Adipose-derived stem cells improve tissue quality in a murine model of delayed wound healing

by

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

**Adipose-derived stem cells improve tissue quality in a murine model of delayed wound healing**

submitted by Diana Forbes in partial fulfillment of the requirements for the degree of Master of Science in Experimental Medicine

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Abstract

Wound repair and regeneration is a multidisciplinary field of research with considerable potential value to the management of deep and large cutaneous injuries such as ulcers caused by pressure, venous and arterial insufficiency, and diabetes. These injuries lack an appropriate tissue scaffold and pro-healing cells making them difficult to heal. An ideal therapy is one that would restore both the essential matrix and cellular components that are lacking in these wounds to expedite healing. In this study, we used a novel liquid dermal scaffold capable of gelation in vivo to show that it is biocompatible with adipose derived cells. Using a validated method of wound splinting in an impaired-healing murine model we showed that wounds treated with adipose derived stem cells and the liquid scaffold had improved epithelialization, angiogenesis, and collagen content. The addition of adipose derived cells showed significantly increased expressions of essential growth factors: vascular endothelial factor (VEGF) and hepatocyte growth factor (HGF) in vivo and that these cells are detectable in the neodermis after wound closure. This liquid dermal scaffold with cells can be considered as a feasible future strategy for the management of complex or deep wounds that are otherwise lacking the appropriate cellular matrix necessary for healing.
Lay Summary

Expediting wound healing has benefits to both patients and the healthcare system. Achieving permanent closure in a non-healing wound remains a challenging endeavor.

The recent development in our lab is a skin matrix substitute that is in a liquid form in low temperature and casts into a gel once applied to the wound. This allows all edges of the wound to seamlessly integrate with the skin matrix to promote healing. To address the lack of cells in these wound cavities, we have decided to combine this matrix with regenerative stem cells harvested from fat tissue. These stem cells have been gaining much attention in the last decade owing to their ability to improve the local wound environment by increasing blood flow, activity of cells necessary for healing and the overall quality of the regenerated skin. This study shows that this combination of matrix and stem cells improves the quality of healed wounds.
Preface

The experiments in this study were conducted under the direct supervision of Dr. Aziz Ghahary and Dr. Reza Jalili at the BC Professional Firefighter’s Burn and Wound Healing Research Laboratory in Vancouver, BC, Canada. The protocol was approved by the Animal Ethics Committee at the University of British Columbia (protocol number: A15-0114).

Dr. Aziz Ghahary is the leading investigator for a patent for the powdered reconstitutable liquid dermal scaffold used in this study.

Dr. Diana Forbes conducted the majority of the *in vitro* and *in vivo* studies in Chapter 2.

Dr. Ali Farrouhki conducted the RT-PCR experiment and analysis

Dr. Reza Jalili performed the statistical analysis of the data from this work.
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<th>Full Form</th>
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<tr>
<td>A2AR</td>
<td>Adenosine 2A receptor</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end-product</td>
</tr>
<tr>
<td>ASC</td>
<td>Adipose derived stromal cell</td>
</tr>
<tr>
<td>bFGF</td>
<td>Fibroblast growth factor 2</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>DFU</td>
<td>Diabetic foot ulcer</td>
</tr>
<tr>
<td>DVT</td>
<td>Deep vein thrombosis</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>I/R</td>
<td>Ischemia-reperfusion</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1B</td>
<td>Interleukin -1B</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>KFG</td>
<td>Keratinocyte growth factor</td>
</tr>
<tr>
<td>LDS</td>
<td>Liquid dermal scaffold</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cells</td>
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<tr>
<td>RT-PCR</td>
<td>Real time polymerase chain reaction</td>
</tr>
<tr>
<td>SVF</td>
<td>Stromal vascular fraction</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming growth factor alpha</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFA/α</td>
<td>Vascular endothelial growth factor alpha</td>
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Finally, I’d like to extend my gratitude to the UBC Plastic and Reconstructive Surgery Program and the Clinician Investigators Program for supporting budding physician scientists. This opportunity has been truly invaluable.
Dedication

To our patients who inspired this work,

To my husband, for your love, humour and our children,

To Chloe and Carter - my precious gems,

To my mother - my rock
Chapter 1: Introduction

1.1 Overview

Wound healing is an essential process and normal healing goes through a series of events including inflammation, proliferation and remodeling. These processes provide hemostasis, the necessary tissue scaffold for cell migration with the end goal of re-epithelialization and wound closure. In chronic wounds, there is a disruption in this normal progression and wounds fail to heal in a timely manner. Some common features among chronic wounds are the persistent inflammation, dysregulated cell signaling, and interruption of keratinocyte migration (1). These factors contribute to the disintegration of the tissue matrix that can result in a physical gap devoid of cells preventing wound closure. Therefore, in order to promote healing, there is a need for novel strategies that can address the lack of a tissue scaffold and cells as well as the appropriate cell signaling molecules in a challenging wound bed.

1.2 Acute wound healing

The skin is the largest organ in the human body and serves many important functions such as providing immunity, sensation, protection against microorganisms and UV radiation, and thermoregulation. Understandably, these functions are critical to the survival of any organism in its environment and a rapid response to insults by the skin is evolutionarily advantageous. In humans, the skin is comprised of an outermost epidermis and an inner dermis. The avascular epidermis can be further divided into 5 distinct layers based on histologic appearance (2). Mitotically active keratinocytes are found in the basal layer, they undergo
constant turnover and become pushed towards the surface, decreasing in mitotic activity and increasing in keratin production as they transition through stratum spinosum, stratum granulosum, stratum lucidum and finally stratum corneum where the keratinocytes become enucleated and interlocking to serve as the outermost barrier (2, 3). In addition to keratinocytes that make up 95% of the epidermis, there are also melanocytes, Langerhan cells and Merkel mechanoreceptor cells (2). Under the epidermis resides the vascularized dermis containing skin appendages such as hair follicles, sebaceous glands, sweat glands and other cells responsible for sensation such as the Meissner, Pacinian, and Ruffini corpuscles for light touch, pressure, and sustained pressure respectively (3). The dermis is also the provider of the structural integrity of the skin owing to its relatively high water and collagen content (3). From 28 types of collagen known to date, human skin contains predominantly Types I and III (4). Insults to the skin triggers a highly regulated sequence of events that is aimed at restoring the functions of the skin. In humans, these steps result in repair of a wound in the form of a scar without regeneration of the skin appendages (5).

Acute wounds go through a predictable and organized course of healing that consists of the inflammatory, proliferative and remodeling phases (1, 6-9). These steps are not distinct events, rather a continuum of overlapping processes. The inflammatory phase is a complex system that activates to recruit a wide range of hematopoietic and non-hematopoietic cells including neutrophils, monocytes, lymphoid cells, natural killer cells, fibroblasts, B cells, T cells, endothelial cells, epithelial cells and stem cells that coordinates the tissue repair process (10). The inflammatory phase is initiated by tissue damage and promotes hemostasis, prevention of infection, and clearance of cellular debris. The coagulation cascade is activated immediately post
injury to achieve hemostasis and an aggregation of platelets at the site of injury forms the initial fibrin plug. The trapped platelets are a source of chemokines and growth factors that propagate the cascade of events essential for healing. Degranulating platelets release platelet-derived growth factor (PDGF) which is a potent chemoattractant for circulating neutrophils, monocytes and local fibroblasts (8, 11). In addition, PDGF has been shown to stimulate fibroblast proliferation, extracellular matrix (ECM) production and fibroblast differentiation into the myofibroblast phenotype (11). Neutrophils are the first nucleated cells to arrive at the wound and within minutes, circulating neutrophils extravasate across the endothelium and migrate to the site of injury where they phagocytose potential pathogenic microorganisms and further release pro-inflammatory cytokines that amplify the inflammatory process (12). The secreted factors by neutrophils in the wound has been extensively described, the key factors being interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α (1, 13-15). Next, blood monocytes arrive at the wound by day 2 and differentiate into tissue macrophages. These cells are capable of phagocytosing foreign material and cellular debris while secreting inflammatory cytokines, chemokines and growth factors such as PDGF, transforming growth factor (TGF) -α and β, basic fibroblast growth factor (bFGF), and VEGF (8, 16). There are 2 main phenotypes identified within the macrophage population: the M1 subtype which is the initial pro-inflammatory phenotype and a subsequent M2 subtype which is an anti-inflammatory, reparative phenotype (8). Although incompletely understood, it is believed that this M1-M2 switch is driven by the unique combination of factors such as IL-4/IL-13, toll like receptors (TLRs) and adenosine 2A receptor (A2AR) expression (12, 17). At any given time point, the wound bed may contain both types of macrophages in different proportions depending on the stage of wound
healing (12). The phenotypic switch is believed to allow the proper transition of wounds out of the inflammatory phase and an impairment in this process is believed to contribute to non-healing wounds (8). What is interesting, although not entirely surprising is that the same cell type can have different and opposing roles depending on the stage of wound healing it is in. For instance, macrophages play a role in neoangiogenesis as well as vessel regression at the start and end of tissue repair respectively (18). Macrophages have been shown to both mitigate and exacerbate tissue fibrosis and cutaneous scar formation (19, 20). The critical roles of macrophages in wound healing have been described extensively in the literature - over 600 articles in a 5 year period (12), however, Martin et al. showed that mice lacking neutrophils and macrophages maintain their ability to heal small wounds with reduced scarring (21) illustrating the biological redundancy and perhaps highlighting our current knowledge gap in this area.

