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Characterizing Hypoxia, Neutrophil Persistence and Revascularization in the Murine db/db Model of Type II Diabetic Impaired Skin Healing

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Abstract

Impaired skin healing represents a significant clinical burden. In the diabetic, inflammatory aberrations, hypoxia and insufficient angiogenesis all result in negative wound healing outcomes - repeated infections, poor perfusion and ultimately amputation. Previous research has reported comparable levels of neutrophils in closed wounds up to 4-12 weeks old. Our study focus was in investigating the dynamics of hypoxia resolution, neutrophil persistence and angiogenic response in the db/db model. Contrary to our hypothesis, we observed significantly higher hypoxic load in the wild types at days 3 and 7. Additionally, we observed significantly elevated neutrophil numbers at day 7 db/db wound bed and an angiogenic deficit at day 3, with wild types exhibiting significantly more $CD31^+$ cells. Our results validate the db/db model as one of impaired healing, and are consistent with literature in the field suggesting a potential deficit in hypoxic adaptation resulting in an overall delayed wound healing process.

Keywords: hypoxia, inflammation, neovascularization, wound-healing

Co-Authorship Statement 1

The pig as a model system for investigating the recruitment and contribution of myofibroblasts in skin healing.

Douglas Hamilton, John Walker, Dylan Tinney, Michael Grynyshyn, Alexander El-Warrak, Emily Truscott, Lauren E. Flynn

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Personal Contribution:

- Wrote the introduction section
- Performed and imaged the a-SMA tissue processing Figure 1 and Figure 2

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Co-Authorship Statement 2

Microcirculation surrounding end-stage human chronic skin wounds is associated with endoglin/CD146/ALK-1 expression, endothelial cell proliferation and an absence of $p16^{Ink4a}$

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Personal Contribution:

- Imaged the CD31 tissue sections in Figure 1
- Imaged the CD146 tissue sections in Figure 2
- Imaged the Endoglin tissue sections in Figure 3
- Performed and imaged the Endoglin/CD146 processing in Figure 4
- Imaged part A) in Figure 6
- Sent tissue to university hospital and imaged it in Figure 9

Estimated Personal Contribution Percentage: 15%

Summary for Lay Audience

Type II Diabetes is a disease that arises from the body's inability to deliver sugar into cells effectively. The result is increased levels of sugar in the blood, often for a very prolonged period of time. Diabetics are also at increased risk for wounds, especially on their feet due to issues with their blood vessels and nerves, which result in poor sensation. Once a wound is created, it often starts a cycle of trauma, inflammation and repeated infection. Diabetics are at a greatly increased risk for foot amputation, and the prognoses following these operations is often poor.

Hypoxia, inflammation and new blood vessel formation are all well-known factors in wound healing. The purpose of this work is to utilize a diabetic and healthy mouse model, to attempt to outline any differences between them with respect to these parameters. The use of such models allows researchers to model complex pathologies in a relatively quick and inexpensive manner. We seek to uncover differences between healthy and diabetic mice in order to further our understanding of what is different between how a diabetic and a healthy person and potentially extrapolate these findings to the discovery of new therapeutics.

Hypoxia, inflammation and vascular are all attractive and promising areas of research for the development of new therapeutics. In the present study we aimed to outline differences in how quickly hypoxia resolves, and whether we could establish a relationship between its resolution, the amount of inflammation present in the tissue and the amount of new blood vessel ingrowth into the wound.

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List of Abbreviations

AGE – advanced glycation end product

ANG -1 – angiopoetin 1

ANG – 2 – angiopoetin 2

ATP – adenosine triphosphate

CD31 – cluster of differentiation 31

DAB – diaminobenzene

DFU – diabetic foot ulcer

ECM – extracellular matrix

FGF – fibroblast growth factor

HIF – 1 – hypoxia inducible factor 1

HClO – hypochlorous acid

IL – interleukin

miR210 – microRNA 210

MMP – matrix metalloproteinase

NF-kB – nuclear factor kappa beta

PEDF – pigment epithelium derived factor

ROS – reactive oxygen species

SEM – scanning electron microscopy

SPRY2 – sprouty 2

TGF-B – transforming growth factor beta

TIMP – tissue inhibitor of matrix metalloproteinases

TNF-a – transforming growth factor alpha

VEGF – vascular endothelial growth factor

Chapter 1

Introduction

The importance of proper wound healing has been recognized as conducive to good health since the dawn of man. Papyrus scrolls dating back to 3200-3000 BC, outlining techniques of compression and hemostasis, point to the recognition of importance of facilitating this process by early medical practitioners. Up until about 100 years ago the biggest health problem facing humanity was infectious disease, owing to issues of sanitation and overcrowding, while further compounded by a lack of available treatments. With the advent of vaccines and antibiotics, we were able to successfully overcome these issues, shifting the current medical focus to addressing other sorts of debilitating, chronic conditions such as cancer, AIDS, neurodegenerative disorders and diabetes. The focus of this manuscript will be Diabetes Mellitus, with a specific emphasis on its commonly seen complication – diabetic foot ulcers (DFUs). Before beginning the comprehensive discussion of the DFU pathology, it is necessary to preface with a brief discussion of the types of chronic wounds and what their medical definition is.

Chronic wounds are defined as wounds exhibiting a disrupted repair process where a sustained anatomical and functional result is not reached within three months [1]. The health issues that often result in chronic wounds are rapidly increasing in most parts of the world, thus clinicians can expect to see an increase in the incidence of non-healing pressure, venous and diabetic ulcers. The majority of chronic wounds fall into three main categories: venous ulcers, pressure ulcers and diabetic ulcers [2]. Venous ulcers represent more than half of all lower limb chronic wounds and will affect 1-2% of the adult population, with a higher prevalence in women and

the elderly [3]. Pressure ulcers tend to afflict those with compromised mobility or sensory perception. Patients who are paralyzed or unconscious for long periods of time cannot feel stimuli which would otherwise tell them it might be time to reposition. Such prolonged unrelieved pressure can lead to ischemia when tissue compression exceeds capillary pressure [4]. Regardless of their etiology, chronic wounds are all characterized by enhanced local hypoxia and inflammation. Sadly, the significance of chronic wounds is often overshadowed by their causes, the costs are poorly documented and appropriate care and education is lacking [5]. Chronic wounds persist as a silent epidemic, adversely impacting the quality of life of over 40 million individuals worldwide [6].

Type 2 diabetes is a metabolic disease, characterized by prolonged elevated blood sugar levels, resulting from the body's inability to effectively shuttle it into cells. There are currently over 382 million people afflicted with this condition worldwide [7]. Unfortunately, due to factors such as an increasingly aging population and high fat diets common in Western culture, these numbers are projected to rise. Diabetic foot ulcers are a commonly seen and serious complication of diabetes [8], with approximately 15% of patients developing one. Of those that develop a DFU, 14-24% will subsequently undergo a lower limb amputation, with a five-year post-op mortality rate from this procedure approaching 50-59% [9–11]. It is clear that therapeutic strategies for promoting the closure of DFU's warrant investigation, due to them being a common and costly complication, and the burden they impart on the patient's life and the healthcare system.

1.1 Mechanism of Initial Ulceration

In diabetics, a pathogenic triad of neuropathy, ischemia, and trauma is traditionally described [12]. A common denominator across DFU's is poor vascular flow, a condition that has been repeatedly implicated in poor wound healing [13]. Peripheral neuropathy results in reduced sensation of the foot, which is often occurring in tandem with reduced perfusion. When this poorly perfused, insensate foot is repeatedly stressed in ways not natural to it, the risk of skin damage and ulceration increases [14]. Once the initial break in the skin occurs, an insidious cycle of repetitive trauma and inflammation begins. This cycle is called ischemia-reperfusion, and it has been proposed to be the precipitating event for several other types of chronic wounds of differing etiologies [15]. Diabetic patients with vascular deficiencies are subjected to cyclic periods of hypoxia in their lower limbs, during activities such as standing. These are followed by periods of reperfusion, when their limbs are elevated and pressure is relieved.

Hypoxia has a strong potential to induce and maintain a pro-inflammatory state, so during periods of reperfusion the patient's wound sees an influx of leukocytes, which produce pro-inflammatory cytokines and reactive oxygen species (ROS), adding to the already accumulated ROS from partial tissue reoxygenation [16]. Nitric oxide – a vasodilator, is downregulated, further exacerbating the inflammatory response [17]. It is evident that as long as the patient keeps attempting to use their feet, these cycles are repeated, along with the deleterious effects of prolonged, repetitive bouts of hypoxia induced inflammation which will be outlined next.

1.2 Inflammation: What is Normal in Skin Healing?

To begin a discussion of the perpetually inflamed DFU pathology, it is necessary to briefly outline the process of normal (acute) inflammation. Upon cutaneous injury, circulating

platelets are exposed to the underlying extracellular matrix (ECM) and are induced to aggregate into a fibrin clot. They also release platelet derived growth factor (PDGF), which plays an important role in initiating the influx of neutrophils, monocytes, smooth muscle cells and fibroblasts to the wound bed. Both platelets and infiltrating leukocytes release interleukins (IL) IL-1a, IL-1B, IL-6, IL-8, Tumor Necrosis Factor Alpha, Platelet Derived Growth Factor and Transforming Growth Factor Beta (TGF-B), the latter of which stimulates cytokine release from macrophages and enhances fibroblast and smooth muscle cell migration [18]. The influx is further facilitated by degranulated mast cells, which release histamine, creating pores in blood vessels, thus aiding extravasation of leukocytes to the wound site and facilitating protein leakage.

The initial leukocyte response is mainly characterized by neutrophils for the first 2-5 days, with macrophages replacing them at approximately day 3. Neutrophils have three main functions: 1) Generate ROS via myeloperoxidase pathway to kill pathogens, 2) Debride the wound by breaking down nonviable tissue with the help of various proteolytic enzymes, 3) Phagocytose dead bacteria and matrix debris [19]. Once these processes are carried out, neutrophils undergo apoptosis and are cleared out by macrophages. This "clearing out" process is termed efferocytosis, and it prevents secondary necrosis of neutrophilic cells, and is thought to be essential for complete tissue repair [20].

As monocytes migrate into the wound, they mature into macrophages and become one of the most important regulatory cells in the inflammatory cascade [21]. They remove any non-functional host cells, bacterial-filled neutrophils (efferocytosis), damaged matrix, foreign debris, and remaining bacteria [22]. Additionally, they release growth factors and cytokines such as Transforming Growth Factors Alpha and Beta, basic Fibroblast Growth Factor and Vascular Endothelial Growth Factor (VEGF) in order to amplify and eventually resolve inflammation.

Some of these secretions serve to recruit endothelial cells and fibroblasts to progress the wound to the proliferative phase.

Under normal circumstances, inflammation resolves within 1-2 weeks of injury, when the leukocyte numbers fall back to their pre-inflammation numbers and phenotypes [23]. Unfortunately, in the presence of a deleterious stimuli, such as persistent hypoxia, accumulation of advanced glycation end products, or repeated trauma, inflammation can become prolonged and/or exacerbated. Additionally, diabetes mellitus leads to hyperglycemia-related metabolic changes that directly impair wound healing [24]. A central component of these changes is accumulation of advanced glycation end-products (AGEs) which can induce oxidative stress, impair skin and inflammatory cell function, increase ECM stiffness, and perhaps most importantly – induce a chronic low-grade, self-perpetuating inflammatory state along with circulatory dysfunctions leading to poor tissue oxygenation [25–28].

1.3 Diabetic Foot Ulcer Inflammation: What is Wrong?

DFU's are known to be locked in a perpetual cycle of inflammation, unable to progress to proliferative and remodeling phases of wound healing [29]. It is also known that several types of chronic wounds occur in the background of local tissue hypoxia due to various vasculopathies [30]. Although the molecular mechanisms vary, generally, hypoxia leads to cell membrane disruption and promotion of deleterious inflammatory cascades [31]. The general relationship between hypoxia and inflammation can be described as mutually reinforcing. It is known that in a healthy person, acute cutaneous injury results in temporary local tissue hypoxia, which is eventually resolved by the orderly phases of wound healing and angiogenesis. Conversely, in a diabetic individual, the local hypoxia in the DFU is never truly resolved, as a result of a number of factors which will be outlined next.

It is established that the accumulation of AGEs results in a chronic, low grade inflammatory state for the diabetic patient. It is also known that hypoxic zones tend to arise in inflamed tissues, as inflammation is intricately linked to oxygen metabolism [32]. Heat, swelling and redness – the three classical manifestations of inflammation, result from enhanced blood flow and vascular permeability and are thus directly associated with reduced oxygen distribution in inflamed areas. It must be noted that enhanced blood flow, while being suggestive of enhanced oxygen delivery, is anything but so. This phenomenon is attributed to reduced oxygen diffusion due to higher interstitial pressure (swelling) and enhanced oxygen consumption by cells as they attempt to cope in the harsh, inflamed environment. While inflammation creating a metabolically demanding environment for cells is deleterious in itself, an additional caveat is that hypoxic conditions in and of themselves are able to induce inflammatory reactions [33]. Studies have shown that mice exposed to 5% O_2 for 60 minutes exhibited significantly enhanced protein expression of interleukin (IL)-6, tumor necrosis factor alpha (TNF-a), and interleukin (IL)-1 in both serum and isolated macrophages. Similar observations were made in healthy human volunteers who showed increased serum levels of proinflammatory factors after three overnight stays at high altitude [34]. This information lends credibility to the idea that hypoxia and inflammation are closely related and are capable of mutually reinforcing one another [35].

1.4 The Rogue Agent – Neutrophil

The cell that seems to draw the suspicion of most investigators looking at pathologically prolonged inflammation in the context of hypoxia is – the Neutrophil. Upon detection of bacteria or mediators of inflammation, neutrophils begin phagocytosing microbes, which is followed by an assembly of an electron transport chain (NADPH oxidase) which delivers

electrons across the membrane to molecular oxygen for the generation of hypochlorous acid (HClO) and ROS leading to the lysis of microbes [36]. This process is called "respiratory burst", and it requires a significantly elevated consumption of molecular oxygen [37]. The respiratory burst accounts for an essential antimicrobial function, allowing neutrophils to carry out their decontamination tasks effectively. In summary, the presence of activated neutrophils at sites of inflammation results in oxygen depletion, a phenomenon aptly termed "inflammatory hypoxia" [38]. While neutrophils are known to be abundant in the early stages of wound healing, and are known to be essential for effective decontamination, a large, body of emerging evidence implicates their overabundance in impaired wound repair; as their persistence has the potential to initiate processes which destroy healthy tissue [39].

Perhaps the most detrimental potential consequence of prolonged hypoxia/inflammation, is the increased survival time of neutrophils [40]. Hypoxia has been shown to inhibit neutrophilic apoptosis via nuclear factor kappa beta (NF-kB) signaling, thus marking NF-kB as a regulator of hypoxic response in neutrophils. Additionally, the neutrophil activating/survival factor MIP-1B was shown to be induced under hypoxic conditions operating as an alternative mediator to neutrophil survival [41]. When this increased survival rate is considered together with the neutrophils ability to shape the tissue microenvironment via depletion of local molecular oxygen and degradation of ECM, the negative implications for sustained hypoxia become clear.

There have been numerous studies implicating increased neutrophil influx/persistence in aggravated disease outcomes. In order to carry out their debridement functions, neutrophils carry cationic peptides, eicosanoids and proteinases. Proteases such as elastase and cathepsin G, are capable of degrading most components of the ECM as well as proteins such as clotting factors, cytokines and immunoglobulins. It is well established that the ECM is an essential

component in the wound microenvironment, serving as a scaffold for infiltrating cells, so any adverse neutrophil modifications to it may have profound consequences for wound repair. Neutrophil overabundance is also associated with an over-production of ROS, causing further damage to the ECM, cell membranes and leading to premature cell senescence [42]. Additionally, neutrophils release serine proteases and matrix metalloproteinases (MMPs) such as neutrophil collagenase; elastase degrades important growth factors such as PDGF and TGF-B while collagenase degrades and inactivates components of the ECM. In addition to ECM damage, neutrophil secreted pro-inflammatory cytokines IL-1B and TNF-a not only increase production of matrix metalloproteinases, but also reduce their tissue inhibitors (TIMPs). This imbalance further augments the degradation of ECM, impairs cell migration, and reduces fibroblast proliferation and collagen synthesis.

1.5 Neutrophil Persistence in Wound Healing – A Different Perspective

A considerable deal of supporting rationale for this project came from the work of Rosalind Butterworth, MD, in 1992. Although the work may seem dated, this must be considered in context of one of the biggest obstacles to human wound research – a substantial lack of human models on which research can be conducted. Although human studies have been carried out since 1992, there is still a recognized lack of detailed information regarding the histological differences between chronic and healthy human wounds. Naturally, this brings up the issue of what it is investigators are trying to "model" in their animal wound healing studies. If sufficient understanding of the differences in cell populations and overall histological presentation has not been attained in human wounds, it can conceivably create difficulty when trying to extrapolate findings and data from animal models. Thus, the starting point for our project was

model validation, as the histological presentation of human wounds differs markedly from the histological findings in similar wounds in laboratory animals.

The primary aim of the Butterworth thesis was to investigate the structure and cellularity of human granulation tissue, via taking punch biopsies from healthy and unhealthy wounds. A secondary aim was evaluation of commonly used animal models of wound healing, and the extent to which data from them can be applied to the understanding of the human wound. There was a total of 24 healthy wounds, and 14 unhealthy wounds assessed, the latter of which were separated into three categories – overgranulating wounds, infected wounds and non-healing wounds. The most striking findings to come out of her research, which were used to form the partial rationale for this project were:

- When counts of all cell types from healthy wounds were considered together, there was a striking persistence of neutrophils and inflammatory cells throughout healing in closed wounds up to 4-12 weeks old.
    - It is normal in healthy human wounds to find an infiltrate of inflammatory cells including neutrophils, macrophages and lymphocytes whose distribution remains similar throughout healing.
- Overall distribution of inflammatory cells in infected or overgranulating wounds was similar to that of healthy wounds.
- The almost complete lack of inflammatory cells observed in non-healing wounds.
- Of the seven cell types examined only capillary endothelial cell number was found to be related to good healing progress.

These findings stand in contrast with the general consensus among many investigators that neutrophil persistence in the context of wound healing is a detrimental outcome. There has

been a considerable amount of work done in attempting to understand the function of this cell, and the potential negative implications associated with its persistence. The main body of the data suggests that beyond the initial acute inflammatory phase, the neutrophil serves little to no beneficial function and further seems to implicate its persistence in a plethora of negative wound healing outcomes. With the data from the Butterworth thesis in mind, our project set out to challenge the negative reputation of the neutrophil and potentially elucidate beneficial functions of this cell persisting well beyond the inflammatory and proliferative phases of healing. As the neutrophil is known to have an enhanced ability to cope and function in a hypoxic environment, we set out to further examine the relationship between the two. Of particular interest were the dynamics of neutrophil persistence, and whether or not they could be related to phenotype, hypoxia and re-vascularization rate.

1.6 Model Validation

While we recognize the current difficulties of generating reliable human data in the field of wound healing, we do believe that any models used must be rigorously validated to ensure adequate recapitulation of the human healing process, if there is any potential therapeutic value to be gleaned from them. Current evidence suggests that healing in mice and humans overlaps sufficiently enough for these animals to be widely used as models of chronic wound healing. While we did not set out with the goal of outright invalidation of this model, we did seek to challenge it with respect to the available data on neutrophil dynamics, and vascular deficiencies widely reported in human and pig studies. Additionally, we sought to validate this model with respect to the expected increased hypoxic load along with impaired re-vascularization which is reported in human studies of diabetic chronic wounds. It must also be noted that while widely

used, this model is not a true representation of chronic wound healing in a sense that all wounds in these animals eventually heal, albeit more slowly.

The finding of similar levels of neutrophil persistence across distinct stages of healing has also been partially validated in a pig model by Dyson et al 1988. This study undertook a quantitative analysis of cell types in the granulation tissue of open pig wounds, with the goal of comparing the effects of different types of bandages. They reported small numbers of neutrophils present at any stage of healing, irrespective of dressing type, and that negligible numbers were observed in wounds after five days. One of the secondary aims of this project was to attempt to confirm this finding with pig wounds from Days 7 and 28.

1.7 The Macrophage

In addition to a multitude of other roles, the macrophage is a critical cell in wounds as it represents the single most effective means of neutrophil clearance [43]. Macrophages are capable of responding to neutrophils and their products and are capable of inducing neutrophil apoptosis [44]. Perhaps most crucially, these cells are able to recognize and actively ingest apoptotic neutrophils, thus directly helping to resolve wound inflammation [45–48]. Several studies suggest that the process of efferocytosis affects macrophage phenotype, causing a switch from pro-inflammatory to a growth enhancing, reparative phenotype [49]. Other recent studies suggest that a failure in removal of inflammatory cells, namely neutrophils, plays a role in the pathogenesis of non-healing wounds [50,51]. Additionally, a deficit in macrophage ability to effectively remove neutrophils was recently reported to be a critical component of the impaired healing seen in diabetes [52]. The investigators implanted sponges into diabetic mice and subsequently isolated the macrophages from these sponges. The isolated macrophages showed a significant impairment in the phagocytosis of apoptotic cells. Interestingly, this

deficit was associated with higher levels of apoptotic cells and pro-inflammatory mediators in wounds, a feature that was further validated in wound tissues of diabetic patients. This data suggests that successful efferocytosis by macrophages may be requisite for appropriate wound healing, both in order to remove apoptotic neutrophils as well as to generate a macrophage phenotype supportive of the proliferative aspects of repair.

1.8 Angiogenesis: What is Considered Normal?

In the unwounded state, endothelial cells in the lumens of blood vessels are in a state of quiescence, evidenced by a basal, balanced expression of pro-anti angiogenic factors in the vascular bed. These factors include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiopoetin-1 (Ang-1) and pigment epithelium-derived factor (PEDF), and their balanced expression results in a vascular network that is neither proliferating nor diminishing [53,54]. Cutaneous injury is accompanied by a sharp increase in local hypoxia, which activates Hypoxia Inducible Factor 1 (HIF-1), which then promotes angiogenesis by upregulating target genes such as VEGF-A. This growth factor stimulates capillaries to form immature loops and branches, a process normally most prominent in the proliferative phase of repair. One of the defining features of normal, early angiogenesis is the formation of disorganized and poorly perfused vasculature, characterized by malformed capillary bed and blind-ended sprouts [55]. Although from a structural integrity standpoint these capillaries are sub-optimal, their number in healing skin is much higher than in normal, unwounded skin and peaks at day 7-10 post wounding [56,57].

