biomaterials and their degradation products should be non-toxic and non-immunogenic. Furthermore, biomaterial properties should be closely matched to the tissues they are replacing. This study has demonstrated that soft collagen protein scaffolds stimulate fibronectin production \textit{in vitro} and future work should be done to assess validity as a soft connective tissue substitute. Rigid PCL scaffolds in contrast support robust cell spreading and proliferation and should be investigated for implementation in the regeneration of stiff tissues (for example, bone), in long-term wound management applications (for example, management of non-healing wounds), and regions of repetitive mechanical loading that require stronger scaffold constructs (for example, wounds at the sole of the foot).
2.5 References


3 Investigating the Effects of Exogenous Galectin-3 in Murine Wound Healing

3.1 Introduction

Skin acts as a barrier between the external environment, facilitates sensation and thermoregulation. Following injury, normal wound healing involves four overlapping spatiotemporally coordinated events, hemostasis to halt bleeding, inflammation to clear the wound of debris and infectious bodies, proliferation of cells and re-epithelialization to restore skin barrier function. However, factors such as advanced age, vascular insufficiency, limb trauma, obesity, and diabetes may result in a reduced ability to heal. If a wound persists beyond 12 weeks, it is classified as a non-healing chronic wound. The most common types of chronic wounds include venous ulcers, arterial ulcers, pressure ulcers, and diabetic ulcers.

Chronic wounds are characterized by increased expression of pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, and tissue necrosis factor alpha (TNF-α), combined with decreased levels of pro-regenerative cytokines including transforming growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF). An imbalance of proteolytic activity leads to excessive degradation of the extracellular matrix, inhibiting cell migration and proliferation. Decreased levels of growth factors, such as keratinocyte growth factor, fibroblast growth factor 2 (FGF-2) and VEGF, further prevents progression towards proliferative and re-epithelialization stages. Chronic wound resolution thus depends on converting the pro-inflammatory microenvironment into a pro-fibrotic one.

Galectin-3, a matricellular protein and member of the lectin family, has been implicated in modulating the inflammatory phase of wound healing. In vitro, galectin-3 treatment increases monocyte and macrophage migration. Galectin-3 is important for macrophage linkage to neutrophils and increases neutrophil phagocytosis. Moreover, this protein is implicated in regulating alternative (M2) macrophage activation, an important process for inflammation resolution. Studies employing galectin-3 knockout mice have shown that macrophages derived from bone marrow of galectin-3 null animals display diminished ability to undergo alternative activation. Thus, the use of galectin-3 in modulating prolonged inflammation of a chronic wound is of interest. The context in which its regulatory activity is preserved, including therapeutic concentration range and delivery system, remains to be elucidated.
Blend electrospinning has previously been employed for the incorporation and delivery of proteins from a biomimetic nanofibrous scaffold. Electrospun scaffolds display high porosity and large surface area-to-volume ratios. Delivery of human recombinant galectin-3 via an electrospun gelatin scaffold in wound healing is of interest, as the scaffold facilitates effective delivery and distribution of the protein into the wound bed microenvironment. In addition, the scaffold would act as a temporary extracellular matrix, directing tissue regeneration by providing a site for cell adhesion, proliferation, and migration into the wound bed. The goal of this research was to generate an electrospun gelatin scaffold loaded with recombinant galectin-3 and assess the effect of exogenous galectin-3 delivery in cutaneous wound healing.

3.2 Materials and Methods

3.2.1 Electrospinning Galectin-3-Loaded Fibrous Gelatin Scaffolds

A polymer solution consisting 50 μg/mL recombinant human galectin-3 (R&D Systems, Minneapolis, MN, USA) and 21% (w/v) Type B Bovine gelatin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 40% (v/v) acetic acid (Thermo Fisher Scientific, Waltham, MA, USA) was aspirated into a plastic 1cc Tuberculin syringe (Terumo, Shibuya, Tokyo, Japan) connected to PTFE capillarity tubing (Saint-Gobain Performance Plastics, France) fitted with a 20 gauge blunt-tip stainless steel needle. The tube and needle were primed by pushing the syringe plunger until the electrospinning solution began to drip from the needle. The syringe was secured in a pump (VWR International, Radnor, PA, USA) and the needle was connected to a high voltage DC power supply (Gamma High Voltage Research, Ormond Beach, FL, USA). The needle tip was positioned 10 cm from a grounded stainless steel rotating barrel. A piece of aluminum foil was wrapped around the rotating barrel and secured with electrical tape to act as the collecting surface. During electrospinning, a 15-kV voltage was applied as the electrospinning solution was pumped at a flowrate of 0.5 mL/hr. Scaffolds were electrospun onto the collection barrel rotating at 100 revolutions per minute (RPM) for 1.5 hours using a total volume of 0.75 mL of solution. Fibrous scaffolds were then crosslinked in a glass desiccator (VWR International) containing drierite (W.A. Hammond Drierite Co. Ltd, Xenia, OH, USA) using the vapour from a 5 mL solution consisting of 1.5% (v/v) glutaraldehyde (GTA) (Sigma-Aldrich, St. Louis, MO, USA) in anhydrous ethyl alcohol (Commercial Alcohols, Brampton, ON). The desiccator was held under
vacuum for 20 min and scaffolds were stored in the sealed desiccator for 48 hr. Following crosslinking, scaffolds were transferred to sealed plastic containers with desiccant at 4°C.

3.2.2 Scanning Electron Microscopy

Gelatin scaffolds (N=3) were electrospun using gelatin polymer solutions of 20, 21, 25, and 30% (w/v) concentration. An 8 mm diameter circular sample was collected per scaffold using a biopsy punch (Integra Miltex, York, PA, USA). Scaffold samples were fixed onto 15 mm aluminum stubs using adhesive carbon discs and sputter coated with osmium (OPC-80T, Filgen Inc., Japan), then imaged using a Hitachi 4300-N scanning electron microscope (Hitachi Ltd., Japan) at the Biotron Integrated Microscopy Facility (Western University). Images were acquired at 1000X, 3000X, and 5000X magnifications at an accelerating voltage of 4 kV. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was employed to assess the diameter of fibers (n=250) from 5 images taken at the same magnification.

3.2.3 Effect of Galectin-3 on Murine Wound Healing In Vivo

Galectin-3-loaded fibrous gelatin scaffolds were recovered from storage at 4°C and 8 mm diameter circular samples were collected using a biopsy punch (Integra Miltex, York, PA, USA). Each scaffold was separated from its tinfoil base and quenched in 0.1 M glycine (Sigma-Aldrich, St. Louis, MO, USA) for 1 hr at 23°C to remove residual glutaraldehyde. Scaffolds underwent three 15 min PBS rinses then were stored overnight at 4°C. Prior to implantation, scaffolds were exposed to ultraviolet (UV) treatment for 2 hr.

All animal procedures followed protocols approved by the University Council on Animal Care at Western University. Nine male wild type (WT) (C57BL/6J; 000664) mice (The Jackson Laboratory, Sacramento, CA, USA), 14 weeks of age at the time of surgery, were employed. Approximately 30 min prior to surgery, 0.05 mg/kg of buprenorphine was administered to each mouse as a pre-emptive analgesic. Animals were anaesthetized using 90 mg/kg ketamine and 5 mg/kg xylazine, with inhalation of 3% isoflurane in oxygen as needed. Hair was shaved from the back of the animal and 1 cc of Nair cream (Church & Dwight Co., Inc., Ewing, NJ, USA) was applied topically for 5 min to remove remaining hair by chemical depilation. The Nair cream was wiped off and the underlying murine skin was rinsed with warm water, then dried. Povidone
iodine was used to clean the area. Four full-thickness dermal wounds measuring 6 mm in diameter were created using a sterile biopsy punch (Integra Miltex, York, PA, USA). Each wound was assigned one of four treatment conditions: empty (control), implantation of a 21% (w/v) gelatin electrospun scaffold containing 50 μg/mL galectin-3, or daily topical application of 10 μL of galectin-3 at a concentration of either 12.5 μg/mL or 25 μg/mL in PBS. Treatments were rotated clockwise in each mouse to eliminate positional effects on wound healing. Following completion of the surgical procedure, atipamezole was administered at a dose of 1 mg/kg. Animals were given buprenorphine again 12 hr post-surgery.