When the wound-healing response is well regulated, the inflammatory phase resolves very quickly, led by neutrophil apoptosis and active depletion of proinflammatory cytokines. The proliferative phase occurring 2-10 days after injury, is initiated by proliferation of keratinocytes and fibroblasts bordering the wound edge. Here, the fibrin plug that was initially formed act as a tissue scaffold and transforms into granulation tissue containing newly formed vessels, sprouted capillaries, macrophages, fibroblasts, fibronectin, and hyaluronic acid (8). The key stimulators of angiogenesis are vessel endothelial growth factor (VEGF) α and fibroblast growth factor 2 (FGF2 also known as bFGF) (5). An impairment in angiogenesis and tissue hypoxia is a major contributor to delayed healing and chronic wounds (7, 8, 16). These changes occur as early as 48 hours post injury with a duration of approximately 10 days (22). This granulation tissue bridges the gap between the wound edges and acts as a scaffold for allowing
migrating and proliferating keratinocytes to re-epithelialize the wound in an uninterrupted fashion from the wound edge. If the injury does not involve a full thickness defect, the remaining hair follicles, and the epithelial stem cells primarily from the bulge region give rise to keratinocytes for re-epithelialization (9). A subset of fibroblasts in later stages of this phase differentiate into myofibroblasts upon stimulation by macrophages. Fibroblasts and myofibroblasts both lay down collagen-rich ECM forming the scar, with the latter also participating in wound contraction (8, 23, 24). Excess collagen synthesis and abnormal ECM turnover are important factors in abnormal scarring seen in hypertrophic scars and keloids (25-27).

Once the new epithelium has formed over the existing granulation tissue, remodeling of the tissue and scar takes place. With the exception of the fetus before the 3rd trimester, cutaneous repair in humans results in scar formation (28). The remodeling phase is initiated 2-3 weeks post injury and can last over a year. During this time, many changes to the wound architecture occur. The interface between the epidermis and the neodermis becomes a definitive basement membrane by the replacement of the temporary ECM with hemidesmosomes and type VII collagen (29). In addition, the majority of infiltrating cells also undergo apoptosis. It appears that there is a feedback inhibition where factors produced in the mature scar environment inhibit proliferation and ECM deposition by other fibroblasts (30).

During this phase, the scar that was initially laid down goes through a maturation process involving the replacement of a type III collagen ECM to one that is predominately type I collagen. Matrix metalloproteinases secreted by fibroblasts and macrophages allow for the maturation process (5). It is also during this phase that the scar gains mechanical strength by the
increase in intermolecular covalent bonding (cross-linking) between collagen fibrils (31, 32) and the replacement of collagen fibers with ones in a different, stronger configuration (33). Myofibroblasts play a role in taking over the mechanical load that was carried by the deposited ECM to add mechanical strength to the newly healed tissue until remodeling is completed (23). This scar evolves to reach its maximum strength (80% of uninjured skin) after approximately 2 months (32) and is devoid of skin appendages such as hair follicles, sweat and sebaceous glands (9, 33). The once cell-populated, vascular granulation tissue becomes a hypovascular, collagen rich scar by the end of the remodeling phase (16). A summary of the stages of wound healing and its major cell types are shown in Figure 1.1 (1).
Figure 1.1. Phases of wound healing. Early stages of wound healing involve the formation of a platelet plug and recruitment of inflammatory cells to the site of injury. Intermediate stage involves cellular proliferation, angiogenesis, ECM deposition and granulation tissue formation. Late stage involves ECM, collagen remodeling, and cellular quiescence. The cell types and their secreted factors are listed below. Eming et al (1) Permission obtained.
1.3 Chronic wound pathophysiology

All chronic wounds begin as acute wounds that have failed to progress through the well-organized cascade of events in normal healing. Although there is some debate, generally speaking, chronic wounds can be defined as wounds that fail to heal within 3 months (34). Various underlying medical comorbidities predispose patients to chronic, nonhealing wounds. It is estimated that at least 40 million people worldwide are affected by chronic wounds and these injuries can persist for months to years before healing occurs (16, 35). According to the Wound Healing Society these wound can be classified into 4 categories: arterial insufficiency ulcers, venous stasis ulcers, pressure ulcers and diabetic ulcers (34). Although there are many causes of chronic wounds, they share similarities such as being halted in the inflammatory phase, bacterial colonization and biofilm formation and local hypoxia with repeated ischemia-reperfusion injury that propagate the deleterious cycle and prevent progression to the proliferative phase of healing (16). Even though the inflammatory process is not found in later stages of normal wound healing, initial aberrancy in inflammation can have profound effects on the ultimate outcome of a cutaneous injury as seen in tissue fibrosis (12, 36). In chronic wounds excessive inflammation is seen secondary to a dysregulation of the proinflammatory cytokines IL-1b and TNF-a (37, 38). These cytokines lead to the increased production of matrix metalloproteinases (MMP) which is a large family of 23 peptidases in humans that influence tissue remodeling by cleaving specific protein components of ECM (39). While MMPs are essential for remodeling, excessive MMP production degrades the scaffold entirely, often seen clinically as a physical gap or cavity, hindering cell migration(40). In addition, the presence of bacteria colonization leads to a continual influx of immune cells, perpetuating the
proinflammatory state. Deficiencies in angiogenesis (sprouting of capillaries from existing vessels) and vasculogenesis (mobilization endothelial progenitors from bone marrow) (41) have been shown to significantly impair healing. In the extracellular milieu of chronic wounds, not only are there reduced levels of proangiogenic factors (i.e. from the VEGF family), there is also an accumulation of proteins with antiangiogenic effects such as myeloperoxidase (15) both contributing to reduced new blood vessel formation. Cellular senescence also occur in chronic wounds as fibroblasts are less metabolically active and recruitment of local stem cells that contribute to wound repair is blunted due to depletion of chemokines and growth factors in chronic wounds (41). The issue of ischemia-reperfusion (I/R) injury is worth discussing in relation to chronic wounds as it thought to contributes to the development and the persistence of pressure, venous stasis and diabetic ulcers (16, 42). In pressure ulcers for example, this phenomenon is when more tissue damage occurs after blood flow is restored (with position change and alleviation of pressure) to a previously hypoxic/ischemic area. Several mechanisms have been described including the damage caused by free-oxygen radical species from reperfusion after depletion of scavenging capabilities in the tissue after a period of nutrient and oxygen depletion and the subsequent activation of the immune system by endothelial cells (42, 43). Other theories include breakdown of cell-cell adhesion and increased permeability of the endothelium after ischemia inciting an inflammatory response (44). In addition to the molecular changes that make healing difficult, physical wound characteristics can also be barriers to healing. Often, chronic wounds have deep cavities and tunneling that extend beneath the skin surface. These are not easily reached by conventional dressings aimed to reduce bacterial burden and wound exudate in order to promote healing. The cavitating physical gap between the wound
edges are devoid of cells and an appropriate tissue scaffold which are necessary for keratinocyte
migration to occur. Lastly, patient factors such as poor nutritional status, metabolic
derangements, systemic illness all contribute to the development of chronic wounds (45-48). The management of chronic wounds focus on addressing the cause, assessing and adequately
debriding the tissue, infection control and achieving good moisture balance of the wound. To
aid decision making, the acronym: TIME, developed over a decade ago is still being used
clinically today. The acronym stands for: Tissue (T), assess the viability of the tissue,
involvement of deeper structures and debriding any necrotic material; Infection/inflammation
(I), assess the need for either topical for systemic antimicrobial therapy and obtain the necessary
surgical specimen when able for guiding antibiotic selection and duration; Moisture (M),
achieving moisture balance by using the appropriate dressing to prevent further skin maceration
from excessive wound exudate; Edges (E) examine the wound edges whether they are advancing
or undermined to determine the effectiveness of the current therapy (49).