After the peak of proliferation has passed, a vessel maturation program begins. This process selects for competent nascent vessels to become durable mature vessels similar to the pre-

injured state. Vessel stabilization is importantly mediated and achieved by pericytes [58]. In healing wounds, pericytes are actively recruited in response to several growth factors, with the best described being – platelet derived growth factor (PDGF) [59,60]. When these pericytes arrive, they interact with endothelial cells as well as the basement membrane. Capillary pruning is mediated by production of several anti-angiogenic, vascular maturation factors, with the two best studied one's being – pigment epithelium-derived factor (PEDF) and sprouty-2 (SPRY2). PEDF has been shown to be one of the most potent anti-angiogenic factors in the vasculature, and has been demonstrated to be capable of inducing endothelial cell apoptosis as well as reducing the permeability of "leaky" neovasculature [61]. In the context of wound healing, studies by Okonkwo et al. have demonstrated its production to be essential for vascular remodeling and maturation [62,63]. Sprouty-2 is an intracellular protein that inhibits mitogen-activated protein kinase signaling, ultimately downregulating the effect of VEGF on endothelial cell proliferation in wounds [63,64]. An additional level of angiogenic control comes from the interactions of Angiopoetins 1 and 2 (Ang-1/Ang-2) with the Tie2 receptor. Ang-1 is a potent maturation factor and stabilizes pericytes and endothelial cells in capillaries, while Ang-2 has an opposite effect and destabilizes vessels [65]. During normal angiogenesis, there are high levels of Ang-2 during the proliferative and pro-angiogenic phases and high levels of Ang-1 during the vessel maturation phase [66]. As the remodeling phase of healing draws to an end, the injured tissue achieves normal vascular permeability, blood flow and shows normal vascular branching [67]. Perhaps most importantly, the high oxygen demand of the early stages of wound healing, a situation that was previously described to induce and potentially aggravate the activation of many pro-inflammatory mediators and pro-angiogenic factors, returns to pre-injury levels [68].

It is worthy of note that angiogenesis overlaps with both the proliferative and remodeling phases of repair [69]. Fibroblast migration, proliferation, and collagen synthesis all occur during the same period as the angiogenic response. Recent studies suggest that capillary pruning and collagen maturation may interact in the remodeling phase, with the pruning of capillaries proposed to have a beneficial influence on the final ECM structure [70].

1.9 Diabetic Angiogenesis: What is Wrong?

The previously discussed Ang-1/Ang-2 – Tie2 complex, is one vascular maturation pathway that has been implicated in the angiogenic deficits seen in diabetic wounds. It has been shown that the ratio of Ang-1 to Ang-2 is decreased, strongly suggesting that the ability of diabetic wound neovasculature to undergo a vessel maturation program is likely disturbed [71,72]. During the maturation and resolution phases of angiogenesis, PDGF is one growth factor that has been identified as being perturbed in the diabetic state. It promotes capillary maturation by recruiting pericytes and slowing down vessel regression [73]. PDGF has been extensively studied in diabetic skin wound healing and db/db mice have been found to express lower levels of PDGF and its receptor in their wounds [74]. As previously discussed, neutrophil overabundance often accompanies bouts of repeated, prolonged inflammation. This cell carries elastase, a protease which effectively degrades PDGF, thus likely exacerbating the PDGF deficit already present in the diabetic condition**.** It is likely that the role of PDGF extends beyond capillary stabilization as it is also a potent mitogen for fibroblasts, thus any perturbations to its normal levels are likely to have profound, widespread effects on different events of the wound healing process, such as re-epithelialization.

A study by Seinz et al. showed that VEGF-A protein and mRNA levels in wounds of db/db mice were significantly decreased, as compared to healthy controls. VEGF-A is a potent pro-angiogenic factor known for increasing vascular permeability. Follow up studies by Galiano et. Al also reported this deficit, and went on to show that VEGF-A treated mice exhibited more of an early leaky, malformed vasculature and more edema until VEGF-A treatment was ceased [75].

In addition to known diabetic deficits in pro-angiogenic factors, several studies have recently identified deficits in anti-angiogenic and capillary maturation factors in wounds. Production of PEDF was examined in the context of diabetic healing, however this study only looked at serum levels rather than tissue expression levels. One such study reported higher circulating levels of PEDF in patients with a DFU, compared to both healthy and diabetic patients without an accompanying DFU [76]. In the context of what PEDF does, this finding is slightly out of line with the others. It must be noted that this study quantified PEDF levels systemically and not within the local wound tissue.

These studies strongly suggest that normal vascular pruning and maturation may be significantly delayed in DFU's, a condition which may greatly contribute to chronicity due to an increased, unresolving hypoxia along with its deleterious pro-inflammatory effects. There is a growing body of evidence that suggest that diabetes results in more tortuous and disorganized vascular architecture. For instance, mice subjected to High Fat Diet induced obesity, a condition meant to closely mimic the diabetic phenotype, present with more tortuous and aberrant vascular network [77]. Such findings are paralleled in human diabetic patients, where corrosion casting and scanning electron microscope (SEM) investigations have shown that those suffering from diabetic microangiopathy in the toe region**,** exhibit damaged capillary architecture and evidence of vascular leakage [78].

1.10 Rationale

Hypothesis: Neutrophil persistence will result in significantly increased oxygen consumption in the wound bed of the db/db mouse, compared to control. This increased stress will manifest as delayed resolution of hypoxia, delayed wound closure, enhanced neutrophil persistence, and reduced formation of diabetic neo-vasculature.

Aim 1: Investigate the relationship between persistence of neutrophils and delayed hypoxia resolution in the context of impaired diabetic healing.

Objective: Confirm that delayed hypoxia resolution correlates with increased persistence of neutrophils in the diabetic wound bed.

Aim 2: Investigate the relationship between hypoxia resolution and the rate of re-vascularization of tissues.

Objective: Confirm that as the wound re-establishes its vascular network, the tissue becomes less hypoxic.

Aim 3: Validate the mouse model as one re-capitulating the major defects seen in impaired human wound healing.

Objective: Confirm that the mouse exhibits appropriately comparable wound healing defects to the human.

Chapter 2

Characterizing Hypoxia, Neutrophil Persistence and Revascularization in the Murine db/db Model of Type II Diabetic Impaired Skin Healing.

Abstract

Typically recruited during early inflammation post-injury, the persistence of neutrophils has been documented in human skin wounds at 4-12 weeks after re-epithelialization is complete. Hypoxia can promote neutrophil survival through an autophagy-like mechanism, with their persistence associated with depletion of molecular oxygen levels in the wound via a process termed “respiratory burst”. Depletion of available molecular oxygen in the context of healing can negatively impact a wide array of necessary processes for healing including cell proliferation, collagen deposition and timely resolution of inflammation. Angiogenesis is also a critical milestone during healing as the re-establishment of vascular supply and adequate oxygenation of tissues is required for cutaneous healing. The current project aims at investigating the relationship between hypoxia, neutrophil recruitment to the wound, and angiogenesis in a murine model of diabetic healing.

Based on previously reported data we hypothesized that db/db diabetic mice would exhibit delayed wound closure, increased neutrophil persistence and reduced angiogenesis in comparison with wild-type mice. Selected timepoints for histology were day 3 and day 7, corresponding to the peak of acute inflammation and the peak of proliferation/beginning of angiogenic phase respectively. db/db mice exhibited a significant delay in closure at days 3, 7 and 12 post-wounding. No significant differences were observed in the relative amount of hypoxia in the epithelium at day 7 assessed through HypoxyProbe. A significant increase in

the number of hypoxic cells in the granulation tissue of wild-type animals was observed at day 7. Additionally, a significant difference was observed in neutrophil numbers, with increased number in granulation tissue in wounds in db/db vs wild-type at day 7. Finally, no significant difference in the number of $CD31^+$ cells was observed between phenotypes. No significant differences were observed in the amount of epithelial hypoxia at Day 3. A significant difference was observed in the number of hypoxic cells at Day 3, with wild-type animals showing a higher number in their granulation tissue and one of the edges. There was no difference observed in the number of neutrophils in the wound at Day 3. Lastly, there were more $CD31^+$ cells in wild-type granulation tissue and at one of the edges.

We conclude that wild-type mice show a minor increase in hypoxia at day 7 post-wounding compared to db/db mice, and db/db mice show a persistence of neutrophils at Day 7. Our current evidence suggests that further validation of the db/db mouse as an impaired wound healing model is needed, as we were not able to reproduce the angiogenic deficit that is often reported in human wounds. The somewhat surprising finding of increased hypoxia in wild-type wounds at days 3 and 7 may further suggest that sustained hypoxia may be a required stimulus for proper wound resolution, however further studies would be required to adequately validate this.

Keywords: hypoxia, inflammation, neovascularization, wound-healing

2.1 Introduction & Significance

Chronic skin wounds are defined as a disrupted or impaired repair process where a sustained anatomical and functional result is not reached within three months [1]. Chronic wounds persist as a silent epidemic, adversely impacting the quality of life of over 40 million individuals worldwide [6]. Impaired skin wounds fall into three general classifications: venous ulcers, pressure ulcers and diabetic ulcers [2]. Venous ulcers affect 1-2% of the adult population, with a higher prevalence in women and the elderly [3], and pressure ulcers are more common in those with compromised mobility or issues with sensory perception; prolonged unrelieved pressure can lead to localized tissue ischemia when applied compression exceeds capillary pressure [4]. Diabetes mellitus is a metabolic disease, characterized by elevated blood sugar levels resulting from the body's inability to effectively transport it into cells. There are currently over 382 million people afflicted with this condition worldwide [7] and diabetic foot ulcers are a commonly seen and serious complication [8]. Approximately 15% of diabetic patients will develop a DFU, with 14-24% subsequently undergoing a lower limb amputation, with a five-year post-op mortality rate from this procedure approaching 50-59% [9–11].

Regardless of their etiology, chronic wounds are all characterized by enhanced local hypoxia and inflammation [79]. Hypoxia results from both damage to the vascular structures in the tissue, as well as cell populations that are recruited to the wound site such as neutrophils. Neutrophils have a high oxygen consumption rate [80], but can also persist in hypoxic conditions [81].

Due to these factors, we investigated the dynamics of neutrophil persistence in the db/db model in the context of hypoxia. Primarily, we were interested in seeing if the temporal resolution of

wound hypoxia would correlate with a decrease in neutrophil numbers. We were also interested in evaluating the db/db model in the context of data presented by Rosalind Butterworth MD, namely the finding of similar neutrophil numbers observed in the wounds that of varying age, between 4-12 weeks [82]. We theorized the db/db model may exhibit a similar neutrophil infiltration pattern to humans, and we investigated whether the db/db injury model possesses parallels with human wound healing. Lastly, we wanted to observe these effects in the context of hypoxia and re-vascularization, as both are known to be able to shape the tissue microenvironment [83]

2.2 Materials and Methods

2.2.1 Mice

All animal procedures were carried out in compliance with protocols approved by the University Council on Animal Care at Western University. Wild-type and diabetic (C57BL/6 and BKS.Cg-Dock7m +/+ Leprdb/J) mice were purchased from the Charles River Laboratories (Wilmington, MA). At the time of experiments mice were 10 weeks old. The mice were housed individually and given unrestricted access to food and water. Blood sugar measurements were not taken, however previous work in our lab has reliably demonstrated that mice of the db db phenotype present with significantly higher body weights and had an average blood sugar level of 18.78 mM when compared to wild types who had an average level of 8.59 mM (n=10) [84].

2.2.2 Excisional Wounding Experiments

For all experiments, 4, 4-mm full thickness excisional wounds were created on the dorsal surface in accordance with AUP 2020-142. Pain was controlled for the first 24 hours via intra-peritoneal administration of Buprenorphine at a dose of 17 uL/10 g. Four injections in total were given, 6-8 hours apart. Mice were euthanized with CO_2 and tissue was isolated at Days 1, 3 7 and 12. For assessing closure kinetics, wounds were photographed at Day 0, and then days 1, 3 7 and 12, using a ruler to standardize image dimensions. Image J software was used to calculate wound area.

2.2.3 Human & Porcine Tissue Samples

All human tissues were collected with written, informed consent from patients under protocols approved by the Western University Review Board for Health Sciences Research Involving Human Subjects and in accordance with the 1964 Declaration of Helsinki. Written informed consent was received from participants prior to inclusion and enrolment in the study. Skin samples collected included those from the wound bed and edge, 2 cm proximal to the wound and a non-involved region at least 10 cm away. The samples were then fixed in 4% paraformaldehyde (Sigma Aldrich, St. Louis, MI) overnight and processed to paraffin for histology. Histological analysis of immune cell infiltration was performed on wound and healthy tissue from 5 patients.

Porcine wounding experiments were performed at the West Valley Animal Unit. All procedures were carried out under protocols approved by the Animal Use Subcommittee at Western University. Assessment timepoints were days 3, 7, 14, 28 and 42, however, in this thesis only days 7 and 28 are included. Full-thickness wounds were made bilaterally along the dorso-rostral back skin in each animal, under appropriate anaesthesia. Wounds were made at least 2 cm apart, in order to prevent potential mechanical interference from

neighboring wounds. Upon experimental endpoint the animals were humanely sacrificed, tissue was harvested and fixed in formalin, processed to paraffin, embedded and ultimately sectioned and processed for immunohistochemistry.

2.2.4 Histology

Mouse tissues were fixed in 10% neutral buffered formalin overnight and processed to paraffin. Sections 10 um thick were cut from paraffin blocks and mounted onto FisherBrand™ Superfrost Plus microscope slides (Fisher Scientific, Toronto, ON). Attempts were made to keep serially stained sections as close as possible, however, in some cases sub-optimal staining required re-runs of stains and additional sectioning. These additional sections were likely some distance away from the originally cut ones. This may account for some of the potential variability seen in wound regions and cell numbers which were analyzed across replicates. Colorimetric staining was performed using the VectaStain HRP – Anti-rabbit detection kit (Vector Laboratories Inc, Burlingame, CA) and a ImmPact DAB Peroxidase Substrate Kit. For Day 3, a total of 8 wild-type and 9 diabetic wounds were analyzed from 3 mice, respectively. For Day 7, a total of 16 wild-type and 14 diabetic wounds were analyzed from 5 mice, respectively. These timepoints were selected for analysis due to their ovelap with the peak of inflammatory phase and beginning of the angiogenic/proliferative phase of healing. Hypoxic cells were identified with the use of Hypoxyprobe Kit (HP1-1000, Burlington, MA). Immune cell infiltration was assessed with an anti-neutrophil elastase rabbit antibody (ab68672) at a dilution of 1:50. Vascular infiltration was assessed with an anti-CD31 rabbit antibody (ab124432) at a dilution of 1:300. For all 3 stains, sections were incubated at 4 degrees Celcius overnight. All stains were run in triplicate.

2.2.5 Cell Counting Quantifications

Hypoxic cell, immune cell and blood vessel infiltration was quantified using QuPATH cell-counting software (Bankhead, P. et al., 2017), as percentage of positive cells per 300x300 um field of view. For counting purposes, two regions of interest were created in the left edge, the wound bed and right edge respectively, totalling six regions per section. The regions of interest were kept in the same locations across replicates and stains. In some cases, the contrast between diaminobenzene (DAB) and hematoxylin was sufficient for the software to recognize positive and negative cells on its own with the default DAB detection vector. However, in other cases where the DAB was clearly present albeit appearing more faintly the software struggled with positively identifying these cells. In these cases, the DAB detection vector was set manually by identifying one clearly labelled well-contrasted cell, drawing a region of interest around it and setting the detection vector to that of the region of interest. This resulted in the software being significantly more accurate in identifying positive cells (Appendix 4.1- 4.3), but a balance had to be struck between optimal detection sensitivity and over-sensitivity in order to ensure that only the truly stained cells were being labelled as positive, as opposed to those displaying background DAB signal.

Total cell counts per field of view were also performed using the QuPATH software, in a similar manner described above. Epithelial hypoxia was assessed with the use of Image J software, beginning at the wound edge, and ending at the first obvious drop-off in signal moving laterally away from the wound edge. The scale was manually set for each image by tracing the scale bar. Additionally, sections were sent to Robarts Research to be stained for Picrosirius Red, Van Gieson and Masson's trichrome. There were 7 sections for Day 7, 4 for Day 3 and 2 for Day 1 from both phenotypes respectively.

2.2.6 Hypoxyprobe™ Assay

Hypoxyprobe (HP1-1000, Burlington, MA) was diluted in solution with 0.9% saline to a final concentration of 6 mg/ml and kept at 4 degrees Celcius in light subdued conditions. At endpoint, Hypoxyprobe solution was administered intra-peritoneally at a dose of 60 mg/kg, 90 minutes prior to euthanasia to allow probe to circulate and bind to targets. Immunohistochemical staining was performed in accordance with the manufacturers protocol, with dilutions of 1:100 used for both primary FITC-MAb1 and secondary rabbit anti-FITC with horseradish peroxidase. Sections were incubated overnight at 4 degrees Celcius with the primary antibody. A logically plausible limitation of this methodology is the destruction of the blood vessels within an intended area of Hypoxyprobe™ labeling. Hypoxyprobe™ has been reliably demonstrated to label the core of tumors (Appendix 4.9), which are known to possess a disorganized vasculature network [85]. These vessels are known to have a diminished ability to deliver nutrients and remove waste [85]. However, these tumors still label strongly for Hypoxyprobe™, confirming a delivery mechanism that is able to overcome any present vascular deficiencies.

2.2.7 Statistical Methods

Statistical analyses were performed using the GraphPad Prism version 10.0.1 for Mac, (GraphPad Software, Boston, MA). Graphs were also generated with the Prism software. Normality testing was carried out, with nearly all data sets showing a non-parametric distribution. Because of this, a non-parametric Mann-Whitney t-test was used and values of $P < 0.05$ were taken to be statistically significant.

2.3 Results

2.3.1 Diabetic mice exhibit a delay in the rate of wound contraction, re-epithelialization and collagen deposition.

Differences in wound closure rates between wild-type and db/db diabetic mice have been well described in the literature, including by our group. In the first experiments, we confirmed whether closure kinetics, re-epithelialization and granulation tissue formation in wild-type and diabetic mice differed. A significant reduction in wound closure was observed in diabetic mice at days 3, 7 and 12 in comparison with wild-type mice (Figure 1). Diabetic mice exhibited reduced re-epithelialization at day 7 compared to wounds in wild type mice (Figure 2). No qualitative differences in collagen deposition were observed at days 1 and 3 between phenotypes (Figure 3), although wounds in db/db mice exhibited less collagen deposition at day 7 versus their wild-type counterparts (Figure 4).

Figure 1 - Diabetic mice exhibit a significant delay in closure at days 3, 7 and 12. (A) – Visual comparison of wounds across timepoints. (B) – Trend graph of percent closure differences between phenotypes. Significant differences were observed at Day 3 ($p=0.015$), 7 ($p=<0.0001$) and 12 ($p=0.0104$); ($p < 0.05$, Mann-Whitney t-test). (C) – Analysis of wound closure percentage relative to Day 0. Calculation performed as [measured wound area] / [original wound area]x100.

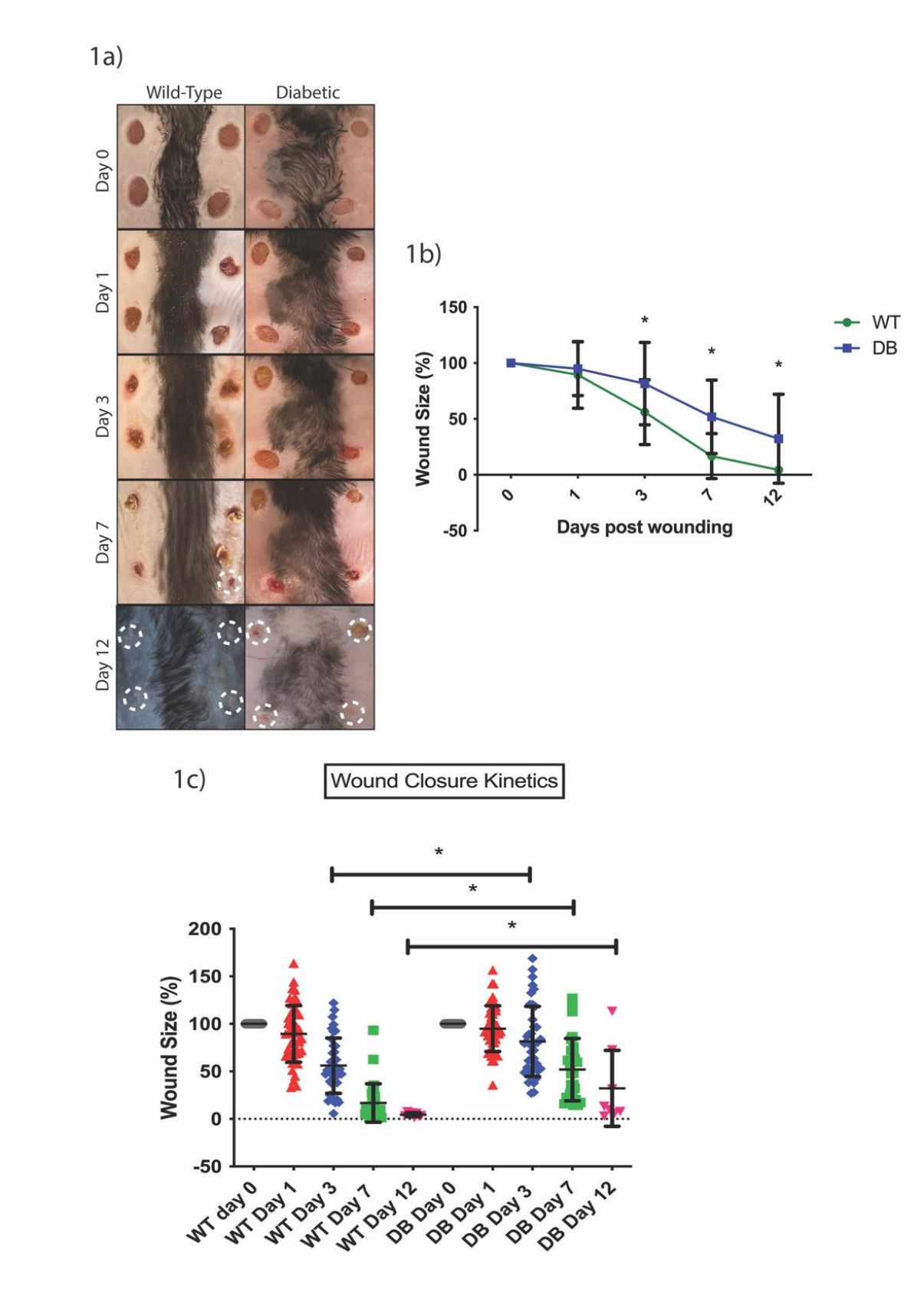

1a)
Wild-Type
Diabetic
Day 0
Day 1
Day 3
Day 7
Day 12
1b)
Wound Size (%)
150
100
50
0
-50
0
1
3
7
12
Days post wounding
*
*
*
WT
DB
1c)
Wound Closure Kinetics
Wound Size (%)
200
150
100
50
0
-50
*
*
*
WT day 0
WT Day 1
WT Day 3
WT Day 7
WT Day 12
DB Day 0
DB Day 1
DB Day 3
DB Day 7
DB Day 12

Figure 2 – Masson's Trichrome Stain showing a failure of re-epithelialization at Day 7 in diabetic animals, with a clear break still present in epithelium. Arrows denoting wound margins.