Mice were sacrificed using carbon dioxide exposure as follows: three mice on day 3, three mice on day 7, and two mice on day 9 post-surgery (one animal was sacrificed early and excluded from the study due to impaired recovery 1-day post-surgery). Wound tissue samples were harvested immediately and fixed in 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) for 24 hr at 4 °C. Tissues were transferred to 70% ethanol (Commercial Alcohols) and were paraffin embedded at the Molecular Pathology Core Facility (Robarts Research Institute, London, ON, Canada). Serial 5 μm sections were taken from the center of the wounds.

3.2.4 Murine Wound Healing Kinetics

On days 0, 3, 5, 7, and 9 post-surgery, each animal was lightly anesthetized by inhalation of 3% isoflurane in oxygen, and the back area containing the dermal biopsy wounds was imaged by digital camera. A ruler was placed next to the animal and included in the digital photographs for scale documentation. Wound bed area was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Serial 5 μm wound sections were stained with Masson’s Trichrome at the Molecular Pathology Core Facility (Robarts Research Institute, London, ON, Canada) and imaged at 5X magnification using an Axio Imager M1 light microscope (Carl Zeiss AG, Oberkochen, Germany). Images were stitched together, and epithelial tongue length and epithelial tongue thickness were evaluated. Epithelial tongue length was measured as the distance from the tip of the epithelial tongue to the unwounded dermis. Epithelial tongue thickness was evaluated as the epithelial tongue area divided by the epithelial tongue length.
3.2.5 Effect of Galectin-3 on Arginase I Enrichment During Wound Healing

Serial 5 μm wound sections were rehydrated, rinsed with PBS for 5 min and subjected to enzymatic antigen retrieval for 15 min at 37°C. Samples were again rinsed in PBS for 5 min at room temperature and blocked using 10% horse serum in PBS for 30 min at room temperature in a humidified chamber. Sections were incubated in primary chicken antibody against arginase I (ABS535; MilliporeSigma, Burlington, MA, USA) diluted at 1:25 in 10% horse serum overnight at 4°C. Sections were rinsed in PBS for 5 min and incubated with Alexa Fluor 488 goat anti-chicken (Abcam, Cambridge, United Kingdom) at a dilution of 1:500 and Hoechst 33342 (Trihydrochloride Trihydrate; Thermo Fisher Scientific) at a dilution of 1:1000 in 10% horse serum for 1 hr at room temperature, protected from light. Sections were rinsed thoroughly in PBS, mounted using Immu-Mount (Thermo Fisher Scientific) mounting medium, sealed with glass coverslips, and imaged using an Axio Observer Z1 fluorescence microscope (Carl Zeiss) using the appropriate filters. Negative controls were also stained without the addition of primary antibodies. These negative control slides were imaged to set the threshold values for the detection of fluorescence (Appendix F). ImageJ software was used to quantify the number of arginase I-positive cells in the wound bed at days 7 and 9 (N=2, n=5).

3.3 Results

3.3.1 Preparation of Electrospun Galectin-3/Gelatin Scaffolds

The effect of gelatin concentration on electrospun fiber morphology was assessed by spinning gelatin polymer solutions at three concentrations, 20%, 25%, and 30% (Figure 3.1). As the concentration of gelatin increased, the resulting fiber diameter also increased. The majority of fibers spun from 30% weight gelatin ranged in diameter from 500-2000 nm in diameter, but there was significant variation in fiber size, with some fibers measuring over 4000 nm in diameter. At 25% weight, the majority of fibers were within the range of 300-500 nm, while lowering the gelatin to 20% resulted in fibers 100-200 nm in diameter. Fiber diameter was refined using 21% weight gelatin in the electrospinning polymer solution, resulting in a tighter distribution of fibers around 200 nm in diameter (Figure 3.2). Fibers spun from 21% gelatin displayed a mean diameter of 181 ± 40 nm.
Figure 3.1. Effect of Gelatin on Electrospun Fiber Morphology. A) Representative scanning electron microscopy images for scaffolds spun from 20%, 25% and 30% gelatin polymer solutions are shown. B) Quantified frequency distribution of fiber diameters for 20%, 25% and 30% gelatin scaffolds.
Figure 3.2. Refined Electrospun Fiber Morphology. A) Representative scanning electron microscopy image of 21% gelatin electrospun fibers. B) Quantification of fiber diameters. Fibers spun from 21% gelatin displayed a mean diameter of 181 ± 40 nm.
A

21% Gelatin

B

Frequency (%)

Fiber Diameter (nm)
3.3.2 *In Vivo* Effects of the Electrospun Galectin-3/Gelatin Scaffold

To investigate the effects of exogenous recombinant galectin-3 in murine wound healing, full-thickness dermal wounds were created on the backs of wild type mice. Independent of treatment, all dermal wounds appeared much smaller by day 9 post-wounding (Figure 3.3). The wound area was measured on days 0, 3, 5, 7 and 9 to calculate wound closure kinetics (Figure 3.4). No differences between each of the treatment groups and the experimental control (empty wound) were observed at all time points assessed. Wound closure increased steadily over the 9-day period for all wounds assessed, independent of treatment. These results demonstrated that neither implantation of a galectin-3/gelatin electrospun scaffold, nor daily topical delivery of galectin-3, delayed wound closure *in vivo*.

Since exogenous delivery of galectin-3, either by slow release from an electrospun scaffold or by topical application did not affect wound healing kinetics, re-epithelialization of the wound was studied. Masson’s trichrome staining for sections obtained from days 7 and 9 post-wounding revealed a great amount of variation between biological replicates, regardless of wound treatment group (Figure 3.5). Wounds re-epithelialized at the same mean rate, and no differences were found between epithelial tongue length or thickness between treatment groups (Figure 3.6). Variation between biological replicates for all treatment groups was apparent. For instance, focusing specifically on the topical galectin-3 (12.5 μg/mL) experimental treatment, a thick tongue and fully re-epithelialized wound was observed for one animal (Figure 3.7A), while another day 7 replicate exhibited a much thinner tongue and significantly less re-epithelialization of the wound (Figure 3.7B). Day 9 animals were similar with one displaying full migration of the epithelial tongue (Figure 3.7C) while another displayed minimal re-epithelialization (Figure 3.7D). While one day 7 animal exhibited a thick epithelial tongue re-epithelializing the wound, both day 9 animals of this group exhibited noticeably thinner epithelial tongues, indicating that the enhanced re-epithelialization observed in a single day 7 mouse was likely not a result of the galectin-3 topical treatment of the wound but rather random occurrence.