The main subtypes of chronic wounds will be briefly discussed in the following sections.

1.3.1 Venous ulcers
More than half of all lower leg chronic wounds are due to venous stasis (50, 51). These ulcers
affect 1-2% of the adult population worldwide (16). Clinically, venous stasis ulcers typically
appear around the medial malleolus, they tend to be relatively shallower and larger with ill-defined borders. The cause of venous stasis is multifactorial; key components being deep venous
thrombosis (DVT) in the lower leg causing valvular insufficiency and high venous pressures
transmitted back into the capillaries (16, 52). Other factor such as protein-S, protein-C and anti-
thrombin III deficiency that increases a person’s risk for DVT are indirect risk factors (50). This increase in pressure causes edema as it increases the permeability of the vessel walls and forces red blood cells (RBCs) and other intraluminal macromolecules into the interstitial space (51, 53, 54). The presence of these components outside their normal domain also recruits neutrophils and triggers a local inflammatory response (16). It has been shown that macrophages that are iron-overloaded assume a M1 proinflammatory phenotype, and that the macrophages that are found in venous stasis ulcers tend to contain excessive iron content (12). Fibrosis of the tissue secondary to prolonged edema impairs tissue oxygenation and nutrient delivery which further damages the already compromised area (53). The venous congestion and secondary local ischemia are relieved by leg elevation and blood flow is temporarily improved in the affected area. This reperfusion may initially appear to be beneficial, however in reality, it can worsen the tissue damage. This I/R phenomenon has been well described in the literature and is one of the factors driving the deleterious cycle of inflammation and tissue damage in chronic wounds (42, 44, 55). Current treatment strategies focus on eliminating the cause of venous hypertension if one can be found such as addressing venous varicosity, and underlying venous thrombosis (51, 54). Non-surgical management involve custom-made compression stockings to reduce venous pooling, regular cleansing of the area to decrease bacterial burden, debridement of devitalized tissue, routine dressing changes combined with topical therapy to improve healing. Surgery in the form of skin grafting can be performed in the right individual as this treatment is considered to be safe and efficacious, more so for venous leg ulcers than those from other etiology (56). Unfortunately recurrence rates for venous stasis ulcers are tremendously high at up to 70% within the first 3 months of initial wound closure (35, 51).
1.3.2 Arterial insufficiency ulcers

Arterial ulcers are less common than venous insufficiency ulcers, in fact, ulcers that are formed purely due to arterial insufficiency are unusual (57). Frequently, arterial insufficiency is a contributing factor to poor healing in ulcers secondary to another etiology such as diabetes or venous congestion (57). Arterial ulcers have been traditionally described as forming around bony prominences distally and are often caused by peripheral vascular disease (53). Atherosclerosis, thromboemboli, radiation induced skin changes can all lead to the development of arterial ulcers in the lower extremities. Risk factors for peripheral vascular disease overlap with those of atherosclerotic and cardiovascular disease such as diabetes, smoking, obesity, hypertension, and dyslipidemia (58). The “angiosome” anatomic concept which was introduced in 1987 by Taylor and Palmer (59) states that the blood supply to the body, particularly to the integument can be considered as 3-dimensional blocks of tissue –“angiosome”, each being supplied by a single source vessel. Adjacent angiosomes are interconnected by smaller communicating vessels that can dilate and compensate against ischemic conditions (59, 60). In patients with longstanding diabetes and end-stage renal disease, these micro-vessels can be jeopardized, putting certain regions of the skin at risk for necrosis and ulceration (60). Clinically, these ulcers appear deeper, well-demarcated and often will have a “punched out” appearance with atrophic skin changes (53). The natural history is one of disease progression leading to eventual loss of the limb (57) thus management is the centered around the reestablishment of blood flow to the limb as this intervention is the most likely to lead to ulcer healing (50). Revascularization can be in the form of an open bypass surgery or with endovascular techniques. There is evidence to suggest that
targeting angiosomes in revascularization leads to better ulcer healing rates (61, 62). Adjuvant therapies such as topical treatments, although not able to correct the underlying etiology, should be combined with surgery to maximize healing (57).

1.3.3 Pressure ulcers

Pressure ulcers typically develop in areas over bony prominences as a result of direct pressure to the tissues or pressure in combination with shear forces (63). There are many risk factors predisposing individuals to pressure ulcers, these are listed in Table 1.1 (64).

Table 1.1 Risk factors associated with the development of pressure ulcers.

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Description</th>
<th>Example</th>
</tr>
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<tbody>
<tr>
<td>Reduced mobility or immobility</td>
<td>Pain is a warning sign that the tissue is undergoing ischemic changes due to prolonged pressure and triggers movement. Patients who cannot move independently are unable to offload this pressure</td>
<td>Frail elderly, sedated patients with mechanical ventilation</td>
</tr>
<tr>
<td>Lack of sensation</td>
<td>Patients who have blunted or absent sensation may not be aware of sustained pressure in an area causing tissue damage</td>
<td>Patients with spinal cord injuries, unconscious patients secondary to a medical condition or drug/alcohol intoxication</td>
</tr>
<tr>
<td>Malnutrition</td>
<td>Malnutrition delays wound healing and is also an independent risk factor for pressure ulcer development</td>
<td>Elderly patients, those with chronic substance/alcohol abuse</td>
</tr>
<tr>
<td>Compromised vascular supply</td>
<td>Suboptimal perfusion predisposes tissues to damage from pressure.</td>
<td>Patients with peripheral vascular disease, post cardiac arrest, hypovolemic shock</td>
</tr>
<tr>
<td>Friction or shear</td>
<td>In addition to pressure, these forces further compromise blood supply.</td>
<td>Often over the sacrum and heels for bedbound patients in the supine position</td>
</tr>
</tbody>
</table>

Table 1.1 from Guy, H. 2012 with modifications. Permission obtained.
These wounds are staged according to the depth of involvement. The updated National Pressure Ulcer Advisory Panel staging is listed in Table 1.2 (65).

**Table 1.2 Updated pressure ulcer definition and stages.**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Non-blanchable erythema of intact skin usually localized in an area over a bony prominence.</td>
</tr>
<tr>
<td>II</td>
<td>Partial thickness loss of dermis presenting as a shallow open ulcer with a red or pink wound bed, without slough or bruising.</td>
</tr>
<tr>
<td>III</td>
<td>Full thickness tissue loss. Subcutaneous fat may be visible but bone, tendon, or muscle are not exposed.</td>
</tr>
<tr>
<td>IV</td>
<td>Full thickness tissue loss with exposed bone, tendon, or muscle.</td>
</tr>
<tr>
<td>Unstageable</td>
<td>Full thickness tissue loss in which the base of the ulcer is covered by slough and/or in the wound bed obscuring the depth determination.</td>
</tr>
</tbody>
</table>

Table 1.2 from Black, J. 2007 with modifications. Permission obtained.