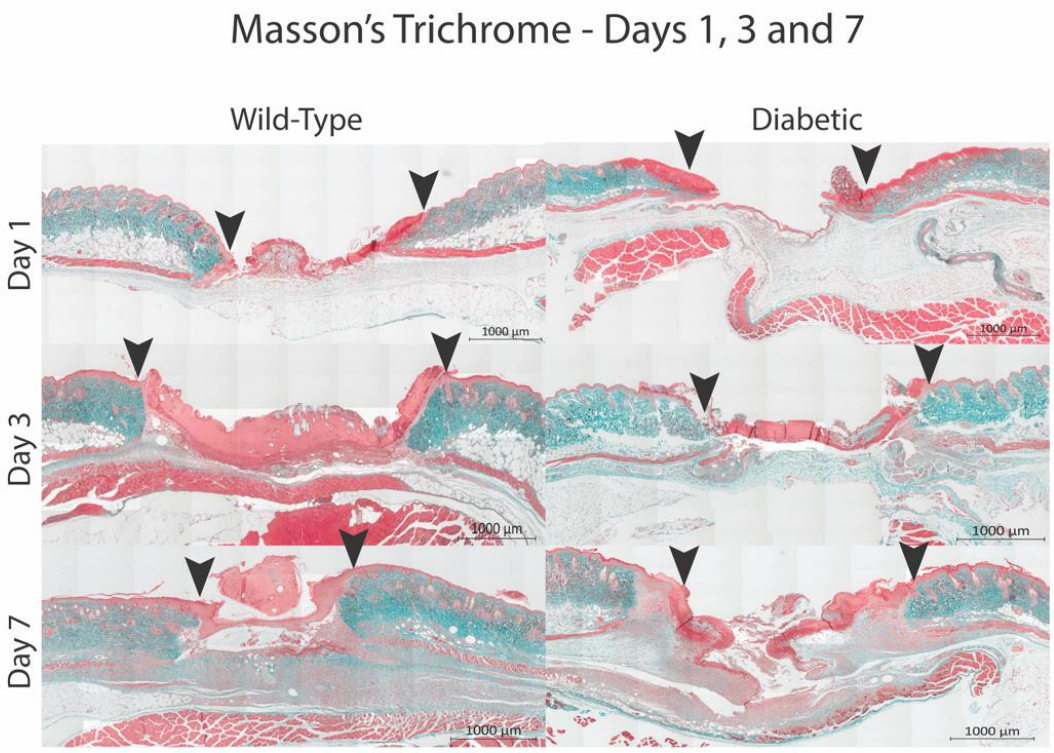

Masson's Trichrome - Days 1, 3 and 7
Wild-Type
Diabetic
Day 1
Day 3
Day 7
1000 µm

Figure 3 – Picrosirius Red Stain showing no visual differences in the amount of collagen deposition between Days 1 (A) and 3 (B). No striking differences in wound morphology. Arrows denoting wound margins.

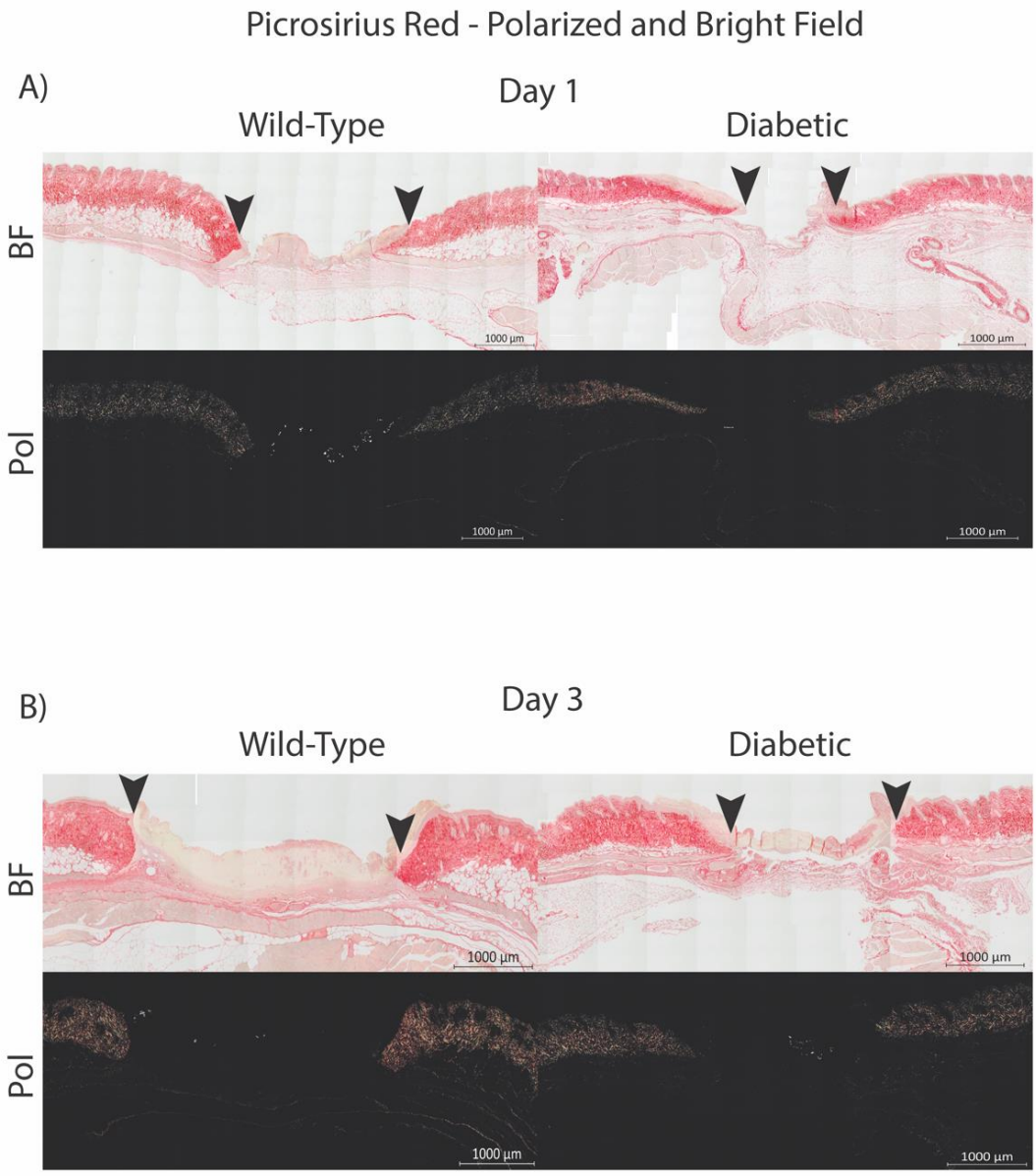
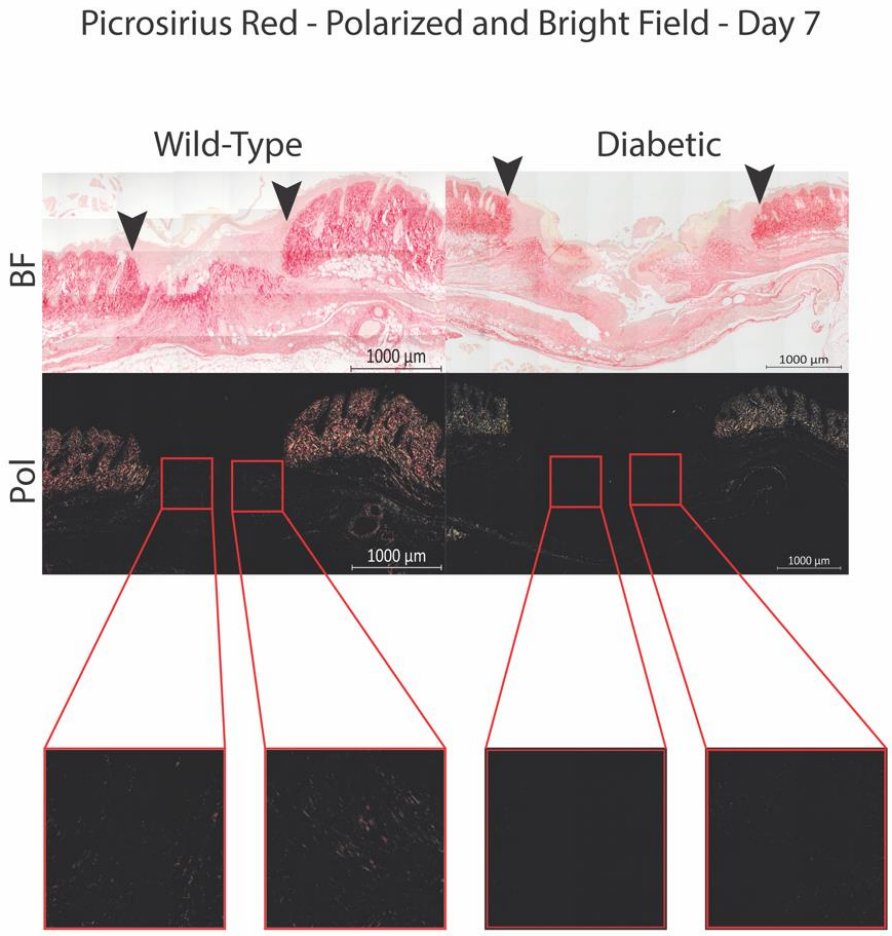

Picrosirius Red - Polarized and Bright Field
A)
Day 1
Wild-Type
Diabetic
BF
Pol
1000 µm
B)
Day 3
Wild-Type
Diabetic
BF
Pol
1000 µm

Figure 4 – Picrosirius Red staining showing more collagen in the wound beds of wild-type animals at Day 7. Re-epithelialization complete at Day 7 in wild-type animals. Arrows denoting wound margins.

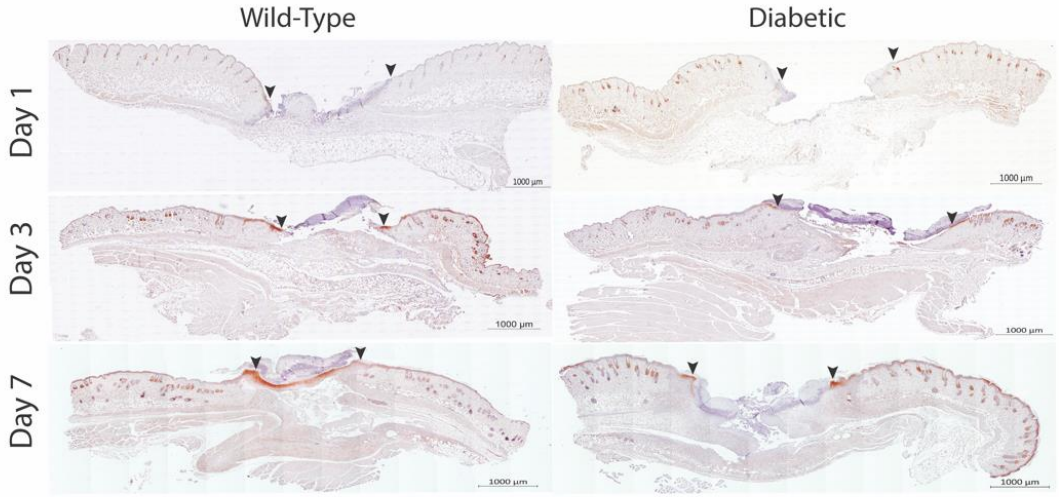

Picrosirius Red - Polarized and Bright Field - Day 7
Wild-Type
Diabetic
BF
Pol
1000 µm
1000 µm
1000 µm
1000 µm

2.3.2 – Wild-type wounds are qualitatively more hypoxic at day 7 post-wounding.

Upon full-thickness dermal injury, it is thought that the wound bed is hypoxic due to the destruction of blood vessels within it. To assess the level of cellular hypoxic load present in wounds, tissues were labeled with Hypoxyprobe^{TM}. No major differences in wound morphology were observed between phenotypes (Figure 5). Wild-type wound beds qualitatively appeared more hypoxic at Day 7, with prominent epithelial staining. Note DAB staining in hair follicles as a positive control. A "wave" of hypoxic cells could be observed at the wound edges, correlating with profound cell infiltration at Day 7. Cells in uninjured dermis were less densely spread, yet still often stained hypoxic.

Figure 5 – General appearance of wild-type and diabetic wounds processed with Hypoxyprobe^{TM}. No striking differences in hypoxic staining localization or wound morphology. Wild-type wound beds qualitatively appear more hypoxic at Day 7, with prominent epithelial staining evident above the wound.

Hypoxyprobe Days 1, 3 and 7



2.3.3 There are no significant differences in the amount and persistence of epithelial hypoxia between phenotypes at Days 3 & 7.

Prior to undertaking the quantitative assessment of the amount of hypoxic cell infiltrate in wounds, we first assessed the persistence of hypoxia in the epithelium of the wounds, moving bi-laterally away from the wound bed. A sharp increase in hypoxic signal was observed at the immediate edges of the wound in both phenotypes, which would then gradually decrease with increasing distance from the wound bed. There were no significant differences in the amount of epithelial hypoxia at either Day 3 or 7 (Figure 6, Figure 7).

Day 3 biological replicates WT: 3, technical replicates WT: 24.

Day 3 biological replicates db/db: 3, technical replicates: 26.

Day 7 biological replicates WT: 5, technical replicates WT: 48.

Day 7 biological replicates db/db: 5, technical replicates db/db: 41.

Figure 6a - Hypoxic signal goes up sharply in the epithelium directly proximal to the wound bed and then drops off. There were no significant differences in the length of persistence of hypoxic signal in the epithelium between phenotypes at Day 3.

6a)

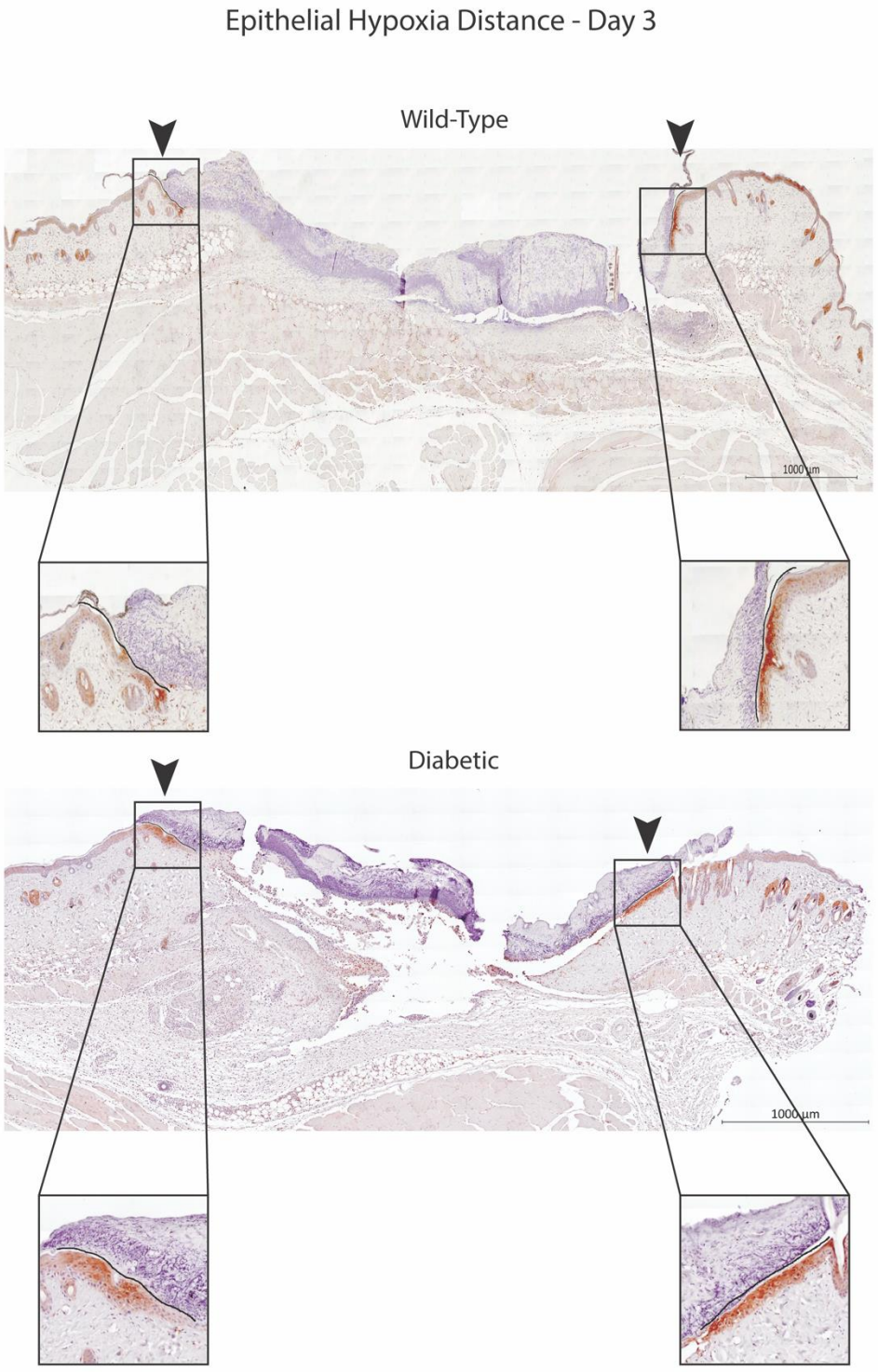

Epithelial Hypoxia Distance - Day 3
Wild-Type
1000 µm
Diabetic
1000 µm

Figure 6b – Hypoxic signal goes up sharply in the epithelium directly proximal to the wound bed and then drops off. There were no significant differences in the length of persistence of epithelial hypoxic signal between phenotypes at Day 7.

Epithelial Hypoxia Distance - Day 7

6b)

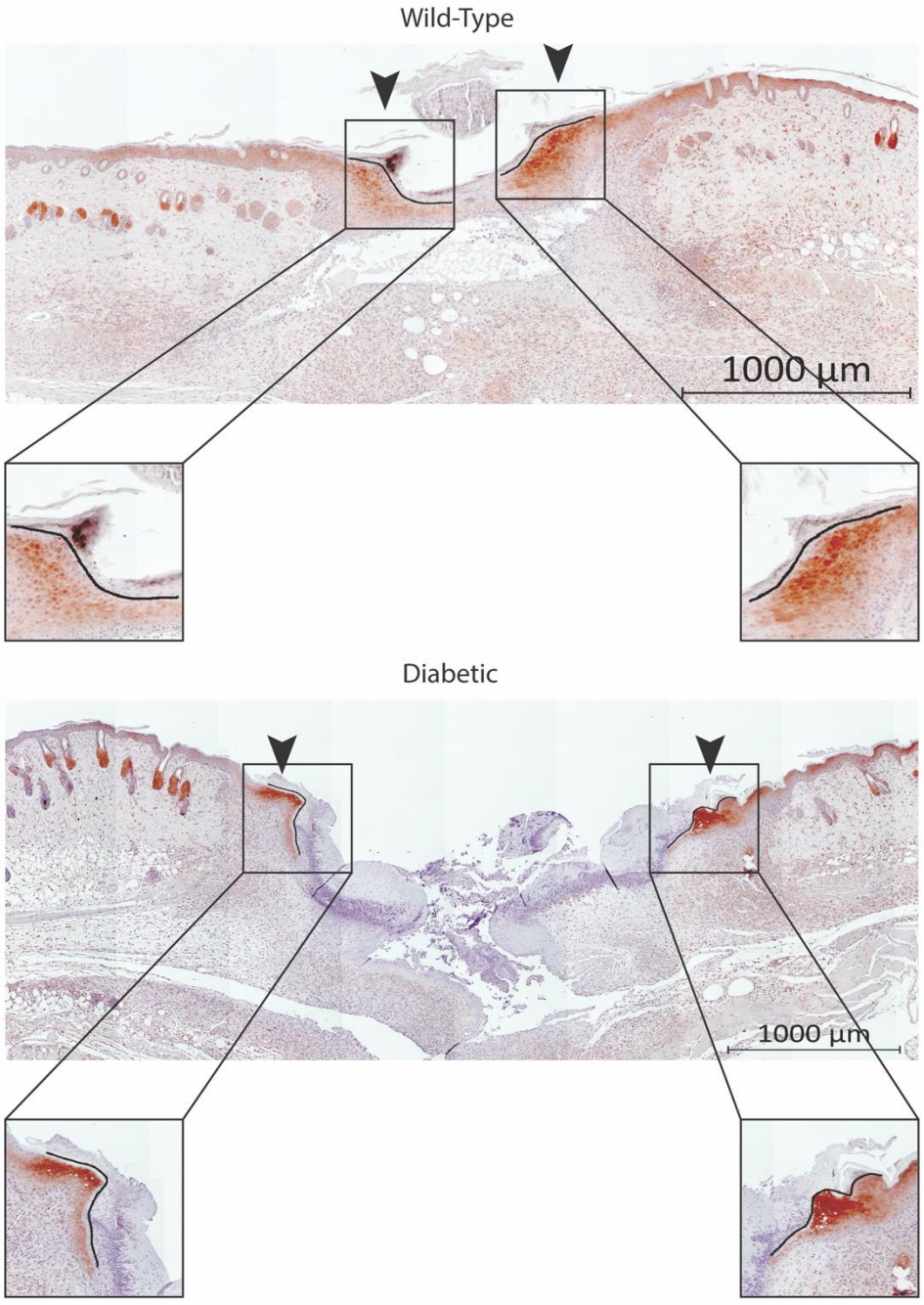
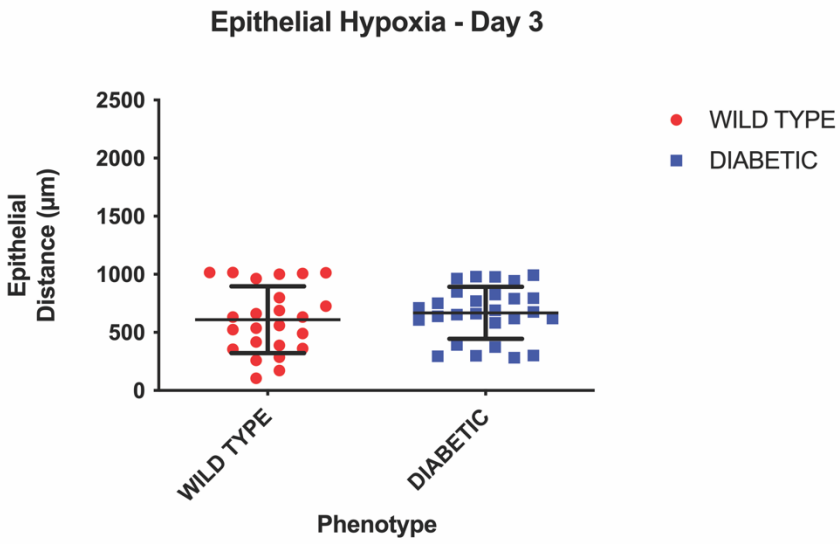
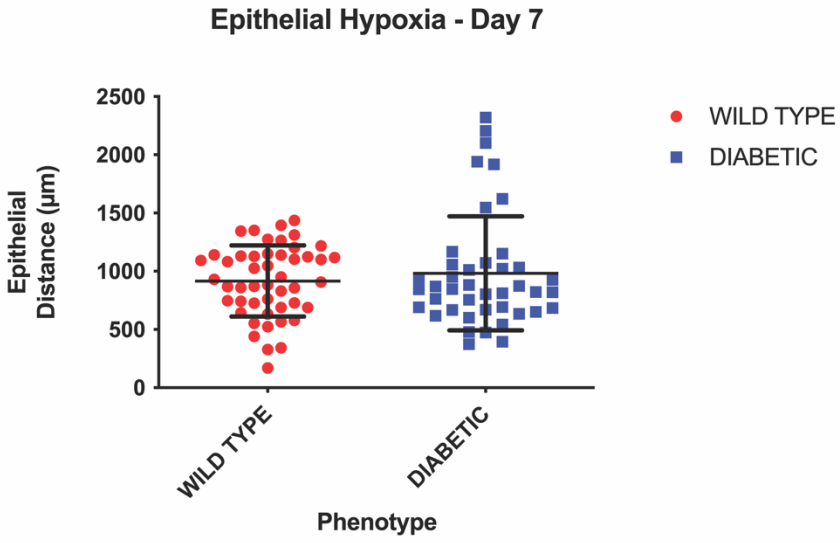


Figure 6c - Epithelial distance (μM) in which hypoxic signal was evident. No significant differences were observed in the amount of hypoxic signal persistence in the epithelium at Day 3 or 7 between phenotypes ($p<0.05$, Mann-Whitney t-test).