The influence of exogenous galectin-3 from an electrospun galectin-3/gelatin scaffold and by daily topical application on macrophage polarity during full-thickness wound healing was also investigated. While qualitatively it appeared that increasing galectin-3 concentration at the wound led to more arginase I-positive macrophages (Figure 3.8), quantification revealed that the
mean density of arginase I-positive cells in the wounds was not different between treatment conditions at day 9 (Figure 3.9).
Figure 3.3. Representative Images of Murine Full-Thickness Dermal Wounds. Full thickness excisional wounds measuring 6 mm in diameter were treated with implantation of an electrospun scaffold composed of 21% gelatin blended with galectin-3 (50 μg/mL), daily topical treatment of galectin-3 in PBS (12.5 μg/mL or 25 μg/mL) or left empty (control). Representative images of the four conditions are shown at days 0, 3, 5, 7 and 9 post-wounding.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 9</th>
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<td>Open</td>
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10 mm
Figure 3.4. Quantification of Murine Wound Healing Kinetics. Full thickness excisional wounds measuring 6 mm in diameter were treated with implantation of an electrospun scaffold composed of 21% gelatin blended with galectin-3 (50 μg/mL), daily topical treatment of galectin-3 in PBS (12.5 μg/mL or 25 μg/mL), or left empty (control). Percentage of wound area closure was calculated over a 9-day period. Data is represented as mean ± SEM.
Wound Closure (%)

Day Post-Wound

- Open
- Gelatin/Galectin-3 Scaffold [50ug/mL]
- Topical Galectin-3 [12.5ug/mL]
- Topical Galectin-3 [25ug/mL]
Figure 3.5. Masson’s Trichrome Staining Following In Vivo Full-Thickness Wounding. Full thickness excisional wounds measuring 6 mm in diameter were treated with implantation of an electrospun scaffold composed of 21% gelatin blended with galectin-3 (50 μg/mL), daily topical treatment of galectin-3 in PBS (12.5 μg/mL or 25 μg/mL concentration), or left empty (control). Sections show the wound edge for each condition and the epithelial tongue. A) Sections from day 7 post-wounding. B) Sections from day 9 post-wounding.
Figure 3.6. Re-epithelialization and Epithelial Thickness Following *In Vivo* Full-Thickness Wounding. Full thickness excisional wounds measuring 6 mm in diameter were treated with implantation of an electrospun scaffold composed of 21% gelatin blended with galectin-3 (50 μg/mL), daily topical treatment of galectin-3 in PBS (12.5 μg/mL or 25 μg/mL), or left empty (control). A) Epithelial tongue length was calculated at days 7 and 9 post-wounding. B) Epithelial tongue thickness was calculated at days 7 and 9 post-wounding. All data is represented as mean ± SEM.
Figure 3.7. Example of Biological Variability in Masson’s Trichrome Staining of Full-Thickness Wounds. All wounds shown were treated with daily topical application of galectin-3 in PBS (12.5 μg/mL concentration). A and B) Two biological replicates from day 7 post-wounding. C and D) Two biological replicates from day 9 post-wounding.
Day 7: Topical Galectin-3 [12.5 μg/mL]

Day 9: Topical Galectin-3 [12.5 μg/mL]
Figure 3.8. Arginase I Enrichment of Full-Thickness Wounds. Full thickness excisional wounds measuring 6 mm in diameter were treated with implantation of an electrospun scaffold composed of 21% gelatin blended with galectin-3 (50 μg/mL), daily topical treatment of galectin-3 in PBS (12.5 μg/mL or 25 μg/mL concentration), or left empty (control). Sections show the relative amounts of arginase I-positive cells (green) in the wound bed for each treatment condition. Cell nuclei are shown in blue. Two biological replicates are shown for each treatment.
Topical Galectin-3 [12.5 μg/mL]

Topical Galectin-3 [25 μg/mL]

Galectin-3/Gelatin Scaffold [50 μg/mL]
Figure 3.9. Arginase I-Positive Cell Density within the Wound Bed. Full thickness excisional wounds measuring 6 mm in diameter were treated with implantation of an electrospun scaffold composed of 21% gelatin blended with galectin-3 (50 μg/mL), daily topical treatment of galectin-3 in PBS (12.5 μg/mL or 25 μg/mL), or left empty (no scaffold control, daily topical PBS). Density of arginase I-positive cells was quantified from 5 sections. All data is represented as mean ± SEM.
Open,
Topical [12.5 ug/mL],
Topical [25 ug/mL],
Scaffold [50 ug/mL]

Arginase$^{-1}$ (cells/cm$^2$)

Galectin-3 Treatment

0 2000 4000 6000 8000 10000 12000 14000 16000 18000

Open, Topical [12.5 ug/mL], Topical [25 ug/mL], Scaffold [50 ug/mL]
3.4 Discussion and Conclusion

Prolonged inflammation, a hallmark of chronic wounds, halts progression to proliferative and re-epithelialization phases, preventing wound resolution and restoration of the epithelial barrier. Galectin-3 has been indicated in wound healing processes, including monocyte migration, alternative macrophage activation, and increased re-epithelialization in corneal wounds. However, the efficacy of exogenous galectin-3 in treating skin wounds appeared to be tissue- and context-specific. Based on its described functions, we hypothesized that local delivery of galectin-3 could regulate macrophage activation and increase re-epithelialization in skin healing. An electrospun gelatin scaffold was employed for the localized delivery of galectin-3. Electrospun nanofibers exhibit a large surface area for distribution of the protein. Type B bovine gelatin is derived from collagen and has suitable biorecognizable properties. While electrospinning of collagen has demonstrated loss of collagen’s tertiary structure, gelatin has been widely employed with results showing biocompatibility and functionality. Electrospun gelatin scaffolds have been shown to enhance wound closure in a full thickness wound healing model in rats. Electrospun gelatin scaffolds, both alone and mixed with other polymers, have also been employed for the delivery of growth factors and bioactive molecules. Moreover, the biodegradability of gelatin can be tuned using glutaraldehyde crosslinking, wherein the aldehyde groups react with lysine or hydroxylysine residues to form aldimine linkages. Subsequent glycine quenching of the scaffolds blocks unreacted aldehyde groups. The electrospinning polymer solution was prepared by dissolving type B bovine gelatin in 40% (v/v) acetic acid. Acetic acid has been used for the electrospinning of collagen, resulting in fiber diameters distributed around 100-200 nm. By electrospinning with acetic acid, the use of highly cytotoxic and protein structure damaging fluoroalcohols is avoided.

Our lab has previously demonstrated the refinement of scaffold manufacturing protocols to create bead-free and ribbon-free fibers with diameters measuring within the range of the native extracellular matrix. The polymer solution flowrate, collector distance, and the concentration of the polymer have all previously been reported to influence the resulting electrospun fiber diameter and morphology. As a result, 0.5 mL/hr was selected for electrospinning since this flowrate decreases the amount of time required for electrospinning as well as the duration that galectin-3 is exposed to the solvent. A collector distance of 10 cm was selected for electrospinning as this facilitates a wider distribution of fibers on the rotating mandrel,
maximizing scaffold surface area. Consistent with other studies, this thesis has demonstrated that increasing the concentration of gelatin results in increased fiber diameter.\textsuperscript{24,30,38} By using a 21\% gelatin polymer solution, electrospun scaffolds exhibited ribbon-like fibers within the range of collagen fibril sizes found in human tissues.\textsuperscript{10} Electrospun scaffolds exhibit densely packed nanofibers and small sized pores, which is not ideal. Scaffolds with pores 100 \(\mu\)m in diameter and overall scaffold porosity of 90\% has been shown to support the infiltration of cells from the surface of the scaffold.\textsuperscript{39} Porosity can be improved using salt or sacrificial secondary polymers, which are deposited during the electrospinning process and subsequently removed.\textsuperscript{40,41}