The mainstay of management is injury prevention. Pressure offloading includes turning a bedbound patient every 2 hours. Using pressure redistribution support surfaces such as air mattresses for high risk patients (66), protective heel and seat cushions, and vibration devices have been associated with less pressure ulcer development. Treatment for pressure ulcers starts with addressing the causal factors followed by methods to keep the area clean, free of stool or urine and wound exudate. Surgical candidates are those who have been optimized pre-operatively from a nutritional, metabolic and lifestyle perspective such as addressing malnutrition, managing diabetes, quitting smoking and/or alcohol and drug abuse. Recurrence rates for these ulcers are reported to be between 11%-39% (67).
1.3.4 Diabetic ulcers

The prevalence of type 2 diabetes worldwide is estimated 6.4%. The lifetime incidence of diabetic foot ulcers (DFUs) have previously been estimated to be from 15%-25% (1, 46), but recent data suggest that this number is close to 34% (68), equivalent to approximately 167 million people. The mortality rates related to DFUs are rather alarming. The 5-year risk of death for patients with a DFU is more than twice as high as diabetic patients without foot ulceration. Most of these ulcers are infected and 1 in 5 will result in some form of amputation. The 5-year mortality risk after amputation exceeds 70% for all diabetic patients (68). Impaired wound healing in diabetic patients is a combination of multiple factors such as chronic hyperglycemia, the accumulation of advanced glycation end-products (AGEs), the eventual insulin depletion, micro and macro circulatory dysfunction, and peripheral neuropathy. Chronic hyperglycemia causes non-enzymatic glycation of lipids and proteins known as AGEs. The accumulation of AGEs happens naturally with aging but is accelerated in diabetes and the presence of these molecules causes intracellular damage and elicits an inflammatory response (69). Hyperglycemia has also been shown to impair macrophage function fibroblast and keratinocyte migration as well as endothelial cell function resulting in inadequate neovascularization after tissue injury (70).

The main principles for treatment involve adequate glycemic control, wound debridement, pressure off-loading, revascularization and infection management (45, 46, 69, 71, 72). Giving systemic insulin therapy has been shown to improve healing in patients with DFUs when other variables were controlled for (71, 72). The barriers to healing are not dissimilar to other chronic wounds; persistent inflammation, repetitive I/R injuries and the physical gap lacking the
appropriate tissue scaffold and cells hindering wound closure. The recurrence for DFUs is high -40% within 1 year of ulcer healing (68).

1.3.5 Financial implications of chronic wounds

The issue of complex wounds has been a longstanding healthcare challenge. Today with the increased incidence and prevalence of diabetes mellitus, more people will be affected by complex wounds and chronic ulcers. It has been estimated that there are over 380 million people with diabetes worldwide and that 1 in 20 patients will develop a diabetic ulcer (16, 68, 73). In addition, patients with burn and spinal cord injuries are often affected by complex and chronic wounds. The estimated health care costs associated with managing challenging wounds can be up to 3% of total health expenditure in developed nations (74). Delayed wound healing increases the length of hospital stay, increases the number of operative procedures and increases patients’ susceptibility to infection. In North America alone, this cost exceeds 20 billion US dollars (70) driving an urgent need to develop new treatments to improve nonhealing cutaneous wounds. In addition to the costs to the healthcare system, patients also suffer financially in the form of lost wages in addition a significant loss of the physical and psychosocial quality of life (75). This issue is amplified when most patients with a history of ulcers tend to have multiple recurrences in their lifetime.
1.4 Bioengineered approaches in wound healing

Rapid wound closure is the cornerstone to minimizing infection and scarring. Full thickness injuries -those that involve the entire depth of the dermis cause the loss of skin appendages which contains cells for reepithelialization. These injuries rely on cell migration from the periphery of the wound to regenerate ECM for wound closure which can be a major barrier to healing in deep or chronic wounds. Current biological therapies aim to reinstate the extracellular matrix framework by which keratinocytes can migrate across to reestablish the epithelium and close the cutaneous defect (76). Most of these artificial matrices are collagen based and are either acellular, thereby relying on host cells to infiltrate the scaffold or are populated with cells such as keratinocytes and fibroblasts from human neonatal foreskin (76-79). Rennert et al (78) published a comprehensive review of the current bioengineered approaches in advanced wound care in phase III clinical trials and beyond and a sample of the current FDA approved approaches can be found in Table 1.3.

Table 1.3 Current FDA approved bioengineered approaches in wound care.

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Product composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apligraf ®</td>
<td>Organogenesis</td>
<td>Bilayered construct containing collagen, expanded human fibroblasts and keratinocytes</td>
</tr>
<tr>
<td>Dermagraft ®</td>
<td>Shire Regenerative Medicine</td>
<td>Collagen/GAG scaffold with human fibroblasts</td>
</tr>
<tr>
<td>Aquacel ®</td>
<td>ConvaTec</td>
<td>Carboxymethylcellulose hydrofibers that gel upon application to hydrate wound</td>
</tr>
<tr>
<td>Bilayer Matrix Wound Dressing ®</td>
<td>Integra</td>
<td>Silicone outer sheet with inner collagen/GAG matrix</td>
</tr>
<tr>
<td>Regranex ®</td>
<td>HealthPoint Biotherapeutics</td>
<td>Topical gel containing recombinant PDGF</td>
</tr>
</tbody>
</table>

Table 1.3 adopted and modified from Nuschke et al (79). Permission obtained.
The current commercially available dermal substitutes are generally available in pre-formed sheets with a pre-determined thickness. The purpose of these dressings is to fill in the cavity of the wound, bridge the edges and allow skin to migrate in to reestablish the ECM. Wounds, however, acute or chronic often have irregular borders and variable depth that do not necessarily match the conformation of these skin substitutes. This mismatch in the clinical setting makes bridging wound gaps difficult and treatments potentially ineffective. The ideal topical therapy would be one that is capable of integrating with the irregular wound edges seamlessly, can address both the lack of a tissue scaffold and cells to promote healing.

1.5 Adipose-derived stem cells and their role in chronic wound healing

Stem cells are defined as cells capable of differentiating into other cell types and unlimited self-renewal (77). These stem cells are categorized according to their differentiation potential; either toti-, pluri-, multi-, or unipotent and their source; embryonic, fetal (placental/umbilical cord), adult or induced pluripotent stem cells (77, 80). Over the last decade, there has been a growing interest in using mesenchymal stem cells (MSC) in wound healing. The term MSCs refers to multipotent progenitor cells capable of differentiating into chondrocytes, myocytes, neurocytes, osteocytes, adipocytes and other cell types under different condition media in vitro (77). Unmitigated hyperinflammation is the most substantial barrier to treatment in chronic wounds (79) and MSC have been shown to alter the local wound environment by augmenting the immune response by reducing host levels of TNF-α, IFN-γ and natural killer (NK) cell activity in the inflammatory phase (81). The mechanism is largely through the induction of the anti-inflammatory cytokine IL-10 in macrophages and T cells by MSCs (81).
MSCs can be isolated from multiple sources such as umbilical cord, placenta, skin, bone marrow, and adipose tissue (77), the most attractive source being adipose tissue as it is relatively abundant, often regarded as medical waste, easily obtainable in large quantities, and has relatively minimal ethical implications compared to other sources such as fetal or embryonic tissue (80). In addition, a functional genomics study comparing various sources of MSCs showed that adipose-derived mesenchymal stem cells (ASCs) seem to have the most abundant immune-related genes expression profile (82). Adipose tissues are derived from the mesodermal layer in embryonic development and consist of adipocytes, and stromal vascular fraction (SVF). ASCs can be found within the SVF making up only 1-5% of adipose tissue. The rest of SVF contains preadipocytes, fibroblasts, smooth muscle and endothelial cells, macrophages and lymphocytes (80). Morphologically, ASCs lack lipid droplets in their cytoplasm and express various markers on their membrane. In the literature, there lacks consensus regarding specific cell surface markers expressed by ASCs that distinguish them from the surrounding SVF. According to the International Federation for Adipose Therapeutics and Science and the International Society for Cellular Therapy (83), ASCs isolated from adipose tissue should be negative for CD 31, CD45 and positive for stromal markers CD 13, CD73 and CD90 (83). Other traditionally accepted defining features of ASCs are that they give rise to colony forming units (CFU) when isolated and cultured and can be induced into other cell types such chondrocytes, adipocytes and osteoblasts in vitro. Over the years, several studies have attempted to characterize these cells. Despite best efforts, there are still no widely accepted criteria for identification of MSCs. In fact, in 2016 Denu et al (84) found that mesenchymal stem/stromal cells and fibroblasts are phenotypically indistinguishable; they share similar cell surface markers, are capable of forming
CFUs, secrete growth factor and can have immunomodulatory effects. The exact definition of these cells is continuing to evolve based on ongoing research.