6c)


Epithelial Hypoxia - Day 3
Epithelial Distance (µm)
2500
2000
1500
1000
500
0
WILD TYPE
DIABETIC
Phenotype
WILD TYPE
DIABETIC


Epithelial Hypoxia - Day 7
Epithelial Distance (µm)
2500
2000
1500
1000
500
0
WILD TYPE
DIABETIC
Phenotype
WILD TYPE
DIABETIC

2.3.4 Wild-type wounds present with more hypoxic cells in their wound beds and edge at Day 3 and more hypoxic cells in wound beds at Day 7.

We assessed the number of hypoxic cells in the wound beds and edges of wild-type and diabetic animals. At day 3, wild-type mice had significantly more hypoxic cells at one of the edges, and wound beds (Figure 8b). At day 7, there were significantly more hypoxic cells in the wound beds of wild-type animals. The edges were not significantly different. (Figure 9b).

Day 3 biological replicates WT: 3, technical replicates WT: 24.

Day 3 biological replicates db/db: 3, technical replicates db/db: 26.

Day 7 biological replicates WT: 5, technical replicates WT 48.

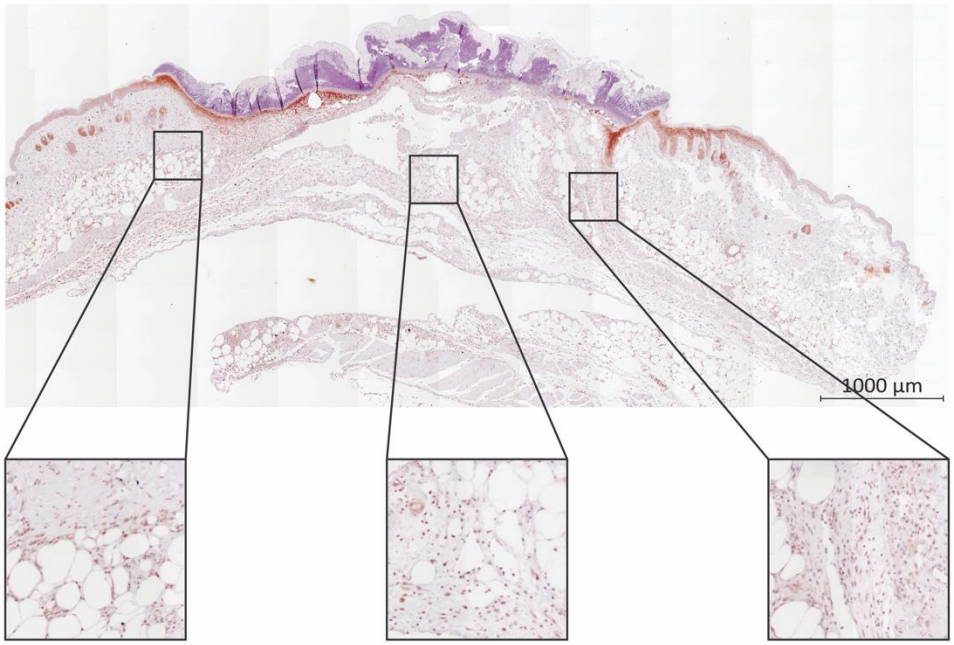
Day 7 biological replicates db/db: 5, technical replicates db/db: 41.

Figure 7a – Wild-type mice exhibited a higher hypoxic cell load at Day 3 at the right edge and wound bed.

Day 3 - Hypoxyprobe

7a)

Wild-Type



Diabetic

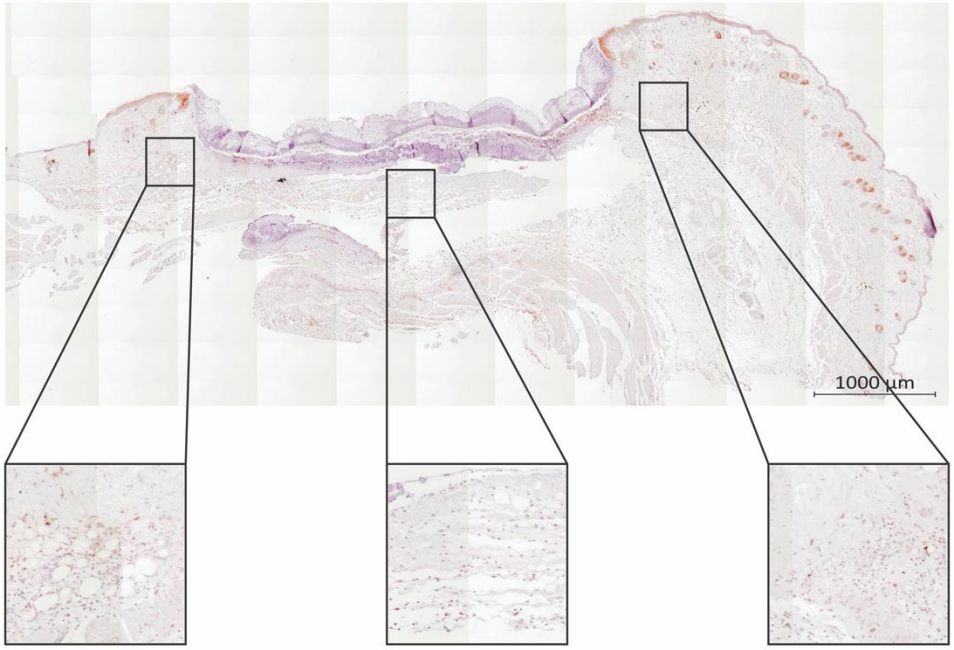
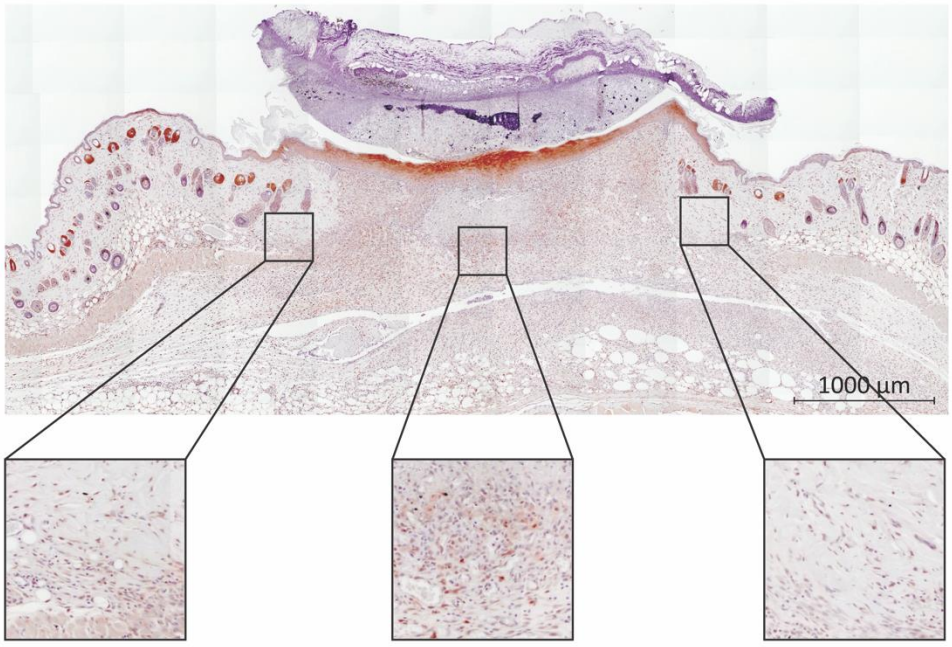


Figure 7b – Wild-type mice exhibited a higher hypoxic cell load at Day 7 in the wound beds. There were no significant differences in hypoxic cell load present at the edges in either phenotype.

Day 7 - Hypoxyprobe

7b)

Wild-Type



Diabetic

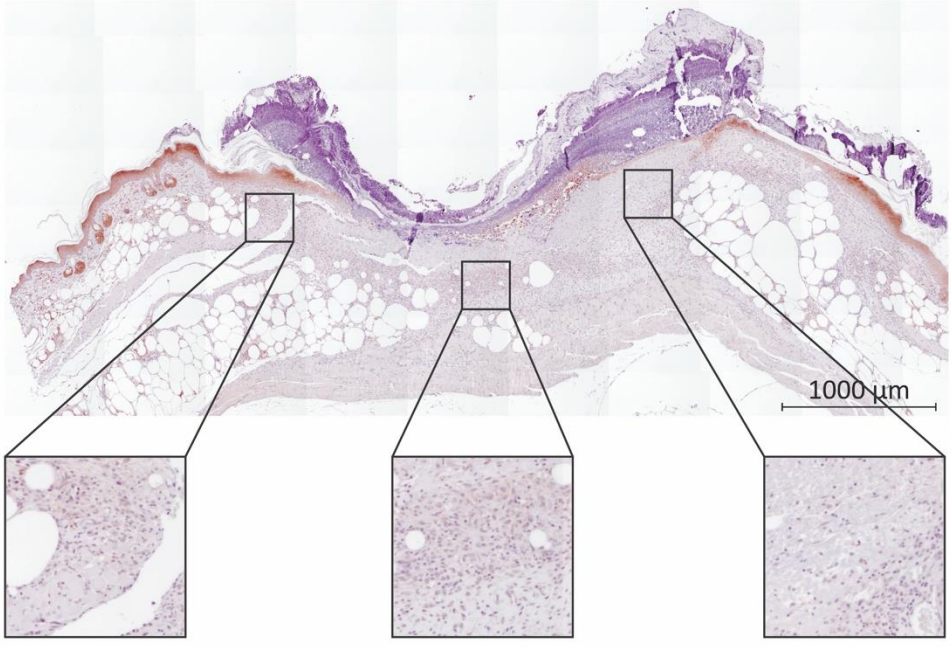
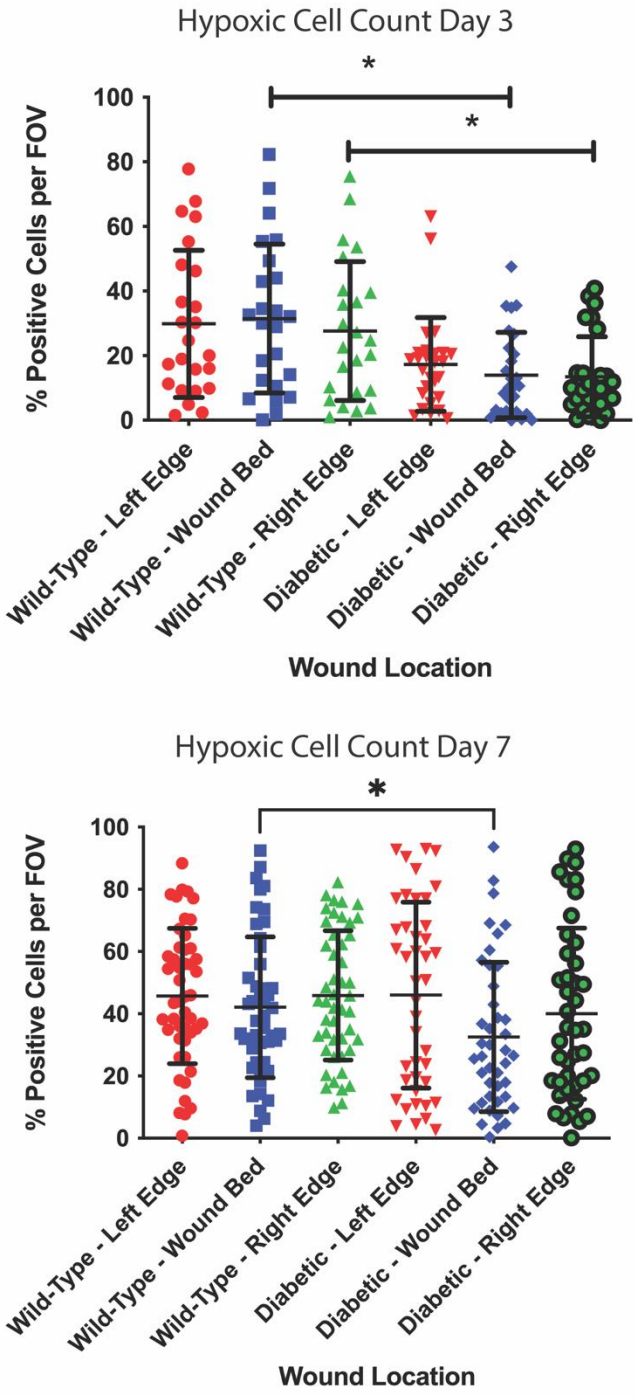


Figure 7c – Different wound regions exhibited distinct profiles in regard to hypoxic cell load at Day 3. There was a significantly higher percentage of hypoxic cells at the wild-type right edges ($p=0.0179$, Mann Whitney t-test) and wound beds ($p=0.0048$, Mann Whitney t-test) per field of view assessed. At day 7 wild-type wounds exhibited a significantly higher percentage of hypoxic cells in their wound beds ($p=0.0238$, Mann Whitney t-test) per field of view assessed.

7c)


Hypoxic Cell Count Day 3
*
*
100
80
60
40
20
0
% Positive Cells per FOV
Wild-Type - Left Edge
Wild-Type - Wound Bed
Wild-Type - Right Edge
Diabetic - Left Edge
Diabetic - Wound Bed
Diabetic - Right Edge
Wound Location
Hypoxic Cell Count Day 7
*
100
80
60
40
20
0
% Positive Cells per FOV
Wild-Type - Left Edge
Wild-Type - Wound Bed
Wild-Type - Right Edge
Diabetic - Left Edge
Diabetic - Wound Bed
Diabetic - Right Edge
Wound Location

2.3.5 There are no significant differences in neutrophil load at Day 3. Diabetic wounds present with a higher neutrophil load in their wound beds at Day 7.

Due to their enhanced ability to cope with hypoxic environments, as well as the widely reported dysfunction in neutrophil clearance in the context of diabetic healing, next we assessed the differences in neutrophil load between phenotypes in the context of a hypoxic environment. At day 3, there were no significant differences in neutrophil load (Figure 10b), however, wild-type wounds did present with a slightly elevated neutrophil count, although this was not statistically significant.

At day 7, a significantly elevated neutrophil count was observed in the wound beds of diabetic mice (Figure 11b). This finding was in line with the majority of reported data in the field, which tends to describe a neutrophil overabundance in diabetic wound beds along with a number of deleterious effects their prolonged presence causes.

Day 3 biological replicates WT: 3, technical replicates WT: 24.

Day 3 biological replicates db/db: 3, technical replicates db/db: 26.

Day 7 biological replicates WT: 5, technical replicates WT: 48.

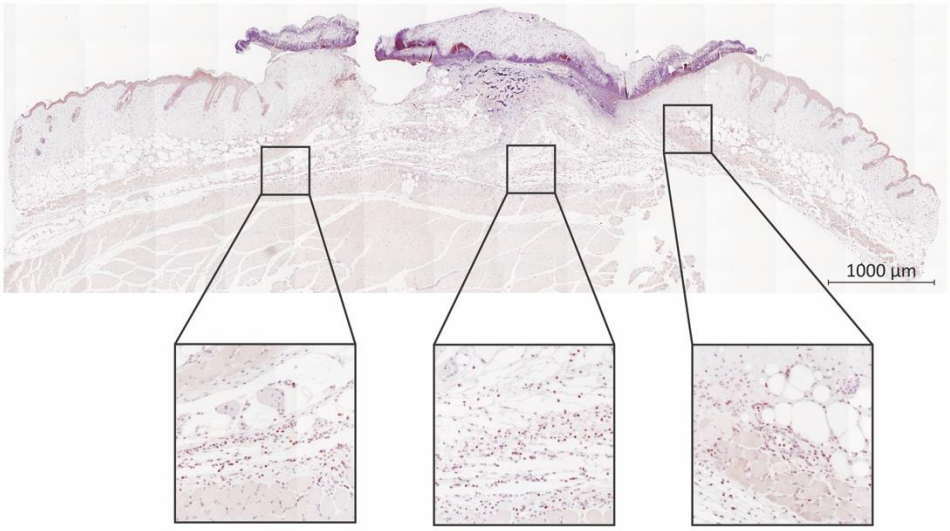
Day 7 biological replicates db/db: 5, technical replicates db/db: 41.

Figure 8a – There were no significant differences between phenotypes in neutrophil load at Day 3.

Neutrophil Elastase Count - Day 3

8a)

Wild-Type



Diabetic

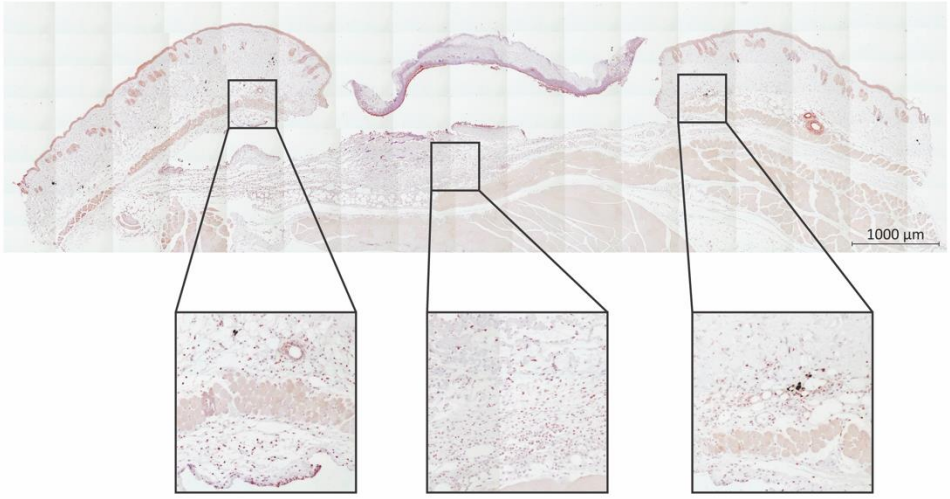


Figure 8b – Diabetic mice exhibited a higher neutrophil load in their wound beds at Day 7.

8b)

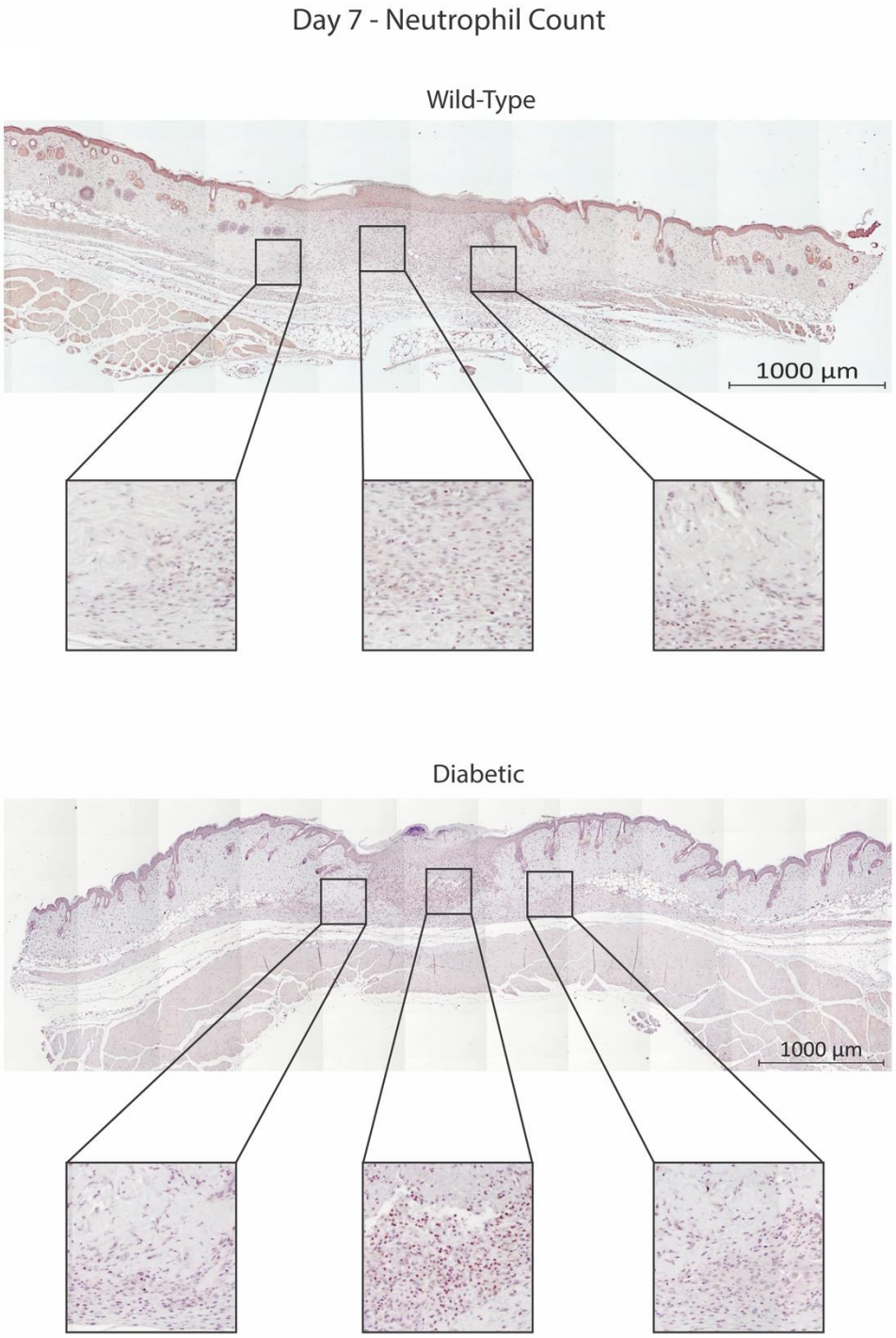
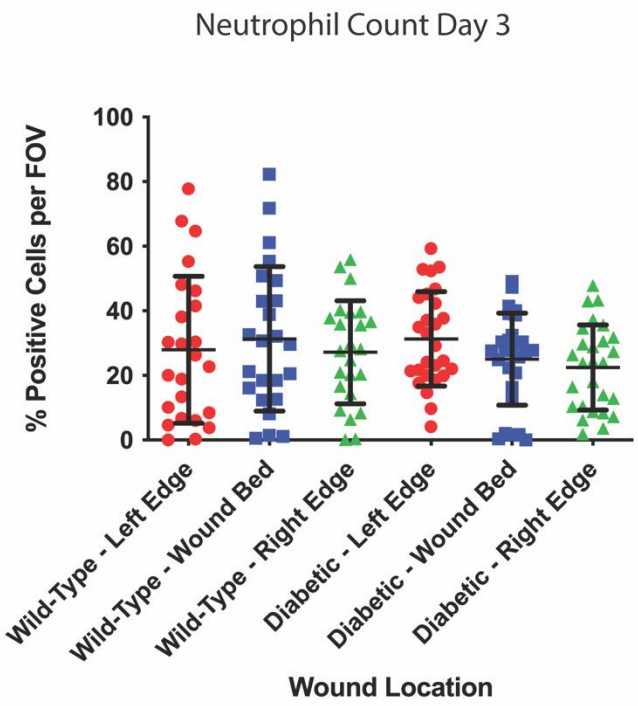
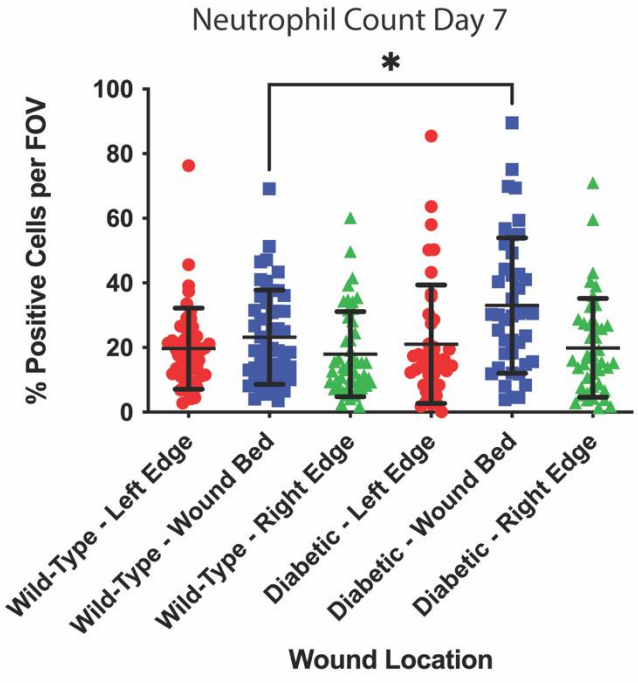


Figure 8c – No significant differences in percentage of neutrophil elastase labelled cells per field of view at Day 3. Wild-type mice exhibited a slightly higher percentage of positive cells at their wound beds but this was not statistically significant ($p<0.05$, Mann-Whitney t-test). At Day 7 Diabetic wounds exhibited a significantly higher percentage of neutrophil elastase labelled cells per field of view assessed at Day 7 ($p=0.0308$, Mann Whitney t-test).