Our lab has shown that macrophage polarization was not affected by treatment with an electrospun scaffold containing 6.7 \(\mu\)g/mL galectin-3 in murine full-thickness wound healing.\textsuperscript{34} Thus, we sought to elucidate the conditions where galectin-3 can affect macrophage polarization \textit{in vivo}. In this thesis, recombinant human galectin-3 was added to the electrospinning gelatin solution to a final concentration of 50 \(\mu\)g/mL, while daily topical delivery was also investigated using 12.5 \(\mu\)g/mL and 25 \(\mu\)g/mL galectin-3 solutions in PBS. These concentrations are much higher than those needed to achieve effects \textit{in vitro}, where concentrations as low as 1 \(\mu\)g/mL have been used to enhance keratinocyte migration\textsuperscript{42} and 0.001-0.01 \(\mu\)M (0.026152-0.26152 \(\mu\)g/mL) to create a concentration-dependent effect on monocyte recruitment.\textsuperscript{43} Additionally, while 6.3 \(\mu\)g/mL galectin-3 promoted human keratinocyte migration, use of 50 \(\mu\)g/mL limited migration \textit{in vitro}.\textsuperscript{44} However, galectin-3 activity is understood to be context-specific, and \textit{in vivo} activity is not always accurately reflected \textit{in vitro}. The use of galectin-3/gelatin scaffolds and topical deliver of galectin-3 did not alter wound closure kinetics during the 9-day period. In contrast to our findings, the use of gelatin scaffolds alone has been reported to increase wound closure in full-thickness skin healing in rats.\textsuperscript{21} However, several factors could have contributed to these discrepancies including the use of rats instead of mice, the difference in size of the initial wounds, and the use of Tegaderm\textsuperscript{TM} (3M) secondary bandaging to cover the wounds.\textsuperscript{21}

During the proliferative/epithelialization stages of wound healing, keratinocyte proliferation and migration over the dermis restores the epithelial barrier.\textsuperscript{1} Studies of dermal healing have demonstrated that galectin-3 deficient mice exhibit impaired re-epithelialization, which manifests in decreased length of the epithelial tongue, and therefore decreased re-epithelialization at days 2\textsuperscript{42} and 7\textsuperscript{45} post-wounding. However, when recombinant human galectin-3 was added to wounds of wildtype mice topically or using a gelatin scaffold, no
differences in epithelial thickness were observed at days 7 and 9 post-wounding. This result was consistent with previous reports showing no defect in epithelial thickness in galectin-3 knockout mice.\textsuperscript{42,45} In contrast, studies of corneal healing have shown that exogenous human recombinant galectin-3 can increase re-epithelialization in mice\textsuperscript{13} and in monkey corneal explants,\textsuperscript{14} highlighting the issue of the context-dependent roles of matricellular proteins.\textsuperscript{46}

During the inflammatory phase, monocytes are recruited to the wound by chemoattractants and differentiate into macrophages.\textsuperscript{47} Macrophages mediate wound healing through the release of regulatory molecules.\textsuperscript{48} Classically activated (M1) macrophages produce nitric oxide and secrete proinflammatory cytokines including TNF-\(\alpha\), IL-1, IL-6, and IL-12, while alternatively activated macrophages (M2) are implicated in tissue remodeling and secrete TGF-\(\beta\).\textsuperscript{49} Control of macrophage activation and whether macrophages can switch between M1 and M2 phenotypes is incompletely understood. Galectin-3 has previously been implicated in macrophage function.\textsuperscript{8,9,43} Thus, we sought to investigate whether galectin-3 treatment using topical galectin-3 and galectin-3/gelatin scaffolds could increase the number of M2 polarized macrophages. While day 9 wound sections qualitatively appeared to contain denser M2 macrophage populations, quantification showed no differences in M2 macrophage density across the four treatment groups. This result was unexpected given that bone marrow derived macrophages (BMDMs) from galectin-3 knockout mice show a defect in IL-4 and IL-13 M2 macrophage polarization \textit{in vivo} and \textit{in vitro}.\textsuperscript{9} However, this study did not investigate the effects of exogenous galectin-3 addition, therefore there is no indication galectin-3 would be sufficient in rescuing the deficient M2 polarization of BMDMs in galectin-3 null mice. It is possible that exogenous galectin-3 alone is not sufficient in upregulating the expression of surface-bound galectin-3, the secretion of galectin-3 or upregulating CD98 which are each implicated in the suggested autocrine loop that controls M2 activation.\textsuperscript{9} Moreover, human and murine galectin-3 are only 80\% similar homologously,\textsuperscript{50} which might contribute to the lack of functionality of exogenous human galectin-3 in murine wound healing.

In conclusion, blend electrospun galectin-3/gelatin scaffolds have been developed which to not delay wound healing in a full-thickness murine model. Use of topical galectin-3 and galectin-3/gelatin scaffolds did not affect wound closure, epithelial thickness, or re-epithelialization, or influence the density of M2 macrophages in the wound. Future work should explore the contexts in which \textit{in vivo} inflammation can be modulated.
3.5 References

4 General Discussion

4.1 Summary of Thesis Objectives

**Aim 1: Three-Dimensional Bioprinting for Tissue Engineering**

**Establish a protocol for soft collagen printing**

In this thesis, collagen bioink scaffolds were printed by positive-displacement extrusion and embedded into a gelatin microparticle support bath. Optimization of the gelatin slurry preparation protocol was performed. Gelatin microparticle size was found to be dependent on the preparation method. Thus, longer durations of mechanical blending resulted in smaller gelatin microparticles that displaced during embedded printing with higher resolution. Rheological testing of the gelatin slurry demonstrated a shear-sensitive support slurry that behaved similarly to a Bingham plastic fluid. Computer-aided design was employed to generate 3D scaffold rectangular prisms (10 x 10 x 0.5 mm) which were sliced using rectilinear and hexagonal infill patterns at 20% density. Scaffolds were printed using a high concentration bovine type I collagen bioink and exhibited high print fidelity and reproducibility.

**Polycaprolactone scaffold printing**

Computer-aided design was employed to generate a 3D scaffold rectangular prism (50 x 50 x 2 mm) which was sliced using a rectilinear infill pattern at 50% density. Polycaprolactone (poly(ε-caprolactone); PCL) constructs were printed using low temperature polymer fusion printing. From the printed construct, 6 mm diameter circular samples were cut and functionalized by argon plasma treatment.

**In vitro proof-of-concept ASC biocompatibility and differentiation**

Three-dimensional printed scaffolds were assessed *in vitro* using human adipose-derived stromal cells (ASCs). ASCs seeded onto PCL scaffolds showed the greatest proliferative potential (96% of cells), while ASCs on either rectilinear or hexagonal collagen scaffolds exhibited less EdU incorporation (~10% of cells). Treatment of ASCs with TGF-β1 stimulated cell spreading, and fibronectin production after 3 days post-seeding. In the absence of TGF-β1, scaffolds supported bioactivity and after 14 days on scaffolds, ASCs exhibited increased cell density, spreading, and fibronectin deposition. Together, these results demonstrated that ASC
activity can be stimulated using TGF-β1 for rapid (3 days) tissue regeneration while scaffolds alone are capable of maintaining viable ASCs for prolonged growth periods (2 weeks). Lastly, ASC phenotype was assessed after treatment with osteogenic and adipogenic induction factors for 3 weeks. ASCs demonstrated a PPARγ-positive phenotype.

**Aim 2: Effects of Exogenous Galectin-3 in a Murine Wound Healing Model**

**Electrospin galectin-3-loaded gelatin scaffolds**

The effect of gelatin concentration on electrospun fiber morphology was assessed by spinning gelatin polymer solutions at three concentrations, 20%, 25%, and 30%. The majority of fibers spun from 30% weight gelatin ranged in diameter from 500-2000 nm in diameter, with significant variation in fiber size. At 25% weight, the majority of fibers were within the range of 300-500 nm, while lowering the gelatin to 20% resulted in fibers 100-200 nm in diameter. Fiber diameter was refined using 21% weight gelatin in the electrospinning polymer solution, resulting in electrospun fibers with a mean diameter of 181 ± 40 nm.