Nonetheless, recent developments in regenerative medicine have shown that ASCs accelerate cutaneous wound re-epithelialization via paracrine and direct cell-cell contact mechanisms (85). Previous studies have shown that these cells secrete a myriad of growth factors such as hepatocyte growth factor (HGF), VEGF, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), FGF, insulin-like growth factor (IGF)-1, facilitate cell proliferation, increase ECM deposition, enhance fibroblast activity, induce neovascularization, and ultimately enhance healing (86-90). The ability to suppress macrophage infiltration and modulated TNF-α and TFG-β1 expression in vivo by ASCs has an anti-fibrosis effect likely mediated through increased secretion of HGF (91, 92) or ASC-released microvesicles and exosomes (93, 94). In the setting of chronic wounds, ASCs have been shown to overcome chronic inflammatory states in animal models (85) and they have also been shown to able to mitigate some of the detrimental downstream effects of inflammation such as downregulation matrix metalloproteinase (MMP)-1 for which fibrillar collagen is a substrate and potentially halt the excessive ECM breakdown (95).

One of the limiting factors for this promising ASC therapy is the poor rate of engraftment. Fu et al. has shown that less than 20% of human ASCs remain viable after 56 days post injection into immunodeficient mice (96), similar to a rat model of osteoarthritis using ASC therapy (97). Other methods include systemic injection or using a biologic construct such as a collagen-based or ECM scaffold. There is insufficient data in the literature to make an accurate comparison between these modes of ASC delivery currently.
1.6 Our combination approach using a dermal matrix populated with adipose-derived stromal cells

To address challenging wounds, we have previously formulated a biocompatible liquid dermal matrix (LDS) composed mainly of crosslinked collagen, chondroitin sulfate, glycosaminoglycans, and polyvinyl alcohol is capable of gelation with desirable mechanical strength once applied in a wound (73, 98). This material is unique as can go through a phase change in vivo once it has filled the cavity of a wound - akin to liquid gelatin casting inside a bowl, to seamlessly bridge the gap that cells must overcome to reestablish the ECM scaffold. To address the absence of cells in this cavity, LDS was seeded with ASCs. We then tested this construct in a diabetic db/db mouse model (99) with wound splinting which is currently a well-described and accepted model of delayed wound healing (100).

1.7 Hypothesis and Objectives

Our working hypothesis is that a liquid dermal scaffold (LDS) capable of gelation in vivo is non-toxic to ASCs and this unique combination of a cellular liquid matrix can improve healing in a murine model.

Aim 1: Evaluate the wound healing rate in an impaired-healing murine model using a liquid dermal matrix with and without the addition of ASCs.

Aim 2: Compare the quality of the healed wound when ASCs are added to the matrix scaffold.
Chapter 2:

Adipose-derived stem cells improve tissue quality in a murine model of delayed wound healing

2.1 Introduction:

Wound repair and regeneration is a multidisciplinary field of research with considerable potential value to the management of complex and chronic wounds such as pressure and diabetic ulcers as well as extensive and deep burn injuries. Currently, the global prevalence of diabetic foot ulceration is 6.3% and it has been estimated that the amputation rate of those with a diabetic ulcer can be greater than 40% (60, 101). Following amputation, half of these patients die or lose their contralateral limb within 5 years (70). In addition to the huge functional impact chronic wounds have on patients, the total medical cost for treating diabetic foot disease in North America can reach up to $13 billion (101, 102). Burn injuries also have a significant impact on the patients and the healthcare system. It has been said that achieving definitive closure of a chronic burn wound is one of the most difficult tasks in burn care (103). Cost-wise, it has been estimated that the average cost of a burn admission is over $80,000 USD in developed countries and this figure can reach over $700,000 USD (104-107). The length of burn center stay and intensive care days make up over 80% of the total costs (105-107). Clearly, successful strategies for accelerating wound healing in these settings would significantly improve both healthcare cost and patient outcomes. Finding strategies to permanently and consistently close chronic wounds has proven to be a challenging endeavor. Unlike acute wounds which go through a predictable and organized course of healing consisting of the hemostasis, inflammatory, proliferative and remodeling phases (5), chronic wounds are halted in the
inflammatory phase characterized by an influx of inflammatory cytokines, predominance of proinflammatory cells, and upregulation of metalloproteases which lead to the destruction of essential extracellular matrix (1, 16). The consequence is that these wounds are devoid of ECM and cells—two integral components for healing. The commercialized skin substitute products are generally available in pre-formed rectangular sheets with uniform thickness, unfortunately chronic wounds often have irregular borders and variable depth that do not necessarily match the conformation of these skin substitutes. The ideal topical therapy that could effectively promote healing would be one that is at least capable of integrating with the wound edges seamlessly, and can address both the lack of a tissue scaffold and cells.

The latest research reveals enormous therapeutic potential of adipose-derived mesenchymal stem cells (ASCs) in burn and chronic wound therapy (108-111). The ASCs are a particularly appealing group compared to other sources of mesenchymal stem cells (such as from the bone marrow or skin) due to the relative abundance of adipose tissue, ease of harvest and lower immunogenicity (90, 112). These adult multipotent cells can positively influence tissue healing by differentiating into many cell types and mitigating the hostile wound environment by dampening inflammation (108, 113). Moreover, ASCs express a variety of cytokines that regulate cellular proliferation, angiogenesis and fibrosis (114, 115). There has been a variety of published methods of incorporating ASCs into a wound bed such as through direct injection or with materials like extracellular matrix derivatives, hydrogels, dermal substitutes and collagen scaffolds (77, 116, 117). Much effort has been dedicated to developing a dermal substitute in the setting of large surface area burns and deep chronic wounds. Previously, we have shown that a collagen-glycosaminoglycan (GAG) based liquid dermal scaffold (LDS) is capable of gelation
at body temperature with desirable mechanical properties. The composition as well as its ability to go through a phase change in vivo allows the scaffold to intimately conform to the wound bed and accelerate healing (73, 98).

Herein, we present our findings to show that this novel dermal scaffold accelerates deep wound closure and that the addition of ASCs improved the quality of the healed skin in a validated delayed-healing murine model.

2.2 Materials and Methods:

2.2.1 Liquid Dermal Scaffold (LDS)

The contents and mechanical properties have been described in detail previously (73, 98). In summary, a collagen-GAG scaffold crosslinked containing polyvinyl alcohol (PVA) hydrogel was used in this experiment as LDS.

2.2.2 Adipose-derived stem cell (ASC) harvest

The protocol for this experiment was approved by the Animal Care and Biosafety Committee of University of British Columbia. (A15-0114). Four transgenic mice expressing Green Fluorescent Protein (GFP) [C57BL/6-Tg(UBC-GFP)] were sacrificed with isoflurane followed by CO₂ inhalation according to protocol. The subcutaneous fat tissues were excised and processed to isolate ASCs as described previously (113) with slight modifications. In brief, adipose tissues were washed three times with phosphate-buffered saline (PBS pH 7.4) at 4°C. Tissues were finely minced and digested with collagenase type V (0.1%w/v; Sigma-Aldrich, St. Louis, MO) in PBS for 20 mins at 37°C under constant agitation. Cell suspension was filtered with a 70-μm
nylon mesh filter and centrifuged at 600g x 5 mins to isolate stromal vascular fraction from adipocytes. The desired cell pellet containing ASCs were resuspended and cultured in Dulbecco’s modified eagle’s medium (Hyclone DMEM/High Glucose; GE Healthcare, Logan, Utah) supplemented with fetal bovine serum (FBS 10% v/v; Gibco, Grand Island, NY), and antibiotic/antimycotic (1% v/v Anti-Anti 100x, Gibco) incubated at 37°C and 5% CO₂ overnight. Non-adherent cells were eliminated by gentle washing with PBS the following day and adherent cells were expanded in vitro to 80% confluence. Adherent first passage cells were detached with trypsin and cryopreserved in liquid nitrogen prior to use.

2.2.3 ASC Viability Assay

A live/dead assay was used to evaluate the cytocompatibility of LDS. ASCs were mixed with either supplemented DMEM or LDS in liquid form and 100 μL aliquots were placed into standard 24-well plates. The plates were placed in a 37°C, 5% CO₂ incubator. Culture medium was added into the wells with LDS as well as the 2D cultures and changed every 2-3 days. Viability at day 10 was determined according to manufacturer’s instructions (Live/Dead Double Staining Kit, Calbiochem, Gibbstown, NJ). Fluorescence microscopy was used to capture images set to detect FITC and Texas Red.