8c)


Neutrophil Count Day 3
% Positive Cells per FOV
100
80
60
40
20
0
Wild-Type - Left Edge
Wild-Type - Wound Bed
Wild-Type - Right Edge
Diabetic - Left Edge
Diabetic - Wound Bed
Diabetic - Right Edge
Wound Location


Neutrophil Count Day 7
*
% Positive Cells per FOV
100
80
60
40
20
0
Wild-Type - Left Edge
Wild-Type - Wound Bed
Wild-Type - Right Edge
Diabetic - Left Edge
Diabetic - Wound Bed
Diabetic - Right Edge
Wound Location

2.3.6 Wild-type mice exhibited significantly more CD31$^+$ cells at Day 3 in their wound beds and right edges. There were no significant differences in amount of CD31$^+$ blood vessels at Day 7 between phenotypes.

As we were interested in the relationship between hypoxia, neutrophil infiltration and new blood vessel formation, the next population of cells we assessed was CD31$^+$. At day 3, there were significantly more CD31$^+$ cells in the wound beds and right edges of wild-type wounds (Figure 12b). There were no significant differences in the amount of CD31$^+$ cells between phenotypes at Day 7 (Figure 13b).

Day 3 biological replicates WT: 3, technical replicates WT: 24.
Day 3 biological replicates db/db: 3, technical replicates db/db: 26.
Day 7 biological replicates WT: 5, technical replicates WT: 48.
Day 7 biological replicates db/db: 5, technical replicates db/db: 41.

Figure 9a – Wild-type mice exhibited a significantly higher number of $CD31^+$ cells in their wound beds and right edge.

Day 3 - CD31

9a)

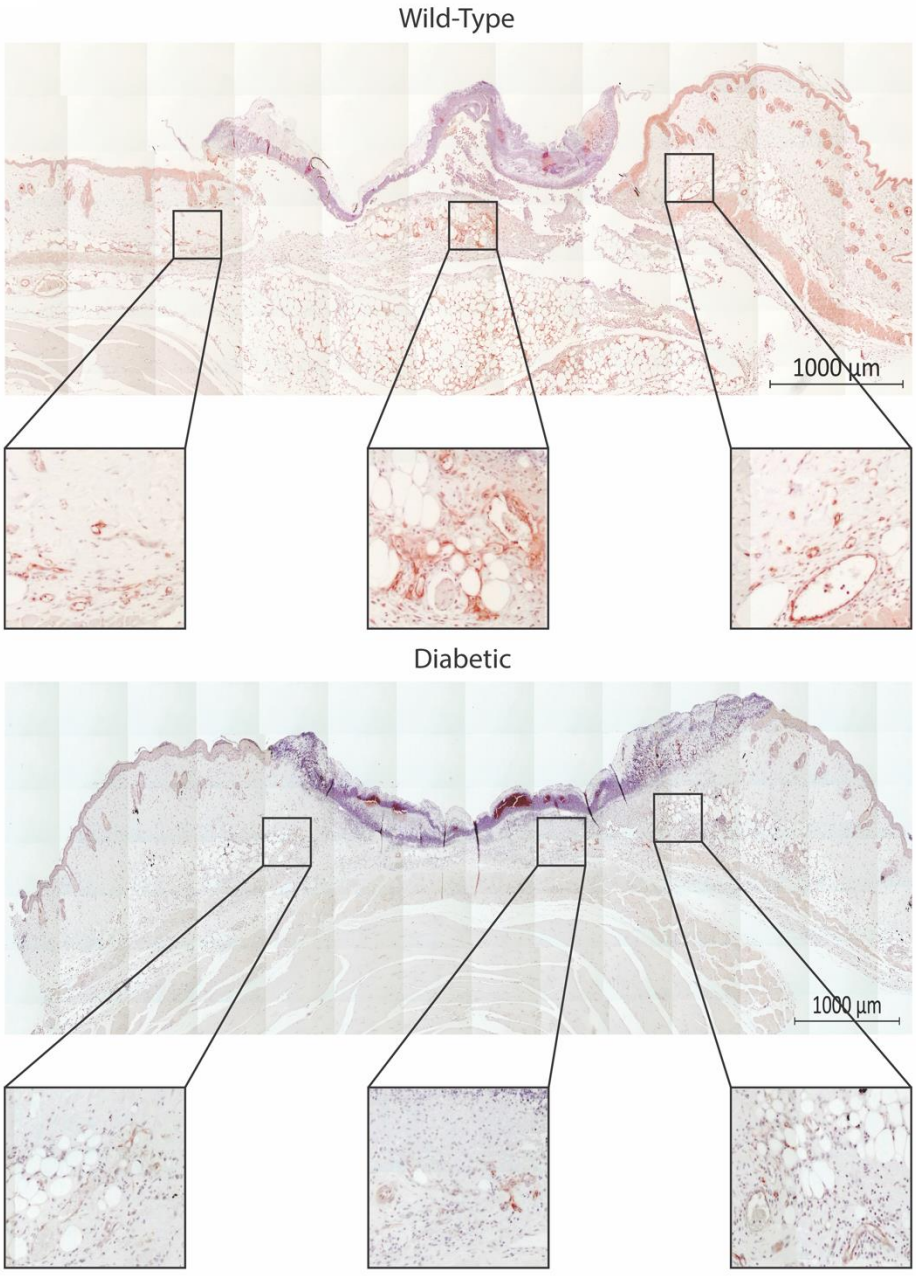


Figure 9b) – There were no significant differences in the number of $CD31^+$ cells at any of the wound locations assessed at Day 7.

Day 7 - CD31

9b)

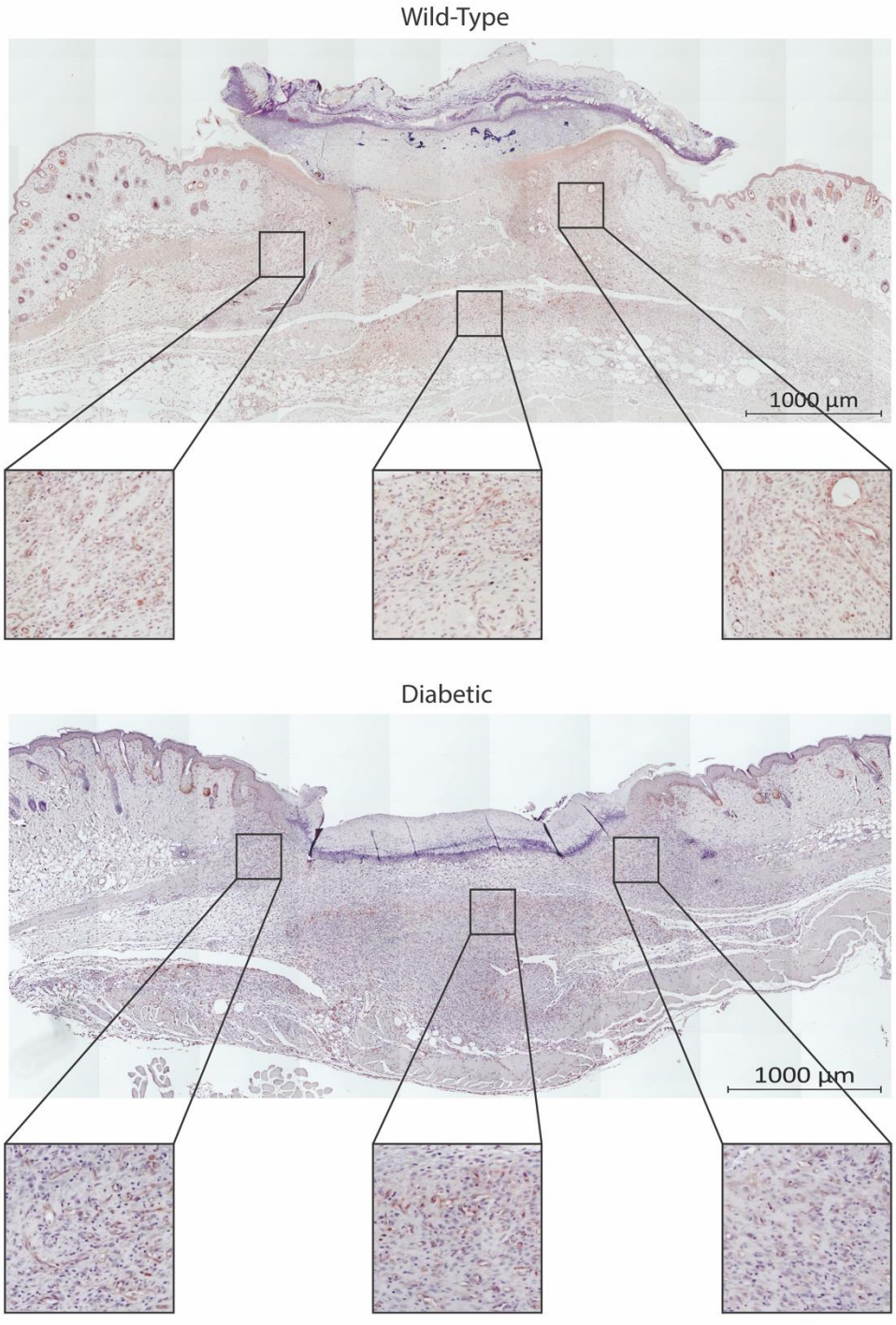
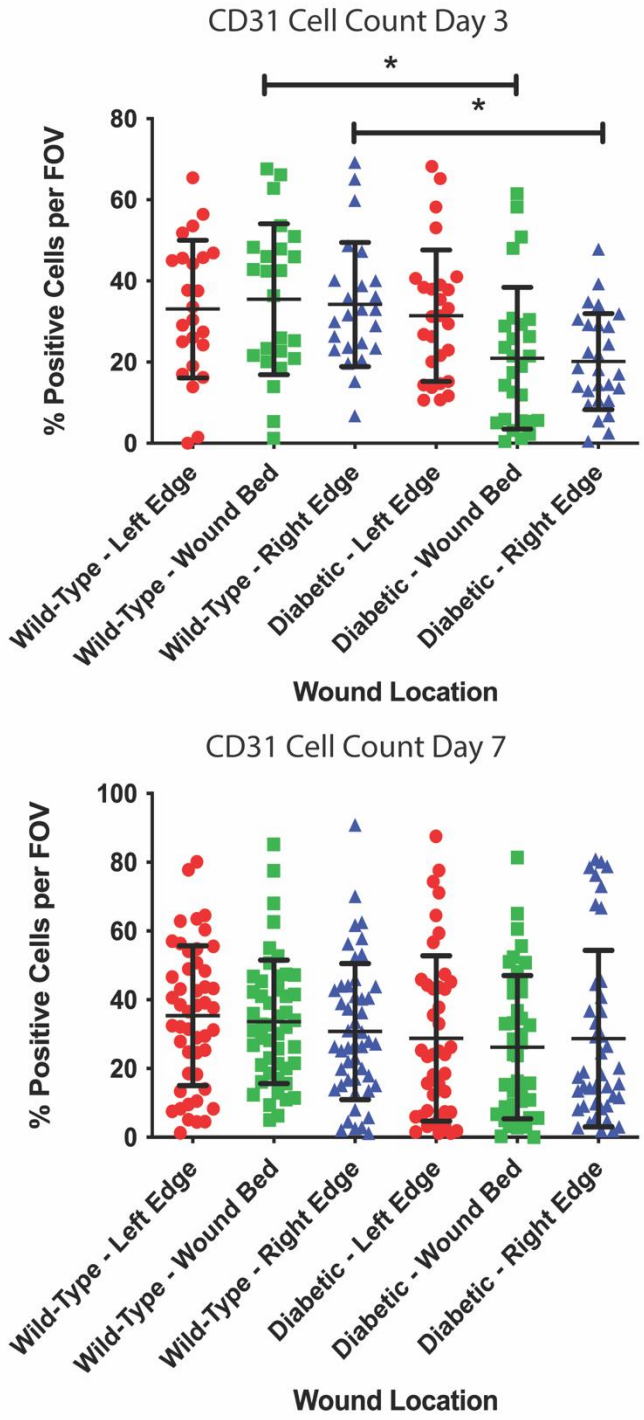


Figure 9c – Wild-type wounds exhibited a significantly higher percentage of $CD31^+$ cells per field of view assessed at Day 3 in their wound beds ($p=0.0107$, Mann Whitney t-test) and right edges ($p=0.0006$, Mann Whitney t-test). At Day 7 there were no significant differences in the percentage of $CD31^+$ cells per field of view assessed ($p<0.05$, Mann Whitney t-test).

9c)


CD31 Cell Count Day 3
*
*
% Positive Cells per FOV
0
20
40
60
80
Wild-Type - Left Edge
Wild-Type - Wound Bed
Wild-Type - Right Edge
Diabetic - Left Edge
Diabetic - Wound Bed
Diabetic - Right Edge
Wound Location
CD31 Cell Count Day 7
% Positive Cells per FOV
0
20
40
60
80
100
Wild-Type - Left Edge
Wild-Type - Wound Bed
Wild-Type - Right Edge
Diabetic - Left Edge
Diabetic - Wound Bed
Diabetic - Right Edge
Wound Location

2.3.7 Neutrophil elastase staining in both human and porcine tissue.

As neutrophils comprised one of the core focus areas of this project, and one of the central aims was evaluation of the degree of correspondence between humans and mice, the following figures (Figure 10 and 11) show the neutrophil elastase staining pattern in both humans and pigs. Note in porcine tissue there is a decrease in neutrophils between Days 7 and 28.

Figure 10 – Human chronic wounds present with an abundance of neutrophils at their wound edges and underneath the eschar.

10)

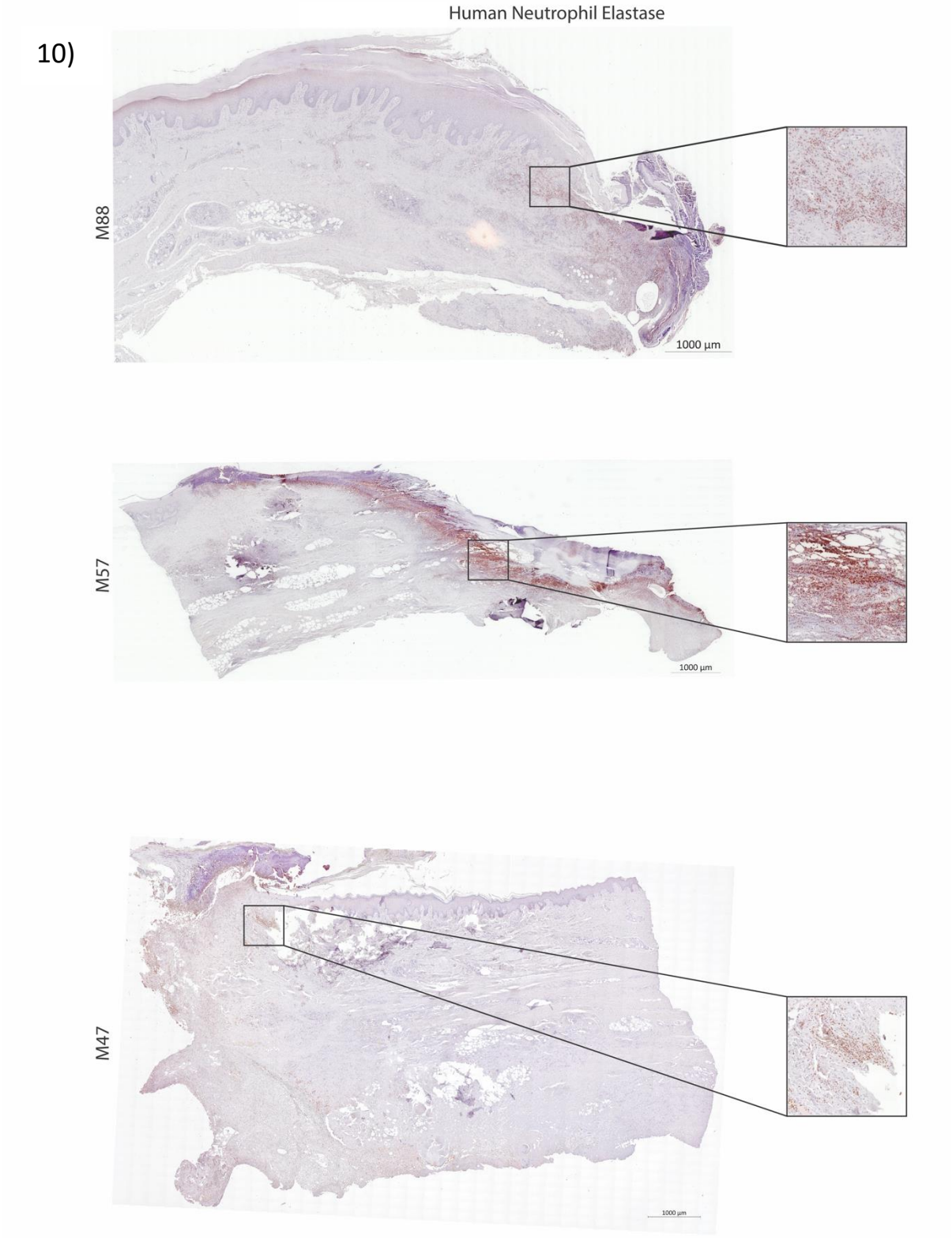
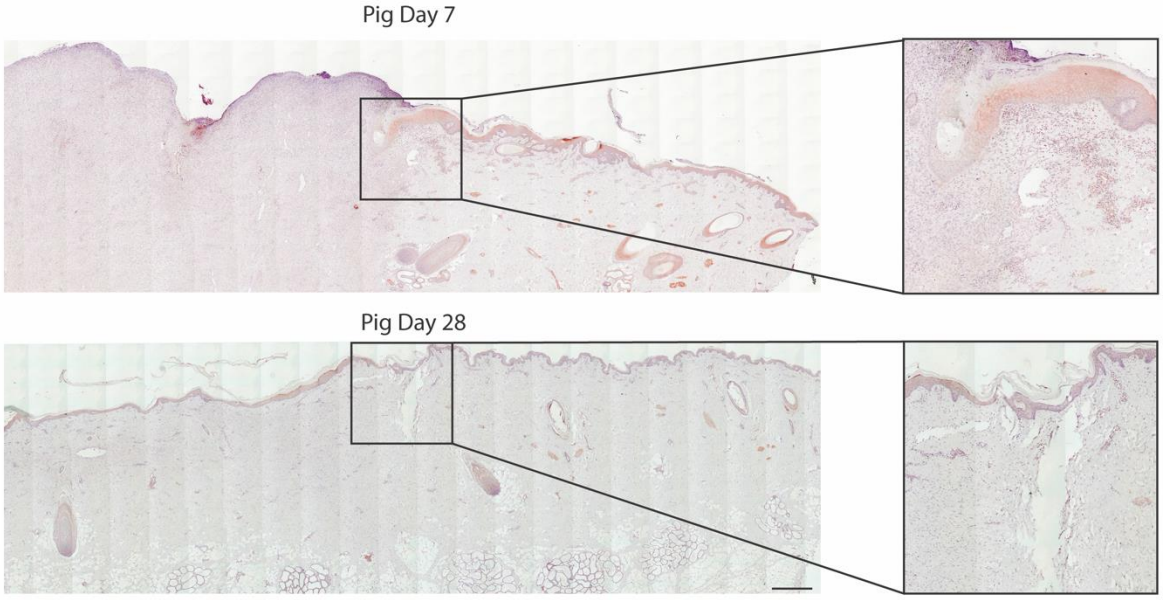

Human Neutrophil Elastase
M88
1000 µm
M57
1000 µm
M47
1000 µm

Figure 11 – Porcine wounds present with an abundance of neutrophils at their wound edges at Day 7. There is a qualitative decrease in neutrophil number evident at Day 28.