**Evaluate effects of exogenous galectin-3 in vivo**

To investigate the effects of exogenous recombinant galectin-3 on murine wound healing, full-thickness excisional dermal wounds were created on the backs of wild type mice. Wounds were treated with either 21% gelatin electrospun scaffold containing 50 μg/mL recombinant galectin-3, daily topical application of galectin-3 at either 12.5 or 25 μg/mL, or no treatment (control). All dermal wounds healed with reduction in size by day 9 post-wounding. The wound area was measured on days 0, 3, 5, 7 and 9 to calculate wound closure kinetics, but no differences were observed between treatments. Epithelial tongue length and thickness was quantified at days 7 and 9 post-wounding; however, no differences were evident between any conditions. Macrophage polarization within the wound was quantified and the mean density of arginase I-positive macrophages in the wounds was found not to be statistically significant between treatment conditions. Thus, treatment of murine full-thickness dermal wounds, either using a galectin-3/gelatin electrospun scaffold or by daily topical galectin-3, did not delay wound healing or re-epithelialization, but did not influence arginase I enrichment within the wounds.
4.2 Summary of Fabricated Biomaterial Scaffolds

Tissue engineering (TE) has emerged as a promising strategy for the replacement of degenerating or damaged tissues in vivo. Also known as regenerative medicine (RM), integral to this therapeutic strategy is biomimetic scaffolds and the biomaterial structural components used to form them. The scaffold needs to have mechanical properties strong enough to withstand the biological forces of the native environment, for example, compression, tension, and shear/torsion forces.¹ Biomaterials should exhibit elasticity such that the scaffold returns to its normal shape after deformation. Moreover, the architectural design should facilitate mass transfer within the scaffold for the exchange of gases, nutrients, and waste, and prevention of necrotic zones.² Further, biomaterials chosen must exhibit biocompatibility, and depending on the biomaterial device itself, biodegradability and bioabsorbability.³ That is, the biomaterial and its degradation products must be non-toxic and non-immunogenic. Clearly, design and fabrication of a biomaterial scaffold is complex, and various fabrication methods have been investigated to fulfill TE requirements.

Controlling scaffold architecture allows the fabrication of scaffolds that are highly porous and provide physical topographic cues to the cell surface. Current approaches to the assembly of three-dimensional (3D) biomaterials employ additive manufacturing (3D printing) to deposit materials layer-by-layer for controlled structure and architecture. Polymer fusion printing is of interest in TE for the fabrication of scaffolds using synthetic materials. For instance, polycaprolactone (poly(ε-caprolactone), PCL), a polyester, has been employed for TE applications due to its biocompatibility.⁴⁻⁶ PCL has a slow degradation rate of approximately two years in the biological environment.⁷⁻⁹ However, by using copolymers such as PCL with dl lactide, a more flexible material with a faster degradation rate than the homopolymer can be achieved.⁷ Moreover, the high degree of permeability has made PCL an important candidate for the development of drug delivery systems and in bone tissue regeneration.⁴,¹⁰⁻¹³ In contrast to synthetic biomaterials, scaffolds synthesized from natural biological materials are highly compatible with the host environment, being of biological origin. Hydrogel printing, including syringe-based extrusion,¹⁴,¹⁵ printing with fibrin,¹⁶,¹⁷ gelatin,¹⁸ type I collagen,¹⁹ and protein mixtures obtained from decellularized tissues,²⁰ has been established as a promising TE strategy. However, due to their viscosity, printing of soft materials poses the challenge of printed materials flowing out of their desired architecture. To overcome this, hydrogels and proteins
must gel in situ in order to prevent their collapse or shape deformation.\textsuperscript{21–23} Embedded printing into a secondary hydrogel that acts as a temporary support bath has been proposed.\textsuperscript{24,25}

In this thesis, fusion printed PCL scaffolds and embedded printed collagen scaffolds were employed to assess in vitro biocompatibility using human adipose-derived stem/stromal cells (ASCs). ASCs are multipotent, with adipogenic, chondrogenic, neurogenic and osteogenic differentiation described.\textsuperscript{26–29} Collagen, being the main structural component of the extracellular matrix,\textsuperscript{30} has a biological molecular composition and is biodegradable.\textsuperscript{31} Independent of the 3D architectural pattern (rectilinear or hexagonal), collagen scaffolds stimulated ASC fibronectin production in vitro while PCL scaffolds supported robust ASC spreading and proliferation. These differences in cell responses indicated that polymer fusion printing and hydrogel embedded printing methods should be employed depending on the TE application. Collagen’s ability to be remodeled and degraded has warranted further investigation into its employment as a soft connective tissue substitute.\textsuperscript{32,33} The scaffold should serve as a temporary cell support that is slowly replaced by newly synthesized matrix components in order to mediate regeneration of the tissue. In contrast, PCL scaffolds supported ASC proliferation and their rigid structure is well suited for applications where stronger scaffold constructs are required, such as in bone regeneration and also wound healing at the sole of the foot, where repetitive mechanical loading would damage soft scaffolds. Future research is needed to validate the RM potential of printed collagen and PCL scaffolds in vivo.

TE constructs that are not pre-seeded with cells rely on the migration of native cells to infiltrate and populate the scaffold. In this study, ASC phenotype was positive for PPAR\textgamma expression. Therefore, future research is needed to determine under which conditions ASCs isolated from pathological tissues can be derived towards adipogenic and osteogenic lineages. Experiments should be performed with a greater number of patient samples to improve sample size and also with non-pathologically sourced ASCs. ASCs were responsive to biomolecule stimulation using TGF-\textbeta1, thus elucidating mechanisms of slow release of this molecule might enhance the outcomes of 3D printed collagen and PCL scaffolds.

While 3D printed collagen and PCL exhibited controlled material deposition and thus scaffold architecture, these constructs were limited in their ability to mimic nanofiber morphology. The extracellular matrix (ECM), comprised of collagen fibers and associated glycoproteins and polysaccharides, fills the interstitial space and supports cell adhesion.
Collagen fibers, the main structural component of the ECM, are typically 100-200 nm in diameter. Cell anchorage is achieved through focal adhesion complexes where transmembrane receptors, such as integrins, link the cytoskeleton to the ECM. Three-dimensional printed struts exhibit much greater diameters, in the range of 0.4-1 mm, depending on the print nozzle. Higher print resolution could improve scaffold morphology, but employment of a smaller-diameter nozzle would create new challenges, mainly nozzle blockage. Thus, other methods of scaffold production are required to produce scaffolds with nanofibrous strut dimensions.

Polymer electrospinning, in contrast to 3D bioprinting, allows for the fabrication of nanofibrous scaffolds. Electrospun scaffolds display high porosity and large surface area-to-volume ratios, facilitating effective delivery and distribution of incorporated bioactive factors. However, in electrospinning, pore size is not controlled, resulting in densely-packed nanofibers. In this thesis, electrospun gelatin scaffolds did not delay healing in vivo, but did not enhance wound closure or re-epithelialization states. Future research should attempt to improve electrospun scaffold porosity. One possible strategy is the incorporation of salt agents or sacrificial secondary polymers within the electrospinning solution, which can be subsequently leached out of the scaffold, resulting in greater void regions.

Together, this thesis has demonstrated that each scaffold fabrication method has its advantages and limitations. Bioprinting allows for controlled scaffold architecture and porosity, but printing resolution is low, on the scale of approximately 400-1000 µm. Higher scaffold resolution can be achieved using electrospun fibers, which exhibit diameters in the range of 100-200 nm. However, electrospun scaffolds are randomly organized and densely packed, limiting the potential for cell infiltration. Design of a TE scaffold must take into consideration the desired physical properties in order to choose a suitable material and fabrication method.