2.2.4 Animals and experimental design

Female diabetic leptin receptor deficient mice (db/db; BKS.Cg-m+/+ Leprdb) were obtained from Jackson Laboratories (Bar Harbor, ME). Five animals were housed per cage before surgery and 3 per cage after surgery. They were maintained in an animal care facility with
a 12-hour light/dark cycle throughout the acclimation (1 week) and test periods. The animals were between 9 to 12 weeks old at the time of surgical wounding. Occurrence of diabetes was confirmed in these animals by measuring random blood glucose being above 15 mmol/L. db/db mice were randomly assigned to 1 of 3 experimental groups with experimental parameters as follows: Group 1: Liquid Dermal Scaffold (LDS) only Vs. no treatment; Group 2: LDS Vs. LDS+ ASCs; Group 3: LDS + ASCs Vs. no treatment. N=4 for each experimental group.

2.2.5 Surgical Procedure

All surgical procedures were performed by one surgeon. Animals were anesthetized with isoflurane and maintained at 2% isoflurane in 100% oxygen, flow rate of 1.0 L/minute. Meloxicam (Metacam®, Boehringer Ingelheim, Ingelheim, Germany), dose 5 mg/kg, and 37°C Ringer’s lactate solution (Lactated Ringer injection USP, Baxter, Deerfield, IL), one-time dose 10 ml/kg, were administered subcutaneously at the beginning of surgery. Each animal was positioned in ventral recumbency, on a 37°C warming pad. Hair was removed from the upper half of the dorsal surface with a shaver and hair removal cream (Nair®, Church and Dwight, Ewing, NJ), Surgical site was prepped with 10% Proviodine -Iodine (Betadine® Purdue Pharma, Pickering, Ontario). The surgical procedure has been described in detail previously (100, 118) with slight modifications. In brief, 2 silicone splints (Grace Bio-Labs, Bend, OR) with an inner and outer diameter of 10 mm and 14 mm respectively were secured using three outer interrupted sutures (6-0 Ethilon Nylon Suture, Ethicon LLC, Cornelia, GA) and six inner interrupted sutures. The location of the splints 40 mm cranial from the tail head and 10 mm
lateral to the spine. Two full thickness 8 mm wounds were created on the dorsum of the animals using an 8 mm sterile skin biopsy punch (Acuderm, Fr. Lauderdale, FL) and Westcott scissors. The skin and the abundant adipose tissue under the wound were completely excised giving a wound depth of approximately 3 mm.

2.2.6 Treatment and Post Surgical Evaluation

Green fluorescent protein (GFP) positive ASCs taken out of cryopreservation, cultured in 175 cm$^2$ sterile, polystyrene flasks (Corning, Oneonta, NY) in supplemented DMEM overnight were resuspended in phosphate buffered saline (pH = 7.4). A total of 100 ul of passage 2 cell suspension [2.5x10$^6$ cells/ml] were injected subcutaneously into the periphery of each wound in the experimental group receiving LDS + ASCs. The same wound also received 250 ul of LDS + ASCs [1x10$^6$ cells / ml] by mixing the cell suspension with LDS with a pipet prior to applying to the wound. Wounds in the LDS Only group received 250 ul without cells. All wounds were covered by a semi-occlusive dressing (Tegaderm Film, 3 M Health Care, St. Paul, MN). The No Treatment group received dressing only. Animals were monitored according to our institution protocol and weighed daily. Photographs were taken on days 0, 7, 10, 14 using a digital camera. Dressing changes were done subsequently. Animals were euthanized at Day 14 with isoflurane followed by CO$_2$ inhalation according to protocol. Skin tissue from the wounded area was harvested for analysis.
2.2.7 Histological and Immunofluorescence Analysis

Masson’s Trichrome (MT) staining

Paraffin-embedded tissue was processed and stained as previously described (119). In brief, 5 μm tissue sections were deparaffinized and rehydrated with ethanol. Slides were incubated in Bouin’s solution for 1 hr at 60°C. After rinsing slides were stained with Weigert’s Hematoxylin solution (Sigma-Aldrich) followed by Biebrich Scarlet-Acid Fuchsin, phosphomolybdic-phosphotungstic acid, aniline blue and 1% acetic acid solution. Collagen appears blue, nuclei black and cytoplasm red.

Immunofluorescence (IF) staining for endothelial cell marker CD31 and GFP

After deparaffinization and rehydration, 5 μm tissue sections were treated using heat-mediated antigen retrieval with sodium citrate buffer pH 6 for 20 mins at 100°C. The tissues were blocked with 5% BSA in PBS for 1 hr before incubation with primary rabbit anti-CD31 antibody (1:500 ab124432, Abcam, Cambridge, UK) or chicken anti-GFP (1:1000 ab13970, Abcam) overnight. Sections were washed with PBS and incubated for 1 hr with 1) goat anti-rabbit secondary antibodies conjugated with Alexa Fluor 546 (1:750 A11010 ThermoFisher Scientific, Waltham, MA) to detect CD31 or 2) goat anti-chicken secondary antibodies conjugated with Alexa Fluor 488 (1:750 ab6877, Abcam) to detect GFP. Nuclei were stained and slides mounted with 4′-6-diamindino-2-phenyl-indole (DAPI Vectashield, Vector Laboratories, Burlingame, CA). Randomly selected 10 high power fields were counted for CD31 positivity by a blinded reviewer and done in duplicates for each group.
Immunohistochemistry (IHC) staining for GFP

After deparaffinization and rehydration, 5 μm tissue sections were treated using heat-mediated antigen retrieval with sodium citrate buffer pH 6 for 20 mins at 100°C. The tissues were incubated with primary chicken anti-GFP antibody (1:200 ab13970, Abcam, Cambridge, UK) overnight. Endogenous peroxidase was blocked with 0.3% peroxide for 15 mins. Primary antibody was detected with biotinylated goat anti-chicken secondary antibody (1:1000 ab6876, Abcam). Staining development was done by the DAB substrate kit (Vector Laboratories).

2.2.8 Image Analysis

All image analyses were performed by a blinded reviewer.

Wound area

Photographs of the wounds were analyzed using the ImageJ software (National Institute of Health, Bethesda, MD) as previously described(118). Open wounds were traced, and the % wound closure area calculated using the equation:

\[
\text{% Wound closure} = 1 - (\text{Open wound area}/\text{Original wound area}) \times 100
\]

Epidermal thickness

Histology sections were analyzed using ImageJ. The entire length of the wound was measured, the reference was the thickness of uninjured, hair bearing normal epidermis at the periphery of the tissue section set as “1”. A midpoint indicating the wound center was selected
and the thickness of the epidermis was determined to be either ≥1 or <1. The number of wounds in each group that had a value of ≥1 was compared.

Collagen content

Histology sections were evaluated for collagen content in the neodermis using digital densiometry recognition by ImageJ according to previous studies (120, 121). The neodermis was the area of interest and was traced out manually. Values are expressed as an area occupied by collagen positive staining.

2.2.9 Real-Time Polymerase Chain Reaction (RT-PCR)

Five main growth factors known to be secreted by ASCs (122, 123) were selected for analysis using RT-PCR. RNA extraction and real-time polymerase chain reaction (PCR) were performed as previously described (124). Briefly, a 0.5 cm x 0.5 cm piece of tissue from the center of the wound was excised at Day 14. Tissue samples (n=5 for all groups) were homogenized in TRIzol (Invitrogen, Life Technologies, Carlsbad, CA) according to manufacturer’s instructions. The total cellular RNA was extracted, purified and DNAse treated. SuperScript First-Strand Synthesis System (Invitrogen) was used converted the RNA into cDNA using random primers for the reverse transcription reaction. The ABI 7900 real-time PCR thermal cycler (Applied Biosystems, Foster City, CA) was used to perform RT-PCR. Primers (Table 2.) for vascular endothelial growth factor alpha (VEGFα), hepatocyte growth factor (HGF), transforming growth factor beta (TGFβ), platelet-derived growth factor (PDGF) and fibroblast growth factor beta (βFGF) were amplified using the TaqMan gene expression assay
(Applied Biosystems). Internal normalization was achieved by using the housekeeping gene glyceraldehyde 3-phosphate dehydrogenate (GAPDH). All samples were performed in triplicate.