11)

Neutrophil Elastase in Porcine Wounds Day 7 & 28



2.4 Discussion

Following full thickness injury to skin, hypoxia increases in the damaged tissue which initiates a cascade of inflammatory and angiogenic cellular processes to facilitate tissue repair. Neutrophils which are initially recruited and are present in hypoxic conditions have been shown in tissues such as the intestine to induce hypoxic signaling at the transcriptional level in epithelial cells [80]. Furthermore, activated neutrophils at sites of inflammation can further increase tissue hypoxia due to their high oxygen consumption, a process termed inflammatory hypoxia [86]. It was shown in 1991 by Rosalind Butterworth that neutrophils persist in the healing dermis of human skin wounds even after re-epithelialization is complete, but the underlying reason is not known. Associated with and known to cause hypoxia, studying neutrophils and their relationship to oxygen levels in humans is problematic due to ethical considerations and the inherent variability between cellular and tissue level hypoxia. The aim of this study was to investigate on the cellular level the relationship between hypoxia, neutrophil persistence and re-vascularization using a murine diabetic model of delayed skin wound healing.

We first focused on quantification of hypoxic signal in cells at the wound edge as well as in the developing granulation tissue. To our knowledge, this is one of the first studies utilizing Hypoxyprobe™ to assess hypoxia in a full-thickness excisional murine skin healing. Previous studies have used Hypoxyprobe™ in analysis of endometrial shedding, where they demonstrated a temporal gradient of hypoxia arising as the endometrial vessels regressed and the lining was shed [87]. In the context of tissue repair, work by Kang et al. demonstrated that following a gastrocnemius or hindpaw incision at days 1, 2, 4 and 10, a temporal hypoxic gradient develops. Staining was most intense at days 1 and 2, and by day 4 staining had become

diffuse and little staining was reported by day 10 [88]. In our study, we showed that wounds in both WT and db/db mice showed Hypoxyprobe™ signal localized strongly in the epithelial edges directly proximal to the wound bed. Hair follicles in both phenotypes uniformly stained positive, providing a positive control to our assay as the cells of hair follicles are known to be hypoxic [89]. Tracing the signal from the wound edge laterally using Image J until the first obvious drop in signal intensity showed no significant differences in epithelial hypoxia between phenotypes at either day 3 or 7. In a subset of wounds irrespective of phenotype, a hypoxic "trail" was observed at the end of the epithelium, extending into the wound bed under the eschar (See Figure 7a). The presence of hypoxia in the epithelium is not unexpected as it is avascular and damage to the skin removes the vascular supply [90]. No significant differences in epithelial hypoxia were observed between phenotypes at either day 3 or 7, with both phenotypes exhibiting an increase in the number of hypoxic epithelial cells between days 3 and 7. This effect was larger in diabetic mice, which had a lower number of hypoxic cells at day 3. Interestingly, the significantly increased hypoxic epithelium at day 3 and 7 observed in the wild-type mice, correlated with significantly faster wound closure kinetics at days 3, 7 and 12 (Figure 1), increased re-epithelialization at day 7 (Figure 2) and increased wound bed collagen deposition at day 7 (Figure 4). We hypothesize the defects in these healing parameters in the db/db mouse to be a possible manifestation of insufficient hypoxia adaptation in the db/db mouse, more specifically an insufficient recruitment of early reparative cell populations because of a failure to respond and adapt to hypoxia.

Of great significance for wound healing and impaired healing models, we demonstrate for the first time that wild-type mice have significantly higher total number of hypoxic cells at day 3 at the wound edges and wound bed as well as in the day 7 wound bed compared to db/db mice.

As previous research highlighted a reduction in cell infiltration in db/db mice [91], we confirmed that the increase in hypoxic cells in WT mice wounds in comparison to db/db wounds was not due to differences in total cell number (Appendix 4.4-4.5). Although wounds in wild-type mice trended towards a higher total average cell infiltrate compared to db/db wounds, it was not significant. Our data suggests that WT wounds exhibit a greater degree of adaptation and responsiveness to hypoxia, compared to db/db mice, casting doubt on the db/db mouse model representing delayed healing due to increased hypoxia. This data is consistent with previous work in the field showing less micron RNA-210 (miR-210) expression in db/db wounds as compared to wild-type. miRNAs are a group of tightly conserved non-coding RNAs, and their dysregulated expression has been shown to have the potential to drastically alter translation of multiple genes in a cell [92]. miR-210 in particular, has been shown to be the highest miRNA upregulated in hypoxic cells, and is also known to upstream of the regulation of a number of genes involved in energy metabolism and tissue repair [93]. Narayanan et al., demonstrated that induction of miR-210 is significantly downregulated in the wounds of diabetic patients and db/db mice, and further demonstrated this effect to be dose dependent due to hyperglycemia. When mice were treated with an miR-210 clone, inflammation was reduced yet granulation tissue formation, proliferation and angiogenesis all improved. As such, they attributed the defects observed in the db/db mouse to miR-210 insufficiency. Further work by Catarina et al., demonstrated a failure of stabilization of a critical hypoxia response regulator transcription factor HIF-1α, and linked this defect to hyperglycemia [94]. Our data is in agreement with current evidence in the field, namely that db/db animals upon injury to the skin exhibit a delay in adaptation to hypoxia resulting in delayed wound healing outcomes.

In both phenotypes, at day 3, most cells infiltrating the granulation tissue at the edges were positive for Hypoxyprobe™, suggesting that these wound regions were likely perceived as

more hypoxic by the cells in comparison to the wound bed. This effect became even more pronounced at day 7, as the overall number of infiltrating cells increased significantly in both phenotypes, with most cells at the wound edges and the wound bed being hypoxic. We observed a clear “volcano shape” of hypoxic cells infiltrating from deeper in the tissue under both sides of the wound margins (Appendix 4.6). It is noteworthy that although there were far less cells in uninjured tissue, many of them still stained positive for Hypoxyprobe^{TM} especially deeper in the reticular dermis, hypodermis and areas around the panniculus carnosus muscle. This suggests that damage to the skin in one area may impact on the vascular trees in adjacent regions that would be seen as "intact" or injured skin with hypoxia occurring.

The finding of increased hypoxic sensing in wild-type mice, seemed somewhat counter-intuitive at first, as we initially hypothesized that due to the widely reported angiogenic deficiencies and an exaggerated inflammatory response [95,96], we would observe an increased hypoxic load in the diabetic wounds. Although counter to our initial hypothesis, our observations could plausibly be explained by a more metabolically active and thus energy demanding environment in the wild-type wounds, potentially accounting for better healing outcomes i.e., increased closure kinetics and collagen deposition seen in this population. Our evidence suggests that the sharp, early increase of acute hypoxia may be a positive prognostic sign for normal healing dynamics, as it’s a well-recognized critical regulator of early recruitment of multiple populations of reparative cells [79]. Further research is needed to better understand at what point acute, beneficial hypoxia becomes chronic in skin healing, and what’s perceived as acute versus chronic on a cellular level.

Due to their ability to function in hypoxic microenvironments we next assessed the level of neutrophil infiltrate in wounds at day 3 and day 7, and whether their persistence correlated with the presence of hypoxia in the surrounding tissue and wound bed. Overall, in both phenotypes at both day 3 and day 7, a few neutrophils were observed in the papillary dermis, with most infiltrating neutrophils emerging from below the panniculus carnosus muscle. Day 3 and 7 wounds in both WT and db/db mice exhibited a pronounced staining pattern directly under the base of the eschar, which in a subset of wounds extended the entire length of the wound. There were no significant differences observed in neutrophil numbers at day 3 between phenotypes, however wild-type mice had slightly higher counts in their wound beds and at one of the edges. In general, the middle core of the wound bed exhibited a lower neutrophil load compared to the superficial portion, the wound edges and the deep portion. The finding that neutrophils are invading from the base of the wound as opposed to the edges bears significance in the context of Hartwell's work, which showed that the wound edges and upper dermis immediately adjacent to the wound are quite inert and do not participate in any significant way in the reparative process [97]. This finding has significance for any biomaterial wound treatment therapies, and suggests that any exogenous materials placed in the wound may have greater success if placed deeper in the healing tissue as opposed to superficial placement.

We report a finding of significantly elevated neutrophil numbers in the day 7 wound beds in db/db mice. These findings are consistent with the widely reported increased neutrophil persistence in both murine and human diabetic healing literature [98]. As expected, in contrast to db/db wounds, we observed a reduction in neutrophil numbers in the wild-type mice from day 3 to day 7. This finding is in line with previous studies showing that neutrophil presence and activity peak between days 3-5 of the late inflammatory phase of healing, and subsequently

drop as the wound moves to the proliferative phase [99]. Our data suggests that the mouse does not show a similar neutrophil count pattern as described in humans in wound bed tissue regardless of the timepoint assessed. Our data further suggests a potential deficit in early hypoxia adaptation in the db/db mouse, as hypoxia is a known powerful mediator of early neutrophil recruitment and energy metabolism [100]. We did not observe a significant difference in neutrophil number until day 7 in the db/db, suggesting that the entire wound healing process in the db/db mouse is delayed with significant neutrophil presence manifesting at day 7 by which time it is generally postulated that their numbers should be decreasing. If a defect in hypoxia adaptation exists in the db/db mouse, and hypoxia is a known regulator of neutrophil recruitment and energy metabolism, we postulate that the significantly elevated neutrophil numbers at day 7 in the db/db, are a manifestation of an overall delay in neutrophil recruitment due to dysregulated hypoxia adaptation in the db/db mouse. This data parallels findings in the human, which show that inflammation persists or occurs slower in the wounds of diabetic patients [101].

The previously discussed defect in HIF-1α stability in db/db mice during wound healing described by Catarina et al., bears implications for angiogenesis as well. HIF-1α binds to the hypoxia response element (HRE) in the gene promoter region of vascular endothelial factor (VEGF), which in turn stimulates angiogenesis [79]. Thus, if a defect exists in HIF-1α stability in the diabetic, a potential manifestation of this could be a delayed recruitment of necessary amounts of endothelial progenitor cells. Immediately following any insult resulting in full thickness dermal injury, blood vessels are damaged which initiates a cascade of reparative events, along with a sharp rise in local tissue hypoxia [79]. It is known that hypoxia serves among several other factors to increase recruitment of many different cell populations

including neutrophils, fibroblasts, and endothelial progenitor cells to the site of injury [79]. Previous work has shown that hypoxia is a necessary physiological event for early repair to proceed successfully [79]. As hypoxia arises from local disturbance and damage to the microcirculation, we next quantified vascular density and blood vessels infiltration of the granulation tissue using CD31 as a marker. In general, we did not observe any major differences in CD31 localization between phenotypes at either day 3 or 7. In both phenotypes, extensive vasculature was observed in the panniculus carnosus layer, and the layer just above it that resembled fatty pockets. Most positively stained rounded and elongated cells, presumed to be monocytes or endothelial progenitors, seemed to come from below the panniculus carnosus, much deeper in the tissue, which has been previously identified in humans as an area from which cells are recruited [97]. At day 3, increased endothelial progenitor populations in the wound beds was observed, although endothelial tubes with lumens were also present. At day 7, we observed very dense vasculature with clear lumens at both the immediate edges and the granulation tissue itself, consistent with previous work demonstrating extensive vascularization during healing in the human. Work by DiPietro et al., showed that as adult human skin wounds heal, a period of rapid capillary growth occurs with many more capillaries forming in the wound bad than are normally present in uninjured tissue [102]. This group also showed that as healing progresses, most of these newly formed capillaries regress and the terminal vascular density becomes similar to that of uninjured tissue. Our data was in line with this work, showing a lot more $CD31^+$ vessels in the granulation tissue than in uninjured skin. These vessels also appeared to contour and align parallel to the wound edges.

Although the localization pattern of the stain did not appear dramatically different between phenotypes, we report a significant difference in the amount of $CD31^+$ cells in the wild-type

right edge and wound bed at day 3. Although this timepoint is well before the defined angiogenic peak, this data could be explained when considered together with the evidence obtained from the Hypoxyprobe™ assay; at day 3 we observed more hypoxic cells at both the wild-type wound edge and the right edge. Hypoxia is a powerful initiator of angiogenesis, via the transcription factor HIF-1α [79]. When activated, it binds to the gene promoter region of the VEGF gene, which in turn upregulates VEGF [79]. This factor is a major angiogenic factor which stimulates endothelial progenitor cells to migrate and form capillaries [79]. Thus, it is possible that the higher level of $CD31^+$ cells could be explained by a more elevated level of local cellular hypoxia. This postulation is made stronger by the fact that the significant difference in degree of vascular infiltration at day 3 (Figure 9c), was coupled with a significant difference in hypoxic cell load in the same wound locations at the same timepoint (Figure 7c). No significant differences were observed between phenotypes at day 7. This could suggest that if an angiogenic deficit existed at day 3, the db/db mouse may have been able to compensate or recover angiogenic processes by day 7. Our current data suggests that the db/db impaired healing model does not consistently re-capitulate the angiogenic deficit commonly reported in the diabetic healing literature [95] . Recent work from our lab suggests that it is not a failure of angiogenesis to occur, but rather a premature stabilization of the developing vessels by pericytes and mural cells [103]. Other work in the field has outlined the existence of a vascular "plasticity window", during which newly formed vessels mature and become remodeled [104]. This work further postulates that it is the acquisition of a vascular pericyte coating that marks the end of this plasticity window. We postulate that it is not necessarily the number of new blood vessels in the granulation tissue that constitutes successful angiogenesis, but the amount of these vessels that undergo further remodeling and maturation that accounts for physiologically desirable wound healing outcomes. If the vessels in the db/db mouse become

prematurely wrapped, and are thus unable to undergo any further remodeling, this can plausibly create issues with their future functionality and levels of perfusion. Interestingly, the significance co-relation observed at day 3 between increased hypoxia and increased vascularization, was absent at day 7. Wild-type wounds presented with more hypoxia, but this was not correlated with an increased number of $CD31^+$ cells. This data is suggestive of an earlier window for the beneficial effects of hypoxia in stimulating angiogenesis and optimal tissue repair.

Overall, our data supports the theory of the importance of adaptation to early hypoxia in the context of wound healing previously outlined by other research groups [105]. Hypoxia and its insufficient adaptation by reparative cells can logically be implicated in all three areas of interest in our study – perceived hypoxic load in the wound and adaptation to it by resident cells, inflammation and neutrophil recruitment, and angiogenesis [94][100][106]. We postulate that the data gleaned from the db/db model in this present study, suggests not a failure of wound healing processes, but a delayed manifestation, as all the physiologically required processes for wound healing were observed in the db/db mouse, albeit with a significant delay. We suggest that further work should focus on the temporal induction of HIF-1α in the earliest stages of healing, ideally, in aged mouse models as most chronic wounds occur in this population [107]. The mice used in the present study were approximately 5 months old. According to Jackson Laboratories, mice ranging from 18-24 months correlate with humans ranging from 56-69 years of age. This fact brings forth an inherent limitation of our study, namely the age of the mice utilized being 5 months, thus likely limiting the translation of our present data to delayed wound healing in the younger human. Taken altogether, in our view future chronic wound healing studies should aim to utilize aged db/db mice at least 1-1.5 years

old, within that cohort we suggest an insulin treatment group in order to further elucidate the effect of hyperglycemia on cellular hypoxic adaptation, HIF-1α stability, and its subsequent activation in various reparative cell populations.

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Chapter 3

General Discussion

3.1 Summary

In this thesis, we hypothesized that neutrophil persistence would result in significantly increased oxygen consumption in the wound bed of the db/db mouse, compared to control. This increased stress would manifest as delayed resolution of hypoxia, delayed wound closure, enhanced neutrophil persistence, and reduced formation of diabetic neo-vasculature.

Next, we present a summary of our findings in the context of our aims.

Aim 1: Investigate the relationship between persistence of neutrophils and delayed hypoxia resolution in the context of impaired diabetic healing.

Objective: Confirm that delayed hypoxia resolution correlates with increased persistence of neutrophils in the diabetic wound bed.

We were not able to establish a relationship between delayed hypoxia resolution and increased persistence of neutrophils in the wound bed in the db/db mouse. Although we observed a significantly increased number of neutrophils in the db/db wounds beds at day 7 (Figure 8c), this was not associated with increased hypoxic load (Figure 7c). In fact, hypoxia was significantly increased in the wild-type at both timepoints assessed day 3 and 7. At day 3, there were more hypoxic cells in the wound beds and one of the edges of wild type mice and at day 7 we observed a significantly elevated hypoxic load in the wild type wound beds only (Figure 7c).

Aim 2: Investigate the relationship between hypoxia resolution and the rate of re-vascularization of tissues.

Objective: Confirm that as the wound re-establishes its vascular network, the tissue becomes less hypoxic.

We were not able to demonstrate a temporal decrease in the gradient of hypoxia as the tissue became re-vascularized. Although we observed a significant increase in the number of $CD31^+$ cells in the wild type at day 3 (Figure 9c), this was not accompanied by an overall decrease in hypoxic load (Figure 7c). There were no significant differences in re-vascularization at day 7 (Figure 9c), and wild-types again presented with a higher hypoxic load at day 7 in the wound beds.

Aim 3: Validate the mouse model as one re-capitulating the major defects seen in impaired human wound healing.

Objective: Confirm that the mouse exhibits appropriately comparable wound healing defects to the human.

We were able to validate the db/db model as one of impaired wound healing across a number of known skin healing impairments in the human. Db/db mice presented with a significant delay in closure kinetics at days 3, 7 and 12 (Figure 1), exhibited delayed re-epithelialization at day 7 (Figure 2), and exhibited reduced collagen deposition at day 7 (Figure 4). Additionally, db/db mice presented with a significantly elevated neutrophil load at day 7 (Figure 8c), a finding often reported in impaired human healing literature [108]. Lastly, db/db mice exhibited significantly less re-vascularization at day 3 (Figure 9c), a phenomenon well characterized in impaired human healing literature [109].

3.2 Contributions to the Literature

3.2.1 Oxygen and the Healing Wound

Oxygen is known to be a pre-requisite for proper wound healing [83]. Reparative processes such as decontamination/debridement, cell proliferation, collagen synthesis and angiogenesis all impart a significant increase in metabolic demand on the healing wound tissue [83]. Oxygen is critical for the production of adenosine triphosphate (ATP), which is the biological equivalent of energy [83]. Therefore, sufficient tissue oxygenation is a necessary to meet increased energy demands, facilitating proper cellular function. Due to full-thickness dermal injury, blood vessels are damaged or lost, leading to a loss of tissue perfusion accompanied by a sharp increase in local hypoxia [83]. This rise in early acute hypoxia is responsible for initiating the earliest steps in dermal tissue repair [83]. It does so by boosting reactive oxygen species (ROS) activity, by recruiting platelets and endothelial cells and by stimulating cytokine release from platelets, monocytes and parenchymal cells [83]. ROS stimulate cytokine and chemokine release, the primary effects of which are the recruitment of neutrophils and macrophages and activation of fibroblasts [83].

Although what constitutes the exact length of acute hypoxia is not completely characterized, it is generally recognized that acute hypoxia is a necessary initiator of wound repair, while prolonged, chronic hypoxia has a detrimental effect on nearly all aspects of normal wound repair [83]. In our present study, we focused on quantifying any potential differences in the temporal profile of hypoxic cell load in full-thickness murine wounds between the db/db and wild-type murine phenotype. We hypothesized that the hypoxic load would decrease as the wound progressed to the angiogenic phase, and that correlations would be evident between neo-angiogenesis and neutrophil persistence. The secondary aim of the project was validation

of the db/db murine model in the context of diabetic skin healing, and how closely it recapitulated what is commonly described in the diabetic patient. Evaluation of the db/db model from the standpoint of hypoxic healing, neutrophil persistence and decreased angiogenesis is an important factor given the commonly reported wound healing deficits reported in diabetic humans in the literature[110]. Of significance, we also focused on whether murine models of skin healing replicate the data derived by Rosalind Butterworth, namely the finding of neutrophil persistence in wounds up to 12 weeks old even after closure [82]. After taking serial punch biopsies from three groups of pilonidal sinus wounds, she reported the neutrophil numbers to be similar irrespective of the timepoint evaluated. Although we recognize this data to stand in contrast with the general consensus in the field - that beyond the acute inflammatory phase, neutrophils serve little beneficial functions and their levels generally fall after about day 5 of healing [111], we aimed to interrogate our murine model with the goal of observing whether it would replicate the data reported by Butterworth.

3.2.2 Tools for Quantifying Cellular Hypoxia: Hypoxyprobe™

In 1976, Varghese et al. demonstrated that 2-nitroimidazoles form adducts in hypoxic cells *in vitro* and *in vivo*. They subsequently showed that these adducts are formed with thiol groups in proteins, peptides and amino acids in such a way, that the entire structure of 2-nitroimidazole is retained, thus opening the door on the possibility of labeling and detecting these adducts [112]. It was of significance demonstrated that an oxygen tension of $pO_2 < 10$ mmHg *was required* for adduct formation, meaning cells had to be in hypoxic conditions. This binding was dependent on the presence of redox enzymes in cells, however, pimonidazole binding was not dependent on the presence of any specific redox enzyme. In essence, this compound is what Hypoxyprobe™ is and how it functions when injected into an

animal. It is a substituted 2-nitroimidazole whose chemical name and sole component is pimonidazole hydrochloride. Hypoxyprobe™ relies on pimonidazole hydrochloride as its hypoxia marker. It forms protein adducts only in cells perceiving a $pO_2 < 10$ mmHg. It is these adducts, that are subsequently visualized using a specific monoclonal antibody. Pimonidazole detection has been demonstrated to be a reliable and accurate measure of tissue hypoxia [113,114]. Importantly, the probe has also been shown to be resistant to fixation and can still be detected [114].

Validation of Hypoxyprobe™ has been documented in models of endometrial shedding, where Cousins et al. demonstrated intense immunostaining for hypoxia during the breakdown and shedding of the decidual mass within 24 hours of progesterone withdrawal [114]. This group was able to show a gradient of hypoxia developing temporally as the endometrial vessels regressed and the lining was shed. Validation of this methodology in the context of tissue repair comes from the work by Kang et al [113]. This group harvested tissue from rats for pimonidazole processing, following a gastrocnemius incision or hindpaw incision at days 1, 2, 4 and 10, using the opposite side as a control. They reported temporal changes in the amount of hypoxic labeling, with staining being most intense at days 1 and 2, diffuse staining at day 4 and little staining by day 10. Of significance, both models showed that intensity of staining and gradients can be detected using this method.

In our study, we utilized Hypoxyprobe™ to label and quantify populations of hypoxic cells in a murine full-thickness excisional wound. To our knowledge, this is the first published report of the use of Hypoxyprobe™ in assessing hypoxic cells in full-thickness excisional

murine skin healing. Hypoxyprobe^{TM} was administered intraperitoneally, in a saline solution and animals were sacrificed 90 minutes later, to allow the probe to circulate and bind to targets. Positive controls were obtained in the form of murine kidney (Appendix 4.7).