4.3 Galectin-3 to Modulate Inflammation In Vivo

Chronic wounds are halted at the inflammatory stage of healing, presenting decreased levels of growth factors such as TGF-β and VEGF and increased levels of expression of inflammatory cytokines, such as IL-1, IL-6, and TNF-α, which increase local inflammation further. Several underlying factors, such as impaired re-epithelialization, angiogenesis, and granulation tissue formation, imbalances in proteolytic activity, persistent bacterial colonization and the formation of biofilms, and the accumulation of oxidative stress at the wound site, disrupt
the normal wound healing process. Regardless of wound management and intervention, including wound debridement, treatment of infection, application of wound dressings to control exudate and moisture, mechanical off-loading to reduce pressure ulcer recurrence, and tissue revascularization, chronic wounds remain a significant burden on patients and the healthcare system. Current available therapies offer low clinical efficacy in the resolution of continuous inflammation, keeping the wound in a pro-inflammatory non-healing state. Thus, a therapeutic strategy capable of artificially shifting the wound microenvironment from pro-inflammatory to pro-fibrotic is of interest.

In normal wound healing, macrophages are implicated in the regulation of fibrosis, and release signaling molecules such as TGF-β, PDGF-β and galectin 3 to increase myofibroblast matrix synthesis and release of tissue inhibitors of metalloproteinases (TIMPs). Macrophages are a heterogenous population of myeloid cells that exhibit distinct physiological roles. Macrophages respond to pathogens and modulate the adaptive immune response, including the induction and resolution of inflammation, tissue repair, and homeostasis. Macrophages exhibit plasticity in their polarization towards classical M1 (pro-inflammatory) or alternative M2 (anti-inflammatory) phenotypes, depending on the types of stimuli present. For instance, M1 macrophages are induced by stimulation with interferon gamma (IFNγ) and lipopolysaccharides (LPS). M2 macrophages can be further classified into four speculated subdivisions based on the stimuli and the achieved transcriptional changes. Alternative activated macrophages (M2a) are activated by Interleukin (IL)-4 or IL-13, type 2 macrophages (M2b) are believed to be activated by immune complexes and LPS, deactivated macrophages (M2c) may be activated by glucocorticoids or IL-10, while M2-like macrophages (M2d) may be activated by adenosines or IL-6. Macrophage phenotype, whether classical (M1) pro-inflammatory or alternative (M2) anti-inflammatory, determines physiological roles and therefore also the expressed proteome of the macrophage.

Galectin-3 gene and protein expression is higher in M2 polarized macrophages. The protein functions during the regulation of M2 macrophage polarization and as a chemoattractant for monocytes and macrophages, implicating galectin-3 as a potential therapeutic target for inflammatory regulation during skin healing. This thesis has shown that delivery of galectin-3, via an electrospun scaffold or topical application, does not enhance M2 macrophage density at the wound. Biological activity of galectin-3 described in the literature is
highly context-specific, leading to discrepancies in its roles during inflammation. For example, a study by Mackinnon et al. found that expression and secretion of galectin-3 was suppressed in bone marrow-derived macrophages exposed to 100 ng/mL LPS. In contrast, Novak et al. reported a significant increase in galectin-3 expression from human blood-monoocyte derived macrophages exposed to 100 ng/mL LPS and 20 ng/mL IFNγ. Moreover, in vitro effects of galectin-3 do not always translate in vivo since important factors including cell maturation, matrix composition and chemoattractants, are often absent from in vitro studies.

In this thesis, exogenous galectin-3 did not significantly influence murine wound healing, re-epithelialization or inflammation. Treatment of full-thickness excisional wounds with recombinant human galectin-3, delivered by an electrospun gelatin scaffold or topically applied, did not delay cutaneous wound healing in vivo after 9 days. Galectin-3 treatment did not affect epithelial tongue length or thickness at days 7 or 9 post-wounding. Arginase I-positive cell density was also unaffected by exogenous galectin-3 treatment. As such, galectin-3 in a recombinant, extracellular presence may not be an effective therapeutic for the clinical treatment of chronic skin wounds. Future experiments may assess the efficacy of other bioactive constituents, such as TGF-β and IL-4, in shifting the chronic wound microenvironment from pro-inflammatory to pro-fibrotic.

4.4 Future Directions

4.4.1 Improving 3D Printed Scaffolds

Collagen protein scaffolds were printed from a high concentration type I collagen bioink. The Young’s modulus of the bioink, reported by Advanced Biomatrix, was approximately 1050 Pa after 30 min in situ gelation. In future studies, the softness of this gel could be tuned by mixing the collagen bioink with a second component to increase scaffold stiffness. For instance, by adding methacrylated type I bovine collagen bioink, gels could be prepared at various concentrations and UV crosslinked after printing to provide tunable gel stiffness. Increasing in situ gelation may affect ASC bioactivity on the scaffolds and improve cell adhesion, spreading and proliferation.

Osteo-inductivity of synthetic PCL scaffolds should be investigated further. Future experiments employing fusion printed PCL scaffolds should investigate improving scaffold biocompatibility via mineral functionalization. For instance, PCL scaffolds containing mineral
additives, such as Bio-Oss (BO) or decellularized bone matrix (DCB), have demonstrated enhanced calcium continent, collagen I deposition, osteocalcin expression of ASCs after 3 weeks. Improving the osteo-inductive properties of PCL scaffolds might improve their efficacy in tissue engineering applications. A second future direction to improve printed PCL scaffold efficacy is by employing a PCL composite. For instance, future work should study photocrosslinkable polycaprolactone dimethylacrylate (PCLDMA) and poly(ethylene glycol) diacrylate (PEGDA), which can be mixed together to prepare an ink for ink-jet 3D printing. This PCLDMA/PEGDA ink has been shown to improve material properties and the quality of the resultant scaffolds. However, a nitrogen atmosphere is needed during printing, thus our current bioprinter set-up would require modification.

4.4.2 Improving Electrospun Scaffolds

Research in our lab has previously demonstrated that electrospun galectin-3/gelatin scaffolds were biocompatible in vitro; electrospun scaffolds supported the adhesion, proliferation, and fibronectin secretion of human dermal fibroblasts. Electrospun galectin-3/gelatin scaffolds generated in this thesis exhibited fibers within the desired 100-200 nm diameter range, but were densely packed. Within a TE scaffold, large pore sizes are necessary to ensure the penetration of cells. One technique to improve scaffold pore size is to co-electrospin polymer fibers with sacrificial fibers that can subsequently be removed. For instance, the co-electrospinning of gelatin scaffolds with sacrificial polyethylene glycol fibers has been shown to generate pores ranging 10-100 μm in diameter. Likewise, poly-ethylene oxide (PEO) is another sacrificial material candidate as it is highly water soluble, facilitating easy removal following gelatin crosslinking. This method has been employed for the blend electrospinning of a collagen/PCL scaffold with PEO as the sacrificial fiber component. Soaking the scaffold in water, dissolved PEO fibers, resulting in a scaffold with improved void pores for cell infiltration. This scaffold design demonstrated complete infiltration of scaffolds after 4 weeks in a model of neoangiogenesis in rats. Moreover, micropores can be generated by simply piercing the electrospun scaffold with a micro-diameter needle. For instance, using an acupuncture needle, 160 μm pores were created in a 70:30 collagen/PCL electrospun scaffold. When implanted into full-thickness skin wounds on the backs of Sprague-Dawley rats, the microporous scaffolds promoted faster skin regeneration in vivo than unprocessed
electrospun scaffolds. Pre-seeding of these microporous scaffolds with fibroblasts further promoted tissue regeneration following wounding. In comparison to other methods for increasing scaffold pore size, the generation of a stamp/press device with a defined needle diameter and scaffold piercing capabilities offers a technically straightforward and cost-effective approach for introducing organized and reproducible micropores into electrospun scaffolds. Improving porosity in the electrospun scaffolds could enhance cell infiltration in vivo during murine wound healing, facilitating fibrosis, vascularization, and re-epithelialization.