**Table 2.1. Primer sequences**

<table>
<thead>
<tr>
<th>Primers:</th>
<th>Forward strand</th>
<th>Reverse Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-α</td>
<td>TCCGAAACCATGAACCTTTCTGC</td>
<td>GGCACTGGCTCTGCTGGTAGAC</td>
</tr>
<tr>
<td>PDGF</td>
<td>TCTGCTGGGAACAACTCAACAG</td>
<td>CACAGTGACACCTTCATCACC</td>
</tr>
<tr>
<td>bFGF</td>
<td>CAAGCGGCTCTACTGCAAGAAC</td>
<td>GTTGGCACACACTCCCTTGATAG</td>
</tr>
<tr>
<td>HGF</td>
<td>GCCAACAGGTGTATCAGGAACAG</td>
<td>GCCCTCTTTACCAATGATGCAG</td>
</tr>
<tr>
<td>TGFβ</td>
<td>AGCCCGAAGCGGACTACTATG</td>
<td>TAATCTGCAAGGCGACGCTC</td>
</tr>
</tbody>
</table>

**2.2.10 Statistical Analysis**

Statistical analyses were done using the IBM SPSS 24 software (Chicago, IL). Data are presented as a mean ± standard deviation (SD) and analyzed using the analysis of variance (ANOVA) Tukey-Kramer multi-comparisons test to compare experimental groups and their controls. A Student’s T-test and Chi Square test was used to compare two experimental groups. A p value of <0.05 was considered as statistically significant.
2.3 Results:

2.3.1 Liquid Dermal Scaffold (LDS) is non-toxic to ASCs.

In order for LDS to be used as an ASC delivery matrix, we first had to ensure that ASCs can survive within the scaffold. Live/Dead assay at day 10 showed that there was no significant difference in the viability between ASCs seeded into the 3D scaffold compared to those grown in 2D culture medium (Figure 2.1).

![Live/Dead assay of ASCs in (A) 2D culture or (B) 3D LDS at Day 10. Live cells in green, dead cells in red. Scale bar = 500 μm. No statistical significance between the two groups (C).](image)

**Figure 2.1.** Live/Dead assay of ASCs in (A) 2D culture or (B) 3D LDS at Day 10. Live cells in green, dead cells in red. Scale bar = 500 μm. No statistical significance between the two groups (C).

2.3.2 LDS alone and in combination with ASCs accelerate healing of full thickness Wounds.

The use of the diabetic mouse model with splinted wounds is a well described model for studying delayed-healing wounds. The wounds with LDS alone and LDS in combination with ASCs showed significantly faster healing than the no treatment group (Figure 2.2). The LDS +
ASC group had a statistically significant smaller wound sizes compared to control at all 3 time points. At day 7, LDS+ASC and No Treatment had a mean open area of 69.4±7.0% compared to 83.5 ±3.7% (p=0.008) respectively, at day 10; 23.8±12.5% Vs 55.4±11.5% (p=0.006) and at day 14; 2.0±3.2% Vs 31.6 ±11.2% (p<0.001). For the LDS only group, the wound closure rate was significantly faster than No Treatment control at day 14; 5.5 ±3.8% Vs 31.6 ±11.2% (p=0.0014). The two experimental groups had similar rates of wound closure with 2D analysis. Histological findings with Masson’s Trichrome staining further support photographic evidence of earlier reepithelialisation in the treatment groups as well (Figure 2.3). At day 14, a layer of epidermis can be seen on the wounds treated with LDS and LDS + ACSs whereas the control group had only partial reepithelialisation with the leading epithelial edges visible.
Figure 2.2. Photographs of full-thickness 8mm diameter wounds. Faster reepithelialization in both LDS alone and LDS + ASCs group (A). Graph depicting wound healing percentage (B). * statistical significance reached between LDS + ASC vs No treatment. ** statistical significance reached between LDS alone vs No treatment.
Figure 2.3. Masson’s Trichrome staining of the wounded area at Day 14. Incomplete reepithelialization was seen for the No Treatment group (a) and complete reepithelialisation of the LDS alone (b) and LDS + ASC (c). Black arrows indicate the edges of the original wound. Top panel scale bars = 1000 μm, bottom panel scale bars = 200 μm.
2.3.3 Wounds treated with LDS+ASCs have a thicker epidermis and more collagen content

Normal, uninjured skin was visible at the periphery of all samples and the epidermis thickness of this area was standardized to 1. The epithelium at the center of the wounds were measured. The proportion of healed wounds with an epidermal thickness of ≥1 was plotted. In the LDS + ASCs group, 83.3% of healed tissue that had an epidermal thickness of ≥1 which was significantly higher than LDS which had only 40% (p<0.001) (Figure 2.4A). No epidermis was seen at the center of the wounds in the no treatment group.

2.3.4 Collagen content is the highest in the healed wounds treated with LDS+ASCs.

To determine collagen content of the healed tissue at Day 14, Masson’s Trichrome stained sections of fully epithelialized tissue was analyzed with ImageJ. A significantly larger area occupied by positive collagen staining was seen in the LDS+ ASC group; 55201.3+/- 12525.1 Vs LDS alone; 24216.2+/-11397.3 (p = 0.009) (Figure 2.4B). Wounds in the No Treatment group did not achieve closure at Day 14 thus were not analyzed for collagen content.
Figure 2.4. Graphic depiction of epidermal thickness and collagen content. The percentage of healed wounds in each group with an epidermal thickness equal to or greater than uninjured skin at Day 14 (A). *Higher percentage of wounds with thicker epidermis in the LDS+ASC group (p<0.001). **Greater collagen content in the neodermis of healed wounds in the LDS+ASC group (p<0.009).

2.3.5 Increased angiogenesis in healed wounds treated with LDS and LDS+ASCs.

To assess angiogenesis, immunofluorescence (IF) staining with CD31 at Day 14 found increased CD31 positive capillary structures in wounds treated with LDS and those treated with LDS+ASCs; 4.3/HPF ± 1.2, 6.2/HPF±1.2 respectively (p<0.001), and compared to no treatment; 1.8/ HPF±0.9 (p<0.001) (Figure 2.5). A qualitative analysis showed that the wounds treated with LDS + ASCs had CD 31 positive structures resembling more mature vessels compared to the other groups (Figure 2.5). Normal tissue was obtained for reference with 3.6 capillary structures /HPF ± 1.1.
Figure 2.5 Immunofluorescence staining for CD31 at Day 14 (A). CD31 positive cells in red, cell nuclei stained with DAPI in blue. Graphical representation (B) of the mean number of capillary structures / high power field (HPF). *Higher number of capillary structures between LDS and No Treatment (p<0.001). **Higher number of capillary structures in LDS + ASC vs LDS alone and No Treatment (p<0.001).
2.3.6 ASCs added to wounds are viable in vivo and become incorporated into the neodermis.

All ASCs used for this study were GFP positive. While GFP remains fluorescent in frozen sectioned tissue, the fixation process disrupts this signal thus a more reliable way to detect GFP positivity would be with an anti-GFP antibody. In this case, immunohistochemistry (IHC) staining at Day 14 shows that GFP positive cells are present in the neodermis in the LDS + ASCs group but not in LDS only or No Treatment groups (Figure 2.6.). These GFP positive cells are found in clusters in the dermis as well as surrounding vessel-like structures (Figure 2.6.). Immunofluorescence staining for GFP detected positive cells in the neodermis, confirming these findings (Supplemental figure S1.)
Figure 2.6. Immunohistochemistry staining for GFP. Compared to No Treatment (A), the LDS + ASC group (B) shows GFP positive ASCs in the previous wound bed appearing in clusters and forming vessel like structures (black arrows). Top panel scale bar = 500 μm, bottom panel scale bar = 50 μm.
2.3.7 Expression of VEGF-α and HGF are elevated in tissues supplemented with ASCs

RT-PCR analysis of the tissue in the healed wound bed extracted at Day 14 showed that the LDS + ASC group had a significant fold increase in gene expression for VEGF-α; 3.1+/-0.9 and a HGF; 4.4+/-1.1 (p>0.001) compared to No Treatment (Figure 7). No statistical difference could be found between the acellular LDS group and No Treatment.

Figure 2.7. RT-PCR gene expression analysis of wound tissue obtained at Day 14. The LDS+ASC group had increased expression of VEGF and HGF compared to No Treatment. There were no differences seen in the LDS group.