3.2.3 Hypoxia and Tissue Response During Skin Healing

Our work demonstrated that wild-type mice presented with a significantly elevated number of hypoxic cells at day 3 on the right wound edge as well as in the wound bed itself (Figure 7c), and in the day 7 wound bed alone (Figure 7c), when compared to db/db mice. The differences were greater at day 3 ($p=0.0179/p=0.0048$) versus day 7 ($p=0.0238$), consistent with previous work in the field describing the impaired adaptation to hypoxia in hyperglycemic wounds [115]. Although it is commonly reported that both human and diabetic mouse wounds are more hypoxic when compared to healthy tissues or wild-type animals respectively [83], if a defect exists in the mechanism of cellular hypoxic sensing/adaptation, wounded tissue in diabetics may be hypoxic, but this may not be sensed by the resident cells, thus accounting for the lower hypoxic signal seen in this population. At day 7, we observed a significantly greater percentage of hypoxic cells in the wound beds of wild-type animals however this significant difference was small compared to day 3. We postulate that by day 7, the db/db mice may begin to recover, similar to the wild-types with regard to hypoxic sensing and adaptation. However, due to the delay observed at day 3, their entire wound healing process may already be delayed due to insufficient initial recruitment of reparative cell populations as a result of insufficient early hypoxia recognition and subsequent adaptation. Interestingly, the increased hypoxic load at day 3 and 7 in wild-types, correlated with improved healing outcomes including significantly faster wound closure kinetics at days 3, 7

and 12 (Figure 1), increased re-epithelialization at day 7, as evaluated by Mason's Trichrome stain (Figure 2) and increased wound bed collagen deposition at day 7, as evaluated by Picrosirius Red stain (Figure 4).

We next assessed specifically the degree of epithelial hypoxia via Hypoxyprobe™, by tracing the length of the signal from the edges of the wound, until the first obvious drop in signal intensity. Generally, the epithelium stained strongly at the immediate wound edges of both phenotypes, and the signal gradually diminished laterally away from the wound towards uninjured tissue. Isolated pockets of hypoxic signaling were evident in epithelial areas far from the wound, but these were generally associated with the presence of closely underlying hair follicles, which are known to be hypoxic [89]. We did not see significant differences at either day 3 (Figure 6c) or 7 (Figure 6c) between phenotypes, suggesting that the proposed defect in hypoxia adaptation may not play as prominent of a role in epithelial cells, as both phenotypes exhibited a closely comparable pattern of staining. Our hypoxia related findings with respect to epithelium were inconsistent with previous work in the field, namely the postulation that diabetic wounds are generally more hypoxic [116]. Although this may be true on a tissue perfusion level, this may not necessarily translate to the resident surface cells of the tissue [117]. It must be noted that we don't suggest that either left or right sides show significant differences in cellular infiltration. These regions were assigned the left or right label based solely off of the slide image on the screen for quantification purposes. Work by Brem et al., demonstrated that distinct wound regions have specific histological profiles [118]. As such although we considered combining the data from the edges, we decided to keep it separate so as not to lose any potential differences existing in different wound locations. We do not postulate that the left or right side accounts for the difference, but rather we demonstrate a distinct wound region that exhibited a significant difference.

Our work demonstrates for the first time, an increased number of hypoxic cells in wild-type full-thickness excisional wounds at both timepoints compared to the wounds created in db/db mice. This finding, although counter to what is reported in the field [119,120] is reconciled in the context of well characterized defective diabetic hypoxia adaptation/sensing [115,121,122], a failure of early recruitment of reparative cells, and the delayed healing outcomes we observed in the db/db mouse.

Common causative factors of local wound tissue hypoxia in the diabetic patient include alterations of the vascular bed, atherosclerosis, hypertension, and swelling [83]. Hypoxia plays an important role in many diabetic complications. Hyperglycemia has also been postulated to induce a pseudohypoxia state [123]. This postulation is based on data showing that high glucose concentrations induce a high $NADH^+/NAD^+$ ratio in cells under normoxic conditions [115]. In addition to this effect, studies have shown that hyperglycemia has a strong potential to interfere with cellular responses and adaptation to hypoxia. Adaptive cellular responses to hypoxia are mediated by HIF-1, a heterodimeric transcription factor made up of two subunits[115]. Modulation of HIF-1 activity is critically dependent on the breakdown of the HIF-1α subunit under normoxic conditions [115]. "The molecular basis of its breakdown is the O_2 dependent hydroxylation of one of the two proline residues, in the oxygen dependent degradation domain of HIF-1α by a specific prolyl 4-hydroxylase"[115]. In this state, HIF-1α binds to the von Hippel-Lindau tumor suppressor protein, which acts as an E3 ubiquitin ligase and targets HIF-1α for proteasomal degradation [115]. Under hypoxic conditions, HIF-1α is stabilized against degradation and upregulates a series of genes involved in

angiogenesis, glycolytic energy metabolism, cell proliferation and survival" [115]. Although hypoxia is the main regulator of HIF-1α, other factors involved in diabetic pathogenesis have the potential to indirectly influence cellular hypoxic adaptation [124]. One of these factors is advanced glycation end products (AGEs), involved in many diabetic complications [125]. Work by Catrina et al. 2004, demonstrated that aberrant glucose levels can inhibit hypoxia-induced upregulation of HIF-1α protein levels in human dermal fibroblasts and human dermal microvascular endothelial cells [115]. This effect was also reported to be dose dependent. These cells also showed reduced levels of HIF-1α protein stabilization when compared to those grown in normal glucose concentrations. They were also able to validate their results *in vivo,* by showing that diabetic foot ulcers (DFU) expressed lower levels of HIF-1α, compared with chronic venous ulcers[115]. Overall, this group demonstrated that hyperglycemia interferes with protection from proteasome-mediated degradation of HIF-1α. If HIF-1α is prematurely degraded in conditions of hyperglycemia, this could plausibly interfere with diabetic cellular ability to sense and adapt to hypoxic stimuli. It was interesting that the *in vivo* effects were observed in the DFU, but not in chronic venous ulcers, further strengthening the likelihood of hyperglycemia being the responsible factor. This data suggests an interaction mechanism between two of the most important determinants of the chronic complications commonly seen in diabetes – hyperglycemia and hypoxia [115,119]

A role for microRNAs (miRNAs) in the context of diabetic healing has also been documented with respect to hypoxia[120,126]. miRNAs are a group of tightly conserved non-coding RNAs that are known to have the ability to either enhance mRNA degradation or inhibit post-transcriptional translation [127]. Dysregulated expression of a single miRNA can drastically alter the translation of multiple genes in a cell, with the potential to alter the cell phenotype [127]. In particular, miRNA-210 has been extensively investigated and identified

as a major miRNA induced under hypoxic conditions. Several studies claim a direct interaction between miR-210 expression and hypoxia [127][120,126,128], and moreover several investigative groups report miR-210 as one of the highest upregulated miRNAs in hypoxic cells. miR-210 is also known to be involved in the regulation of expression of a cluster of genes involved in processes highly important for energy metabolism and tissue repair [120]. These processes include angiogenesis, cell proliferation and metabolic adaptation [120]. Work by Narayanan et al., demonstrated that induction of miR-210 is suppressed in the wounds of diabetic patients and in the wounds of db/db mice [120]. In summary, these findings support the theory of an insufficient adaptive response to the presence of hypoxia in diabetic wounds either due to insufficient HIF-1α protein stabilization, aberrant miR-210 expression or potentially undiscovered factors relating to hyperglycemia and the presence of AGEs. Narayanan et al., partially attributed a range of poor wound healing outcomes such as diminished proliferation, angiogenesis, migration and increased inflammation to miR-210 insufficiency, as a localized treatment with an miR-210 clone reduced inflammation, improved granulation, proliferation and angiogenesis in db/db wounds [120].

In conclusion, the current evidence in the field with regard to hypoxic diabetic healing seems to strongly suggest a defect in cellular hypoxic adaptation, along with a variety of resulting undesirable outcomes in skin healing[120–122]. It is interesting that diabetic wounds are generally postulated to be more hypoxic [119], yet if a defect exists in how the reparative cells sense and respond to hypoxia, it is entirely plausible to suggest a cyclical mechanism in which expression of HIF-1α and miR-210 is dysregulated in the context of AGE accumulation, leading to an overall delay in healing, as one of the most important early mediators in healing is not activating in a timely manner, thus temporally delaying the entire wound healing process.

3.2.4 Hypoxia and Inflammation

Our evidence showed no significant differences in neutrophil infiltrate at day 3 (Figure 8c) and a significantly elevated number of neutrophils at day 7 in the wound beds of db/db animals (Figure 8c). Although not statistically significant, wild-type animals had slightly higher counts in their wound beds and edges. This finding is consistent with previous work in the field describing a general increased inflammatory cell load in the diabetic healing context [101]. However, we postulate that this observation is a delay in early neutrophil recruitment, resulting in the need for an increased neutrophil count at day 7 in the wound bed in db/db mice to facilitate wound debridement. It is known that approximately 95% of myeloid cells are recruited to sites of inflammation, as opposed to residing there [122]. Next, we discuss a range of evidence that could potentially account for delayed neutrophil infiltration, or alternatively a fault in neutrophil clearance by macrophages.

Work by Sawaya et al., focused on the transcription factor FOXM1 specifically in the context of decreased inflammatory cell recruitment [129]. This transcription factor was identified as an important mediator of immune cell activation, recruitment and survival in normal healing [129]. This group first outlines an inflammatory transcriptional signature present in acute, healthy oral and skin wounds, and further goes to show that this same signature is deficient in DFUs [129]. More specifically, they found that levels of FOXM1, responsible for activation and recruitment of inflammatory cells was downregulated in diabetic patients. They confirmed these findings with evidence obtained using the db/db model, which showed downregulated levels of FOXM1 concomitant with delayed wound healing and a decrease in neutrophil and macrophage recruitment to the wound site [129].

Our data is also supported by the findings of Wetzler et al., which showed that wound repair in the db/db mouse was characterized by a prolonged inflammatory response and a prolonged expression of macrophage inflammatory protein-2 and macrophage attractant protein-1 [130]. It was demonstrated that a sustained expression of the inflammatory cytokines IL-1B and TNF-α were present in the db/db mouse as late as day 13 after injury compared to the wild-types which exhibited diminishing levels from day 5 onwards [130]. As neutrophils and macrophages are both potent producers of these cytokines, this group hypothesized that healing in the db/db mouse might be accompanied by increased numbers of neutrophils and macrophages during the late phase of repair. Their subsequent data confirmed this hypothesis, with clearly elevated levels of both neutrophils and macrophages at the late stages of repair [130]. This data further supports our postulation that the inflammatory response in the db/db skin healing model is not due to a greater persistence of neutrophils in the healing wound [131], but rather delayed inflammatory response.

Our data is also consistent with previously reported evidence in the field relating to insufficient hypoxia adaptation [122]. Hypoxia has been demonstrated to have a profound effect on a broad range of myeloid cell properties, including migration, cytokine secretion and survival [122]. It has long been described that neutrophils are highly dependent on anaerobic glycolysis for ATP production, and are thus highly adapted to functioning in a hypoxic environment [122]. It has also been shown that inhibition of glycolysis leads to direct inhibition of ATP production and consequently negatively impacts such inflammatory cell properties as adhesion, extravasation, motility and invasion [122]. Hypoxia is also a key regulator of glycolysis via the transcription factor HIF-1α, thus we hypothesize that the previously discussed insufficiency in HIF-1α described in murine and human diabetic tissues can lead to insufficient ATP levels and an overall deficiency in early neutrophil recruitment,

thus delaying the entire skin healing process in the db/db animal [122]. Interestingly, work by Cramer et al. demonstrated that the loss of HIF-1α in the myeloid lineage results in an almost complete lack of the inflammatory response in the skin [122]. They described a pronounced inflammatory response in wild-type animals, with prominent inflammatory cell infiltration and epidermal hyperproliferation, which were absent in the HIF-1α knockouts. This work lends further credibility to the postulation of insufficient hypoxic adaptation having potential downstream effects on neutrophil recruitment and metabolism, leading to a delay in infiltration with significantly higher numbers at day 7 granulation tissue of db/db animals (Figure 8c).

While transcriptions factors activate specific genes in wound healing, work by Barman & Koh., proposes an alternative mechanism accounting for delayed neutrophil clearance. Their work expands on the concept of macrophage polarization, with an imbalance in pro-inflammatory M1 macrophages and pro-reparative M2 macrophages a possible cause for insufficient neutrophil clearance and delayed resolution of inflammation [132]. Although the work attempting to uncover the microenvironmental stimuli that cause altered macrophage polarization is largely unknown in vivo, it is known that this shift has to occur for physiological healing to proceed normally [132]. It is also known that the process of efferocytosis, or neutrophil clearance is primarily carried out by M2 macrophages [133]. Thus, if the polarization towards an M2 phenotype does not occur in db/db skin wounds, this could account for a decreased rate of neutrophil clearance from the granulation tissue. This provides an alternative explanation for the significantly increased neutrophil load at day 7 in the db/db animal we observed, as being not a result of delayed infiltration but a delayed clearance of these cells from the wound bed. Further work is needed to attempt to elucidate

which mechanism may be primarily responsible for the overall sustain in inflammation seen in the diabetic, or whether it is a combination of both.

In summary our findings of significantly increased neutrophil numbers in the wound beds of db/db animals at day 7 are in agreement with the current consensus of potential factors accounting for a delayed inflammatory phase resolution seen in the db/db animal. The potential mechanisms include - insufficient activity in myeloid cell activating transcriptional factors such as FOXM1 or HIF-1α, which are essential in the earliest stages of inflammatory cell recruitment for neutrophils to generate sufficient energy to extravasate, migrate and carry out their functions or a potential defect in the clearance of these cells once their functions are completed.

3.2.5 Hypoxia and Angiogenesis

We observed a significant decrease in the number of $CD31^+$ cells at day 3 in the db/db mouse at the right edge and wound bed (Figure 9c). Interestingly, this observation correlated with a significantly higher number of hypoxic cells in the same regions in the wild type, suggesting a correlation between hypoxia and endothelial cell recruitment. By day 7, the number of $CD31^+$ cells was similar between phenotypes (Figure 9c), suggesting that either the db/db mouse is able to partially overcome any existing angiogenic defects by day 7, or suggesting the possibility of involvement of other factors allowing it to compensate for defective hypoxia adaptation in the early stages of wound healing. This postulation is lent credibility by the fact that the variability in results between phenotypes at day 7 was quite low, with overall similar distributions of $CD31^+$ cells at both the edges and the wound bed. It is interesting that the higher level of hypoxia at day 7 in the wild type did not correlate with significantly elevated $CD31^+$ cell populations, further suggesting that the role of hypoxia

adaptation may be most prominent in the earliest, acute stages of healing after which other factors may become more important in regulating the wound healing events. Our data stands in partial contrast with what is commonly reported in the field, namely lower levels of VEGF with subsequent lower $CD31^+$ cell populations and impaired angiogenesis in the context of diabetic healing at timepoints at timepoints of day 10 and 18 and day 10 respectively [134]-[135].

We suggest that the db/db model should be considered as a model of delayed rather than impaired healing, as most physiological processes occurring in the wild type, also occur in the diabetic at comparable levels, however, they were all delayed temporally. This model is commonly presented as a model of impaired healing, with researchers commonly citing defects in closure kinetics, angiogenic insufficiency and aberrant inflammation as proof of validation [130,134]. Our study was able to recapitulate most of these effects. The db/db mice exhibited delayed closure kinetics at days 3 7 and 12, they exhibited an angiogenic deficit at day 3, and exhibited significantly increased neutrophil numbers at day 7. The only parameter that this model did not recapitulate was the increased hypoxic load, with wild-type mice exhibiting more hypoxia at both days 3 and 7. This could raise potential issues with any hypoxia targeted treatments in the db/db model as we postulate that it exhibits a defect in hypoxic sensing/adaptation. If defects in hypoxia sensing exist in this model, it may not be an optimal model for testing of hypoxia targeted treatments, as the cells in the tissue may not perceive themselves as hypoxic. If hypoxia targeted treatments are to be tested in these studies, it can logically create problems with translation if the cells in the db/db wound are not adapting/responding to the environment that is to be addressed in the study.

Hypoxia activates the transcription factor HIF-1α, which then binds to the HRE in the gene promoter region of vascular endothelial growth factor (VEGF), which in turn upregulates VEGF [83]. This factor is a major angiogenic factor that stimulates endothelial cells to migrate, proliferate and form new capillaries [83]. If hypoxia is one of the central initiators of angiogenesis in the healing wound, and diabetic cells fail to perceive or adapt to it due to defective HIF-1α activity, this can plausibly result in a delay or failure in recruitment of adequate numbers of angiogenic cells. Work by Thangarajah et al. examined whether hypoxic upregulation of VEGF was impaired in diabetes. First, they demonstrated that normal fibroblasts isolated from healthy patients upregulated VEGF protein levels 2.2-fold in response to hypoxia, while fibroblasts from diabetic patients demonstrated no significant increase in VEGF production [121]. To confirm their findings were due to hyperglycemia, they then grew normal fibroblasts from patients with no vascular disease in low and high glucose conditions for 4 weeks, in order to simulate hyperglycemia. These cells were then placed in hypoxic conditions for 24 hours, and VEGF protein levels were quantified. They found that fibroblasts grown in low glucose upregulated VEGF protein levels by about 3.2-fold, while cells grown in high glucose showed a VEGF expression increase of only 1.2-fold. They paralleled these findings in murine cells by demonstrating a similar pattern of VEGF production in dermal fibroblasts grown in hyperglycemic conditions (4 weeks). When they cultured the same murine cells in an acute high glucose environment (<24 hours), they saw no disparities in VEGF upregulation relative to cells cultured in a low glucose environment, suggesting that chronic, and not acute exposure to hyperglycemia is the responsible factor in compromising VEGF expression [121]. They also demonstrated that db/db mice had significantly lower levels of VEGF expression in comparison to wild types at both 24 and 72 hours after injury. Lastly, this group demonstrated that chronic high glucose exposure

decreases HIF-1α transactivation in response to hypoxia, thus plausibly implicating dysregulation of this transcription factor in all three main areas of interest in our project – hypoxia sensing, inflammation and angiogenesis. Interestingly, this group failed to confirm the findings of impaired HIF-1α stability in a hyperglycemic setting that has been reported by other groups [115,122,128]. They went on to suggest that the hyperglycemia associated defect in HIF-mediated gene transcription results from a defect in HIF transactivation and not as a consequence of impaired HIF-1α stability [121]. They supported this postulation by demonstrating that the interaction between HIF-1α and its coactivator p300 is greatly decreased in hyperglycemic conditions. Thus, it may be pertinent to look not only at HIF-1α activity and stability, but also its known co-activators as well.

Other research has highlighted other potential deficits responsible for angiogenic dysregulation commonly reported in the diabetic condition [103]. Recent work in our lab showed that it is not specifically reduced angiogenesis, but a premature terminal stabilization of the vasculature by pericytes and mural cells, thus preventing these vessels from undergoing further necessary maturation and remodeling processes [103]. The idea of a vascular "plasticity window" is not novel. It has been described by Benjamin et al., as a time during which neo-vasculature matures and undergoes remodeling, and this process was shown to be necessary for successful wound healing progression [104] As an alternative explanation to the HIF-1α mediated insufficient recruitment of endothelial cells and induction of VEGF, we propose that it is not necessarily the number of new blood vessels in the wound that accounts for successful wound healing, but rather the amount of these vessels that undergo remodeling and maturation[103] . Additionally, building on the previously discussed imbalance between pro-inflammatory M1 macrophages and pro-reparative M2 macrophages present during diabetic healing, work by Jetten et al., demonstrated that it is the

M2 macrophages which are chiefly responsible for promoting angiogenesis in a mouse model [136]. It is noteworthy that while M1 macrophages rely on glycolysis for energy, M2 macrophages require oxidative energy pathways [137]. If the diabetic wound is not adapting to hypoxia properly, and failing to initiate processes necessary to resolve it, this could provide an alternative explanation for decreased M2 macrophage populations seen in the diabetic condition, with the accompanying angiogenic defects as these cells are known to be major sources of TGF-B and VEGF both critically important for angiogenesis [136].

Although it is early to suggest that dysregulation of any transcriptional factor, cytokine or cell population is solely responsible for the defects observed in the db/db mouse, namely delayed closure kinetics, insufficient angiogenesesis, reduced collagen deposition and reduced re-epithelialization in the present study, it is noteworthy that the great majority of our data could be explained by an insufficient early adaptation to hypoxic stimuli. As the aim of this project was the broad study of the relationship between hypoxia resolution, inflammation and angiogenesis, the intricate study of how cells become adapted/activated by hypoxia fell beyond its immediate scope. However, we suggest further work to focus closely on what transcription factors are temporally upregulated in hypoxic conditions and in what reparative cell populations. Future work should also aim to elucidate the temporal profile of the vascular plasticity window, as such findings could lead to better temporally targeted therapeutic interventions.

3.2.6 Murine Model and it's Validation

The main aspect to be considered when selecting any particular model is the degree of clinical relevancy or translation. The model used in the present study is the db/db strain, created by a point mutation in the leptin receptor gene [138]. Although a great body of work has been carried out with this and other models, murine models as a whole have shown limited translational value to impaired human wound healing conditions [138]. Furthermore, there is a recognized lack of a current consensus on the most suitable mouse model to be used in attempting to model diabetes associated ulceration [139]. The db/db model has reliably been established as one exhibiting impaired healing in an excisional wounding model and has thus achieved widespread acceptance in wound healing studies[140]. However, there are several important limitations of this model which will be discussed next. One of the most apparent limitations of this model is the greater role of leptin in murine appetite control when compared to humans [140]. This raises the concern that the deficiency of the leptin axis in mice may potentially have a wider range of phenotypic changes than what is clinically observed in human Type 2 diabetes. Experiments in leptin-deficient ob/ob mice have shown that leptin may function is an important modulator of the wound healing cascade, including mitogenic activity and angiogenesis [140]. Another major limitation of this model is the etiology of its obesity and subsequent diabetes development, as analogous forms of this severe type of condition in humans are rare [140]. Additionally, in humans Type 2 diabetes is a polygenic disease, involving many factors, while in mice this condition is modelled monogenically. Interestingly, although this model is popular due to its clear and reproducible wound healing defects, such as delayed contraction and re-epithelialization, this delay in wound contraction may in part be attributable to the striking obesity of this model [140]. This obesity maintains a constant stretch on the skin, plausibly leading to a reduction of available

loose skin required for optimal contraction to occur. As such, this particular wound healing deficit may be independent of the diabetic condition of the mouse, and is thus different in etiology to the delay observed in humans. Lastly, a recent report demonstrated that the impaired wound healing parameters in db/db mice may not be related to the level of hyperglycemia, thus suggesting that the impaired healing seen in the db/db mouse may not be readily translatable to the wound healing defects seen in patients with Type 2 diabetes [140]. As such, although the db/db model may provide value as a model of impaired contraction and delayed excisional wound closure, the extent to which it reflects similar defects in human Type 2 diabetes should remain open to question.