4.4.3 ASC Differentiation

ASC multipotency has been widely explored, leading to the generation of various classifications of the cells including adipose-derived stem cells (ADSCs), adipose-derived adult stem cells, adipose-derived mesenchymal stem cells (AD-MSCs), adipose MSCs (AMSCs), and adipose stromal/stem cells (ASCs). To avoid further confusion, the International Society for Cellular Therapy proposed classifying all plastic-adherent cells derived from mesenchymal tissues and showing multipotency as mesenchymal stromal cells, regardless of tissue source. The term stem cell should be reserved for cells showing definitive stem cell characteristics, including adherence to tissue culture plastic, multipotency, and long-term self-renewal capacity. Future experiments should study the culturing conditions which will enhance the differentiation potential of ASCs on 3D printed scaffolds. This potentially might be achieved through the co-culturing of ASCs with differentiated cells, for example fibroblasts, chondrocytes, endothelial cells, or Schwann cells.

4.4.4 Establishing In Vitro and In Vivo Effects of Galectin-3

This thesis has demonstrated that treatment of murine wounds with recombinant human galectin-3, delivered by an electrospun scaffold or topically applied, did not statistically influence wound healing kinetics, re-epithelialization, or M2 macrophage number. The lyophilized protein used in this study has been tested for in vitro bioactivity using rabbit erythrocytes to evaluate galectin-3-mediated agglutination. Galectin-3 bioactivity has also been confirmed by studying cell viability following treatment of acute T-cell leukemia cell lines with galectin-3, a treatment known to induce apoptosis in T-cells.
The findings of this thesis suggest galectin-3 in an exogenous form does not influence acute wound healing processes. Research from our laboratory has shown that galectin-3 knockout mice display closure kinetics of full thickness excisional wounds comparable to that of wildtype groups. Moreover, the bioactivity of galectin-3 is possibly influenced by post-translational modifications, including cleavage and phosphorylation of the protein. Cleavage of galectin-3 by MMPs, prostate-specific antigen (PSA), and parasite-originating proteases has been shown to produce intact carbohydrate-recognition domain and N-terminal peptides of variable sizes that retained lectin binding activity but lost multivalence. Moreover, phosphorylation of serine and tyrosine by Abelson tyrosine kinase (c-Abl), cyclin-dependent kinase inhibitor (CKI), and glycogen synthase kinase-3 beta (GSK-3β) could regulate galectin-3 localization and associated signal transduction. Thus, galectin-3 contains several domains through which protein multivalence, localization, and ligand interactions can be modified; absence of these modifications may render recombinant human galectin-3 inactive in the extracellular environment.

As previously discussed, interleukin 4 (IL-4) and IL-13 treatment of bone marrow derived macrophages from galectin-3 knockout mice resulted in reduced arginase I activity. In vitro analysis of exogenous galectin-3 is required to better understand the influence of the protein on macrophage polarization in a controlled experimental environment. THP-1, an immortalized monocyte-like cell line, could be employed to identify the effects of exogenous recombinant human galectin-3 on upregulating M2 macrophage markers, including TGF-β and the mannose receptor in human monocytes. Such experiments might indicate whether galectin-3 can direct monocyte differentiation towards an M2 phenotype, and under which treatment concentrations galectin-3 bioactivity is optimized. Once the bioactivity of galectin-3 is better understood in vitro, investigation of its role in vivo via topical delivery could once again be pursued. If galectin-3 does influence M2 macrophage density in vivo, future work should also investigate the role of galectin-3 at various time points post-wounding, given that galectin-3 expression is highest day 1 following wounding in wildtype mice.
4.5 Limitations of Results

4.5.1 Human Adipose Derived Stem/Stromal Cells

In this thesis, human adipose derived stem/stromal cells (ASCs) were cultured onto 3D printed scaffolds to study material biocompatibility and the influence of a scaffold on ASC lineage commitment. Initially known as processed lipoaspirate cells or PLA cells, ASCs can be isolated from human lipoaspirates and, similar to mesenchymal stem cells (MSCs), can be differentiated in vitro using lineage-specific factors toward osteogenic, adipogenic, myogenic, and chondrogenic phenotypes. Adipose tissue is easily isolated by tissue resection, ultrasonic-assisted lipoplasty, syringe extraction, or suction-assisted lipoplasty (commonly known as liposuction). From adipose tissue, the stromal vascular fraction (SVF), composed of red blood cells, fibroblasts, endothelial cells, smooth muscle cells, pericytes and pre-adipocytes, can be isolated using enzymatic digestion. Further processing and culturing of the SVF eliminates contaminating cell populations and results in an adherent, multi-potent pre-adipocyte population. Due to their availability and multi-potency, ASCs have been employed in a variety of regenerative models. In vitro bone tissue regeneration has been demonstrated using porous poly(lactic-co-glycolic)acid-β-tricalcium phosphate (PLGA-β-TCP) scaffolds, polylactic acid (PLA)/β-tricalcium phosphate (β-TCP) composite scaffolds, and polycaprolactone-tricalcium phosphate (mPCL-TCP) scaffolds with matrix components, either fibrin glue or lyophilized collagen. Similarly, in vitro cartilage tissue regeneration, adipose differentiation, and epidermal differentiation have also been validated.

Despite previous findings, differentiation of multiple lineages was not observed in this thesis. ASCs in this study were derived from human adipose tissue originating from the lower limb of a 67-year-old woman, and lineage appeared already committed towards an adipogenic phenotype. This finding was independent of ASC treatment with lineage-specific factors for osteogenic commitment, such as glycerol 2-phosphate (commonly known as β-glycerophosphate; BGP) and ascorbic acid 2-phosphate (Asc 2-P). This result was unexpected given that BGP and Asc 2-P are well established osteogenic lineage inducing factors. Reports have shown that MSC isolated from bone marrow exhibit rapid growth and osteogenic differentiation following treatment with 10 mM BGP. Likewise Asc 2-P treatment significantly improved nascent cell growth at both high and low concentrations (0.25-1.0 mM), and also increased expression of osteoblast differentiation markers, including collagen synthesis and alkaline phosphatase.
activity. Osteogenic differentiation has been noted to take 4-6 weeks in previous studies, therefore the 3 week experimental time point of this thesis may have been insufficient. Moreover, this thesis employed ASCs of pathological origin, whereas other reports employed healthy human or animal ASCs. Thus, future work is needed to elucidate the differentiation potential of ASCs originating from pathological, amputated limbs in order to justify their use in regenerative medicine.

4.5.2 Murine Model of Wound Healing

Human chronic skin wounds represent a complex pathological environment with observed heterogeneity among populations. Chronic wounds can arise from a number of factors including malnutrition, advanced age, infection, diabetes, vascular insufficiency, prolonged pressure and edema contribute to amplified inflammation and a non-healing wound. Thus, a major limitation to this thesis is the employment of an animal model that cannot fully imitate the complexity of human chronic wounds. This study utilizes a wild type mouse (Mus musculus) model of full-thickness dermal wound healing. While murine models are simple to maintain and economically accessible, important differences between murine and human skin healing exist. Mice have small bodies which limits modeling of wound sizes relevant for humans, and also have a short life expectancy which limits studies of “chronic” wounds in mice.

With regards to wound healing, murine wounds close mainly by contraction via the subcutaneous panniculus carnosus, a layer of striated muscle. This contrasts with human wounds closure which is achieved through cell migration over granulation tissue and re-epithelialization. In the dermis and epidermis, human and murine skin have unvarying layers of cells, however these layers greatly diverge in physiology. While human skin is over 100 μm thick, firm, and adherent to basal tissues, murine skin is less than 25 μm thick and is unattached and loose. The epidermis is comprised of 5-10 cell layers in humans but only 2-3 in mice. Humans and mice also differ in the role of specific niches of skin stem cells. For instance, murine skin is densely populated by hair follicles while the majority of human epidermis is classified as interfollicular, exhibiting sparse and uneven hair follicle distribution. In a study of split-thickness skin grafting, scalp donor sites rich in hair follicles healed faster than thigh sites which had fewer hair follicles. This difference is due to the important roles hair follicle stem cells play in cutaneous repair, where up to a quarter of newly formed
epidermis is derived from hair follicle stem or progenitor cells. Overall, caution must be exercised when translating murine findings to pre-clinical studies.