2.4 Discussion

Previously, we have shown that a collagen-GAG based liquid dermal scaffold (LDS) capable of gelation in vivo is non-toxic to human fibroblasts. This scaffold alone, when applied in vivo has been shown to have pro-healing effects, increase angiogenesis in the wound bed and improve outcomes in a hypertrophic scar animal model(73). Using a diabetic murine model
mimicking delayed-healing, our current study demonstrated that this liquid scaffold is biocompatible with ASCs and capable of significantly accelerate wound closure under pathological conditions. While there are numerous commercially available products, none are currently able to go through a phase change in vivo. We think this is an essential feature when addressing deep or chronic wounds as an artificial dermal scaffold because it allows the material to intimately conform to the irregular wound edge with variable depths, eliminating any gap that cells must migrate across (76, 98).

In recent years, mesenchymal stem cells have gained significant attention in wound healing research. These multipotent cells, when placed in a wound bed can secrete essential growth factors promoting angiogenesis and cellular proliferation, modulate the local immune response and can differentiate into multiple cell types (73, 76, 77, 116, 125, 126). In the setting of burn injury, autologous ASCs have also gained attention due to their ability to ameliorate the inflammatory conditions of the wound while maintaining their multipotency (111). These cells alone, without a carrier, however, cannot be easily applied to large, irregular wounds. The findings from the current study shows that when ASCs are placed in this liquid dermal scaffold, they can maintain their viability. When they are applied to the wound bed they can be found in the wounded area after the injury has reepithelialized.

This wound healing animal study did not find a difference in reepithelialisation time between the LDS alone and LDS supplemented with ASCs using pictorial 2D analysis. It is possible that the accelerated healing rate seen by the LDS alone group masked the relatively smaller differences caused by the supplementation of ASCs. It would be fair to conclude that
relatively small wounds do not require ASC supplementation and perhaps a larger diameter wound would allow these differences to emerge.

The excessive subcutaneous fat in these obese mice also act as a source of stem cells. In fact, Hu et al showed that murine wounds with minimal subcutaneous fat were slower to heal (94). In our technique, we attempted to address this issue by removing the fat directly beneath the wound without causing excessive morbidity. Nonetheless, a significant amount of adipose tissue remains in contact with the periphery, acting as a source of cells for wound healing.

Although adding ASCs did not seem to alter the time to wound closure in our study compared to LDS alone, we did notice that the group treated with ASCs had smaller and more shallow appearing wounds. A 3D analysis is required to verify this difference. We did not factor this potential need into our study design and the current methods for 3D volume determination with standard photographs are often inaccurate (127). Theoretically, shallower wounds should have more rapid closure, however unlike humans, rodents heal cutaneous injuries mainly by wound contraction (128) and a size and potential depth difference may have been overcome by this powerful, evolutionarily advantageous phenomenon towards the later time points. Splinting the wounds should control for most, but not all of the contraction occurring in the rodent model (129). Further research with larger wounds is needed to elucidate the effects of ASCs on wound healing and that one should consider using not only standard photographs but also a 3D camera for wound volume analysis (127).

In this study, the ASCs were introduced to the wound via both topical application and injection. Injection of ASCs alone has the issue of lower retention (130-132) even when the number of cells injected have reached $1 \times 10^6$ (131). The density of ASC in the LDS used in the
study was $1 \times 10^6$ cells/ml. The wound size was a limiting factor in the amount of LDS+ASC we can add. To increase more cells in the wound, we could in theory increase the concentration of the cells in the LDS, however, we know that in vitro, high cellular density negatively impacts viability, thus subcutaneous injection was used to introduce additional ASCs into the wound to a total of $5 \times 10^5$ cells per wound -half from injection, half from LDS seeding. We recognize that this step affects the final interpretation of the data, future experiments with larger wounds or in a large animal model would allow us to introduce higher numbers of ASCs in the liquid scaffold alone to evaluate the effects of this combination on wound healing.

Despite the aforementioned challenges and limitations, we were nonetheless able to detect positive changes that make the healed tissue in the ASC supplemented group superior on a molecular level. The healed wounds in the LDS+ASC group consistently had a more robust epidermis, similar to other studies using mesenchymal stem cells from human (133) and porcine sources (134). The neodermis in this group also had the highest collagen content. The increase in collagen content is supported by other studies using ASCs in mice (129), burn wounds in the rat (135) and both in vitro (136) and in vivo (126) human ASC studies. Proposed mechanisms include the direct fibroblast stimulation by ASCs to increase procollagen gene expression (137), via exosomes (94) or through de novo differentiation of ASCs into fibroblasts (136).

Angiogenesis is an essential component of wound healing and deficiencies in this process have been found to be a common feature among many types of chronic wounds. Similar to previous studies (126, 129), our results show that supplementing with ASCs led to higher numbers of vessel-like structures indicating possible superior angiogenesis. While adding exogenous growth factors can also produce this effect, ASCs are live entities thus they are able
to tailor their gene expression and secretory function in an environment specific manner. Through immunohistochemistry and immunofluorescence staining, we were also able to visualize the ASCs in the new dermis in clusters and forming vessel-like structures. Similar to previous findings, likely these ASCs became incorporated into the microvasculature to become endothelial-progenitor cells (86, 96, 126, 138-140).

Quantitative gene expression analysis using RT-PCR extracted from cells within the tissue bed provided us with a snapshot of the growth factor expression profile at day 14. There were significantly higher levels of VEGF and HGF over others that were examined. VEGF is a family of potent angiogenic factors (41) and interestingly, HGF has been found to be a factor in reducing tissue fibrosis and excessive scar formation (26, 39, 141, 142). It has been shown that ASCs secrete relatively significant levels of HGF compared to other growth factors in vitro (143). Under hypoxic conditions, there can be a selective increase in VEGF production by 5-fold (143) which matches the wound conditions in our study; deep, relatively avascular and sealed with a dressing.

In terms of the engraftment of transplanted ASCs, this seems to be a limiting factor of current therapies (144). Fu et al (96), using stromal vascular fraction from GFP mice have found that at 56 days, less than 20% of the original cells are detectable with fluorescence imaging. Agrawal et al showed that less than 10% of ASCs were detectable in the wound after 10 days (132). While the number of viable cells in the healed tissue was not quantified in this study, we do know that the downstream effects of having a more robust epithelium, increased angiogenesis and the upregulation of key growth factors in the healed area have favorable long-
term benefits. These effects are likely the result of the paracrine actions exerting influences on
the surrounding cells and not from direct differentiation of ASCs.
Chapter 3: Conclusion

This liquid dermal scaffold accelerates wound healing in a murine model mimicking delayed healing. The addition of ASCs improved the tissue quality. This cell-scaffold combination can address wounds that are lacking in both cells and ECM essential for healing. We think that this combination has therapeutic potential in the treatment of deep cutaneous injuries and chronic wounds and more research is warranted.

3.1 Future direction:

The results of this study show that this combination therapy, alters the tissue at the molecular level when ASCs are added. Large animal studies using purely LDS as a carrier for ASCs would be beneficial in testing the feasibility of LDS in supporting ASCs for potential clinical use. To simplify the preparation of this cellular scaffold dressing for clinical use, a modification would be to supplement the LDS with fresh homogenized whole fat without in vitro manipulation and compare this formulation to ASC. Eliminating the tissue culture step would make this treatment more acceptable in a clinic setting on human subjects.
3.2 Limitations:

One major limitation to the study is the small sample size. Another limitation is in the use of this animal model. In mice, cutaneous wounds heal mainly by contraction as they have a loose skin and a layer of muscle - the panniculus carnosus under the skin. On the other hand, dermal wound in human heal mostly by granulation tissue formation and reepithelialization. The best murine model of delayed healing is using splinted wounds in db/db mice. However, this is not a perfect model. Splinting eliminates some but not all wound contraction. The db/db mice in the study are diabetic due to leptin receptor deficiencies, are insulin resistant, and become hyperphagic and obese. Clinically the pathophysiology of most diabetic patients is not related to leptin receptor deficiencies. In addition, these splinted wounds will ultimately heal without intervention and recurrence unlike human chronic wounds. Advocating for the use of this therapy in humans at this point remains under question.
References


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Figure S1. Immunofluorescence staining of GFP. No GFP cells found in the LDS only group (A), GFP positive cells seen in the neodermis in the LDS+ASC group (B). Scale bar = 100 μm. Green = GFP; blue = nucleus.