Moreover, in this thesis, the validity of the mouse studies conducted was limited by the low number of mice used for each condition and at each time point. Three mice were sacrificed at day 3 post-wounding, three mice at day 7, and two mice at day 9. The inclusion of a larger sample size of mice may have eliminated the significant variability observed in the study, thereby improving the power of the study. All mice were age and sex matched, in addition to being wildtype. Thus, this model of healing was unchallenged and does not accurately reflect the impeded healing associated with human chronic wounds. Additionally, observation of wound area for closure kinetics was affected by the overlaying eschar which concealed healing wounds. In calculations of the wound area, the eschar area was also included, thus measured wound areas could have appeared larger. In order to directly observe the underlying healing tissues, it would have been necessary to remove the eschar. However, this manipulation could potentially disrupt the underlying tissues and thus was not executed.

4.6 Conclusion

This thesis has demonstrated fabrication of three different biomaterial scaffolds for tissue engineering applications, three-dimensional reverse embedding of collagen scaffolds, polymer fusion printing of PCL scaffolds, and electrospinning of gelatin scaffolds. Bioprinting of soft collagen scaffolds required embedding the print within a secondary gelatin support slurry to facilitate in situ gelation. Preparation and validation of the gelatin support slurry demonstrated that gelatin microparticle morphology was dependent on mechanical blend treatment, and that the prepared slurry behaved as a Bingham plastic fluid. Collagen scaffolds of rectilinear or hexagonal infill patterning stimulated ASC fibronectin deposition. Rectilinear PCL scaffolds promoted ASC adhesion, spreading, and proliferation responses. While collagen and PCL scaffolds exhibit in vitro biocompatibility, ASC phenotype was uninfluenced by 3D versus 2D culturing conditions, collagen or PCL scaffold material, or rectilinear versus hexagonal geometric cues. These scaffolds should be investigated further regarding in vivo efficacy for RM applications. Gelatin polymer blended with recombinant galectin-3 was electrospun into a protein delivery scaffold and employed in a murine model of cutaneous wound healing. Treatment of wounds with the galectin-3/gelatin scaffolds, or with topical galectin-3, did not
enhance wound closure, re-epithelialization, or influence macrophage phenotypes in the wound. Future directions include elucidating the conditions where galectin-3 might modulate inflammation in vivo and considering other target molecules such as TGF-β and IL-4.
4.7 References


5 Appendices

Appendix A: Supplementary Rheological Results

Pre-Shear: Structural Regeneration

[Graph showing viscosity (Pa·s) over time (s) for different times t=60, t=90, t=120]

Frequency Sweep: Damping Factor

[Graph showing damping factor over angular frequency (rad/s) for different times t=60, t=90, t=120]
Collagen Hexagonal Scaffolds

- external perimeters extrusion width = 0.16mm
- perimeters extrusion width = 0.22mm
- solid extrusion thickness = 0.22mm
- top infill extrusion thickness = 0.22mm

G1 X12.44 Y12.277 E0.01268
G1 X12.529 Y11.476 E0.01094
G1 X-12.529 Y1.468 E0.13626
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F90.000
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PCL Rectilinear Scaffolds

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## Appendix C: Summary of Early Embedded Printing Attempts

<table>
<thead>
<tr>
<th>Variable</th>
<th>Settings Attempted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Needle</td>
<td>30G, 18G</td>
</tr>
<tr>
<td>Print Speed</td>
<td>5% - 100%</td>
</tr>
<tr>
<td>First Layer Print Speed</td>
<td>10%, 20%, 50%</td>
</tr>
<tr>
<td>Extrusion Multiplier</td>
<td>0.45, 0.5, 0.6, 0.9</td>
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<tr>
<td>Bioink Dilution</td>
<td>None, 5:2, 2:1</td>
</tr>
<tr>
<td>Infill Density</td>
<td>20%, 40%, 70%, 100%</td>
</tr>
<tr>
<td>Infill Pattern</td>
<td>Line, Rectilinear, Hexagonal</td>
</tr>
<tr>
<td>Infill/Perimeter Overlap</td>
<td>0%, 10%</td>
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<tr>
<td>Support</td>
<td>Gelatin blended microparticle slurry, Dry culture plastic</td>
</tr>
<tr>
<td>Gelatin Slurry Preparation</td>
<td>Blend: 60 s, 90 s, 120 s</td>
</tr>
<tr>
<td></td>
<td>Centrifugation: 225G for 5 min, 300G for 5 min, 3800G for 4 min, 4000G for 5 min</td>
</tr>
<tr>
<td></td>
<td>Temperature: room temperature preparation, kept on ice and centrifuged at 4°C</td>
</tr>
</tbody>
</table>
Rectilinear (L) and hexagonal (R) 40% infill density was too dense

Rectilinear (L) and hexagonal (R) 20% infill resulted in reproducible porosity

Unoptimized printing parameters resulted in bioink ejection issues

Compromised print resolution was observed when a gelatin slurry blended for 60 s was employed

Higher print resolution was achieved when gelatin slurry was blended for 120 s

Rectilinear (L) and hexagonal (R) 40% infill density was too dense

Rectilinear (L) and hexagonal (R) 20% infill resulted in reproducible porosity
Appendix D: Autofluorescence of PCL Scaffolds
Appendix E: Example of the Appearance of Individual Z Slices for Scaffold Confocal Microscopy
Appendix F: Arginase-I Immunofluorescent Staining of Day 9 Murine Wound Sections No Primary Antibody Negative Control
Appendix G: Research Ethics Approvals

<table>
<thead>
<tr>
<th>PI</th>
<th>Hamilton, Doug</th>
</tr>
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<tbody>
<tr>
<td>Protocol #</td>
<td>2016-085</td>
</tr>
<tr>
<td>Status</td>
<td>Approved (w/o Stipulation)</td>
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<tr>
<td>Approved</td>
<td>01/09/2017</td>
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<td>01/01/2021</td>
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<tr>
<td>Title</td>
<td>Influence of biomaterials and material physiochemical properties on cell behaviour in vitro and in vivo.</td>
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</tbody>
</table>

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  - Species Strains
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  - Environmental Enrichment
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  - Animal Transfers
  - Environmental Enrichment
  - Animal Holding/Housing and Use Location Information
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  - Acclimatization Period & Quarantine
  - Experimental Agents Information
Date: 27 June 2019

To: Dr. Douglas Hamilton

Project ID: 6311

Study Title: Role of peristin in the repair of skin - 16324E

Application Type: Continuing Ethics Review (CER) Form

Review Type: Delegated

REB Meeting Date: 02 Jul 2019

Date Approval Issued: 27 Jun 2019

REB Approval Expiry Date: 24 Jun 2020

Lapse in Approval: June 25, 2019 to June 27, 2019

Dear Dr. Douglas Hamilton,

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 (WHIPA 2004) and its applicable regulations. The REB is registered with the U.S. Department of Health & Human Services under the REB registration number IRB 00000846.

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

Sincerely,

Daniel Wysoczynski, Research Ethics Coordinator, on behalf of Dr. Joseph Gilbert, HSREB Chair

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

Name
Madeleine Di Gregorio

Post-Secondary
Education and Degrees
University of Toronto
Toronto, Ontario, Canada
2013-2017 BSc

The University of Western Ontario
London, Ontario, Canada
2017-2019 MESc

Related Work
Teaching Assistant
Experience
The University of Western Ontario
2018

Publications