Ulcer development or wound chronicity in diabetics can take decades and is promoted by factors such as years of macro-vascular insufficiency, chronic inflammation and chronic hyperglycemia [121]. Understandably, the mouse lifespan may be too short to allow for accurate recapitulation of these prolonged processes. It is also noteworthy that rodent healing differs significantly from human healing. Mice heal primarily by contraction, whereas humans heal mainly by re-epithelialization [141]. Although researchers often attempt to overcome this by splinting the wound, previous work in our lab showed that the murine model may demonstrate a primarily contractile defect as opposed to one relating to re-epithelialization[84]. Wounds from control db/db animals exhibited less αSMA staining when compared to those in the different scaffold treatment conditions [84]. This finding bears relevance in the context of translation to the human, as it focuses on aspects of wound chronicity in actually modeled in db/db mice. If a fundamental contractile defect exists in the db/db mouse, then any inferences made with regard to slower closure kinetics are confounded by the fact that epithelial cells have to migrate further to close the wound. Considering the findings of the present study, we were able to partially validate the db/db

mouse as a model of hypoxic healing. However, we postulate this model to be defective in a sense that although it exhibits aspects of hypoxic healing, but not more than the wild type. Our preliminary evidence suggests that by day 12 the core of the wild type wound begins to exhibit hypoxia resolution (Appendix 4.8), however with an n=2 for this timepoint, further staining is needed to definitively confirm this. Our findings of increased neutrophil load at day 7 validate this model as one of delayed inflammatory resolution, however, we postulate this to be a manifestation of an overall delay in inflammatory cell recruitment and/or clearance and not of an overall greater recruitment of neutrophils in the diabetic wound. Similarly, this model exhibited an angiogenic deficit at day 3, validating it as a model of insufficient re-vascularization.

A secondary aim of this project was validation of whether neutrophil counts remain unchanged through different stages of healing as described in human data; comparably high levels of neutrophils in healed wounds up to 4-12 weeks old [82]. We report a failure of this model to recapitulate this aspect of human healing. We also performed preliminary experiments is porcine wounded skin tissue to see if it would exhibit similar levels of neutrophil infiltration at days 7 and 28. We observed neutrophils infiltration at the wound edge at day 7, however, their numbers qualitatively were diminished at day 28 (Figure 11). To compare this data to the human chronic wound, we carried out neutrophil elastase staining in chronic wounds of patients ranging from 47-87 years old. We observed a pronounced pattern of neutrophil infiltration at the edges of human chronic wounds, as well as immediately under the eschar (Figure 10).

It must be considered that pathologies can take decades to develop in the human, while in the mouse, researchers are trying to model these processes in a span of 0-1.5 years. It is entirely logical to presume the possibility of insufficient time for diabetes related pathologies to manifest in comparable levels to the human after such a short time. It must also be noted that after such a long period of time of mouse focused research, it is possible that the db/db model has undergone epigenetic changes, potentially confounding any data coming from work with this model.

3.2.7 Future Directions

Due to the fact that altered hypoxia adaptation could plausibly be implicated in every area of interest in our project, we believe that future work should further focus on transcription factors known to be upregulated in hypoxia and their target genes. Aberrant hypoxic signaling in the context of hyperglycemia, persistent inflammation and vascular deficiency are all well described cornerstones underpinning the commonly reported diabetes associated pathologies [115,121,122]. Although all these pathological factors represent promising therapeutic targets, if sufficient understanding is not gained in how cells temporally perceive and adapt to hypoxia in the human, any model tested treatments designed to potentially address them are unlikely to progress to the clinical setting.

An additional caveat is that the great majority of chronic wounds are associated with conditions that tend to manifest in older human demographics, including vascular disease, diabetes, and age-related changes in the inflammatory response [110]. According to Jackson Laboratory, mice ranging from 18-24 months correlate with humans ranging from 56-69 years of age [142]. The mice used in the present study were approximately 5 months old. Thus,

an inherent limitation of our study is the age of our mice, as the data obtained from them would likely correlate more closely with a young human, thus potentially limiting the translatory value of our data to impaired wound healing in the younger adult. To closer mimic what is seen in the clinical setting with regard to the DFU, we suggest that future studies should consider using an aged mouse model of at least 1-1.5 years in order to more accurately recapitulate any wound healing processes occurring in the aged human, that could be masked if using a younger, healthier mouse.

Additionally, we propose that future work should focus on HIF-1α and its activation in the earliest stages of wound healing, with the aim of elucidating what cell populations are chiefly responsible for early hypoxic adaptation. Hyperglycemia has also been extensively discussed as a factor inhibiting HIF-1α stability, thus future studies should aim to expand on this finding and attempt to elucidate whether treatment in aged mice with insulin, has the potential to attenuate the negative effects of hyperglycemia resulting aberrant HIF-1α signaling. If translation is the ultimate goal, an ideal study would utilize an aged wild-type and db/db mouse model of at least 1-1.5 years old, within the db/db cohort there should be a treatment group with insulin to attempt to elucidate if the negative impacts of hyperglycemia on hypoxic adaptation could potentially be reversed. Immunohistochemical co-labeling could then be employed to co-stain cell populations of interest for Hypoxyprobe™ and HIF-1α activity, across timepoints corresponding to known wound healing events such as day 1, 3 7 and 21 to further elucidate the temporal activation profile of this transcription factor and attempt to correlate it with wound healing outcomes such as closure kinetics, inflammation and degree of revascularization. Western Blotting could be used to confirm the protein levels of HIF-1α, VEGF and any other proteins of interest. To complete this study, RT-qPCR could be used to confirm RNA levels of proteins of interest. This data could further be

supplemented with *in vitro* studies, such as growing murine aged cells like fibroblasts, keratinocytes, neutrophils and endothelial progenitors in varying hypoxic and glucose conditions to further elucidate their adaptative response to hypoxia under hyperglycemic conditions. An additional future direction for research could be attempting to elucidate the factors responsible for the macrophage polarization shift from M1 to M2. This concept is relatively new, however it is evident that there exist distinct macrophage phenotypes in the wound [136]. There are recognized shortcomings in techniques available to conclusively identify the two distinct phenotypes [136]. We believe it is worthwile to investigate additional markers that could aid researchers in positively identifying these phenotypes. Additionally, further work is needed to definitively characterize the factors responsible for the polarization shift, as such findings could bear significant implications for wound healing therapeutics.

Refocusing on the issue of clinical translation, we argue that chronic wound studies should ideally be carried out with close involvement of clinicians who have access to human chronic wound tissue. Due to the known differences in healing between mice and humans, any murine data no matter how promising, should be interpreted with caution and ideally validated and replicated in human tissue. Current work in our lab is performed with close involvement of clinicians in the field. Such co-operation allows us to gain access to and analyze tissues from the DFU prior to the terminal endpoint of amputation. Our evidence suggests that by the time of amputation, the wound profile is so dysregulated that any intervention at that stage likely futile. Clinical wound healing research is often limited by ethical considerations, affording most academic investigators only the terminal endpoint from which to draw conclusions. With the help of our clinician partners, we have been able to gain access to debridement tissues, sometimes from the same patient, allowing us to

investigate and track any potential changes in cellular processes longitudinally. We propose supplementing the above-described murine study with age matched cell culture studies, as we have shown that it is possible to isolate and culture cells from diabetic debridement tissues. We propose these future studies to be undertaken with the ultimate goal of further elucidating the temporal profile of cellular adaptive responses to hypoxia, the effect of hyperglycemia in the context of aging, and any effects on inflammatory and angiogenic responses. Additional work should also be undertaken in the area of macrophage polarization and the vascular plasticity window, as these areas represent exciting, promising areas of study for the DFU. Although hypoxia represents an attractive therapeutic target, we believe it is premature to attempt to tackle it head on, without a prior detailed understanding of what cells are most responsive to it, and at what stages in wound healing it plays the most prominent role.

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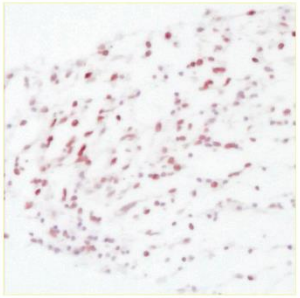
4.0 Appendix

4.1 – Hypoxyprobe™ staining validation figure showing suboptimal detection with the default QuPATH vector, and improved detection with a manually set vector.

Hypoxyprobe Staining Validation

1a)

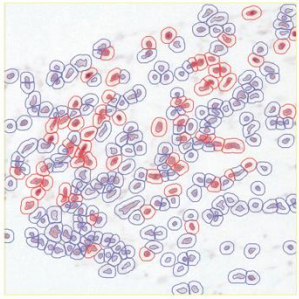
Good Contrast - Pre Analysis



Default Vector

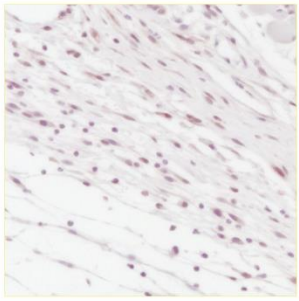
DAB: 0.269 0.568 0.778

Good Contrast - Post Analysis



1b)

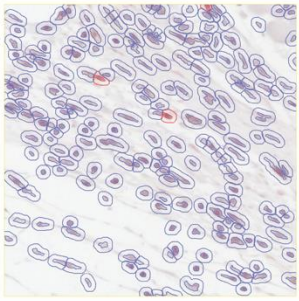
Poor Contrast - Pre Analysis



Default Vector

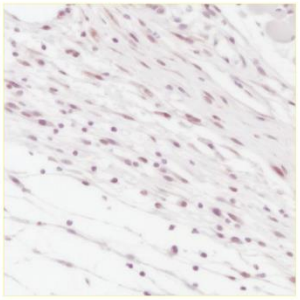
DAB: 0.269 0.568 0.778

Poor Contrast - Post Analysis



1c)

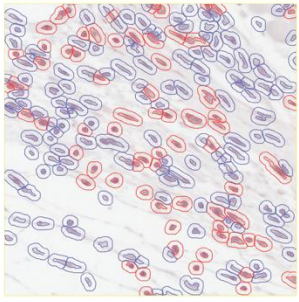
Poor Contrast - Pre Analysis



Manual Vector

DAB: 0.383 0.722 0.576

Poor Contrast - Post Analysis

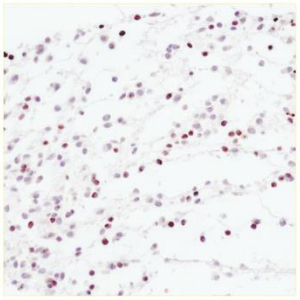


4.2 – Neutrophil elastase staining validation figure showing sub-optimal detection with default QuPATH vector, and improved detection with a manually set vector.

Neutrophil Elastase Staining Validation

1a)

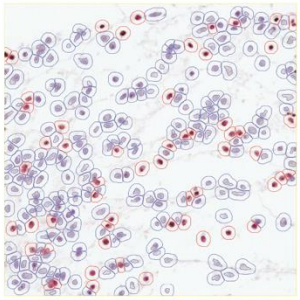
Good Contrast - Pre Analysis



Default Vector

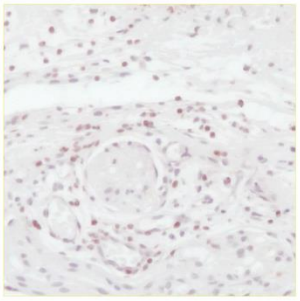
DAB: 0.269 0.568 0.778

Good Contrast - Post Analysis



1b)

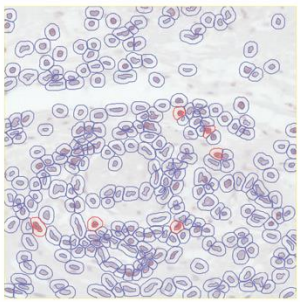
Poor Contrast - Pre Analysis



Default Vector

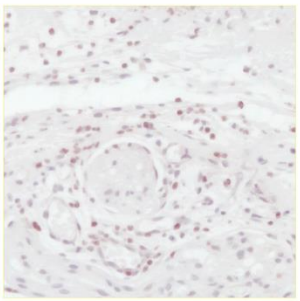
DAB: 0.269 0.568 0.778

Poor Contrast - Post Analysis



1c)

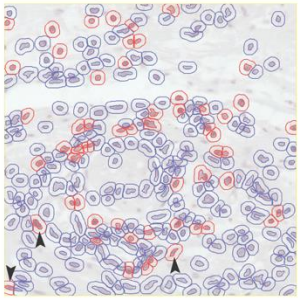
Poor Contrast - Pre Analysis



Manual Vector

DAB: 0.402 0.715 0.573

Poor Contrast - Post Analysis



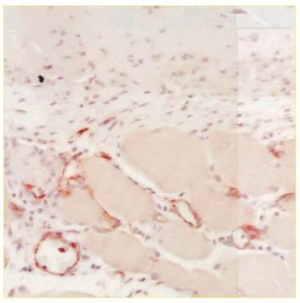
Arrows denoting cells which would be excluded based on morphology

4.3 – $CD31^+$ staining validation figure showing sub-optimal detection with default vector, and improved detection with a manually set vector.

CD31 Staining Validation

1a)

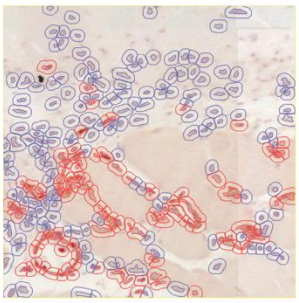
Good Contrast - Pre Analysis



Default Vector

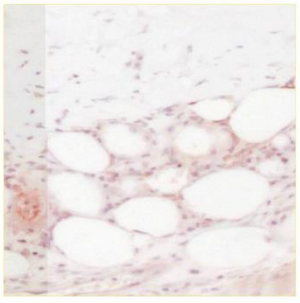
DAB: 0.269 0.568 0.778

Good Contrast - Post Analysis



1b)

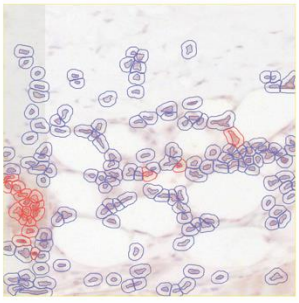
Poor Contrast - Pre Analysis



Default Vector

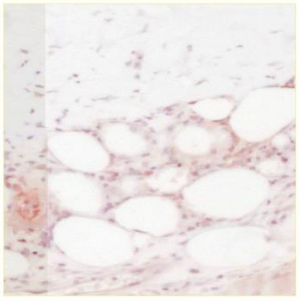
DAB: 0.269 0.568 0.778

Poor Contrast - Post Analysis



1c)

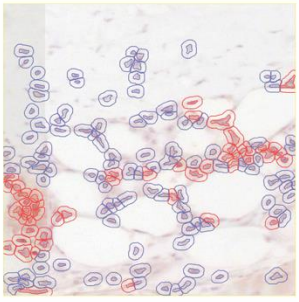
Poor Contrast - Pre Analysis



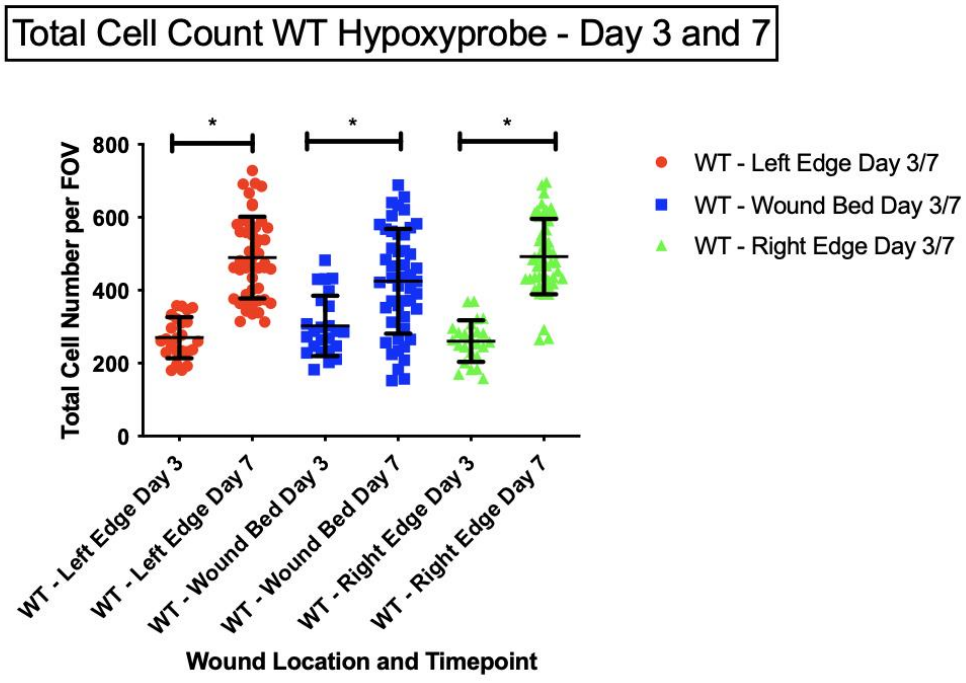
Manual Vector

DAB: 0.3 0.771 0.561

Poor Contrast - Post Analysis

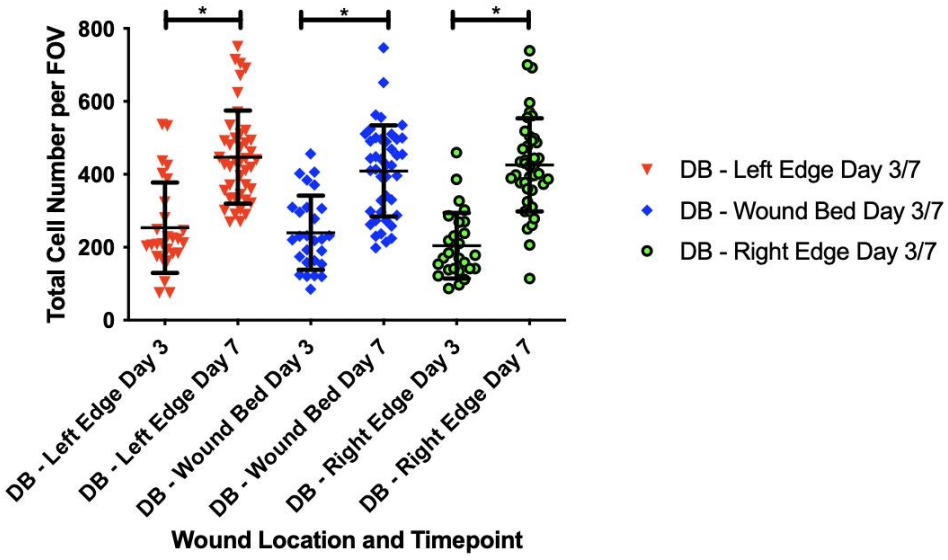


4.4 – Total cell counts at day 3 & 7 for wild type mice. Note the cell increase between days 3 and 7 and the slight trend toward more cell infiltration compared to db/db, especially in wound bed.


Total Cell Count WT Hypoxyprobe - Day 3 and 7
Total Cell Number per FOV
800
600
400
200
0
*
*
*
WT - Left Edge Day 3
WT - Left Edge Day 7
WT - Wound Bed Day 3
WT - Wound Bed Day 7
WT - Right Edge Day 3
WT - Right Edge Day 7
Wound Location and Timepoint
WT - Left Edge Day 3/7
WT - Wound Bed Day 3/7
WT - Right Edge Day 3/7

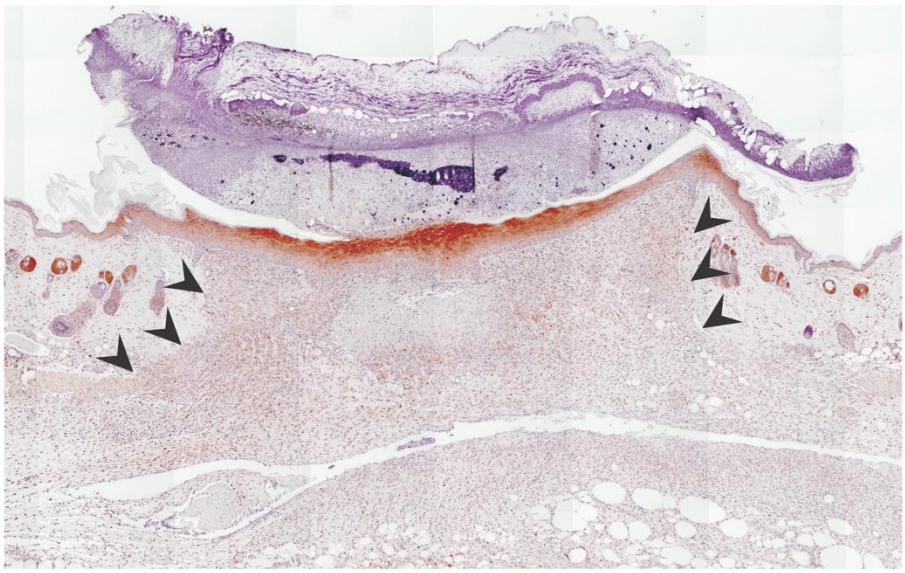
4.5 – Total cell counts for days 3 & 7 for db/db mice. Note the cell increase between days 3 & 7.

Total Cell Count Hypoxyprobe DB - Day 3 and 7



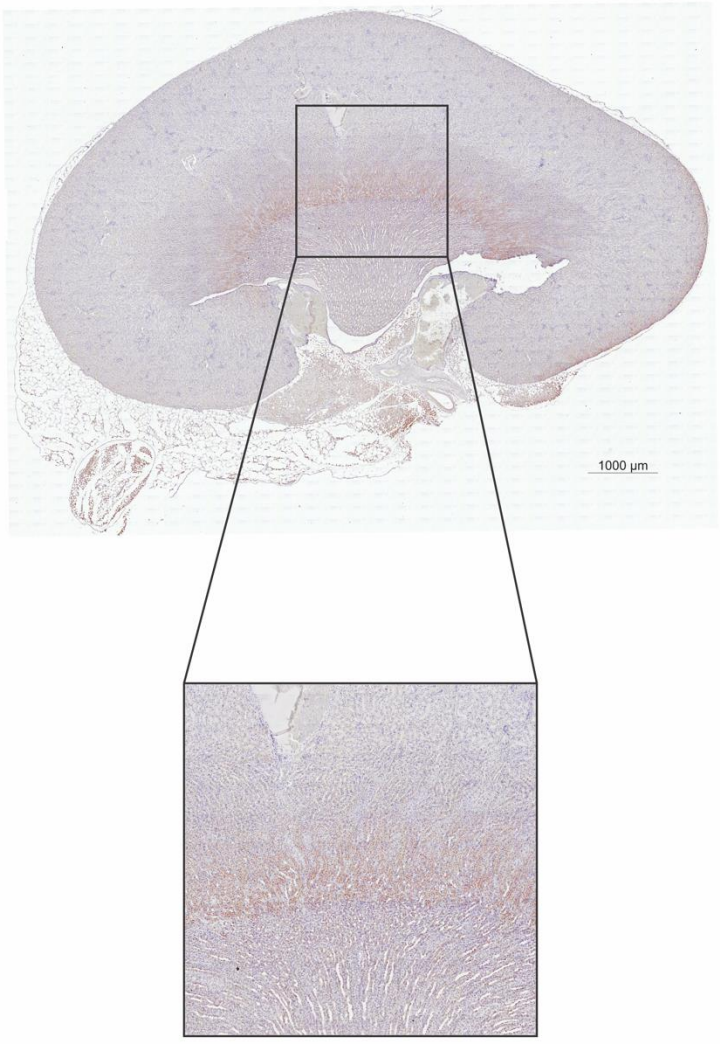
Appendix 4.6 – Figure showing “volcano like” infiltration pattern of hypoxic cells evident in both db/dbs and wild type mice

Appendix 4.6 – Showing volcano like infiltration pattern of hypoxic cells from deep within tissue



Appendix 4.7 – Figure showing positive control for Hypoxyprobe™ in the form of murine kidney.

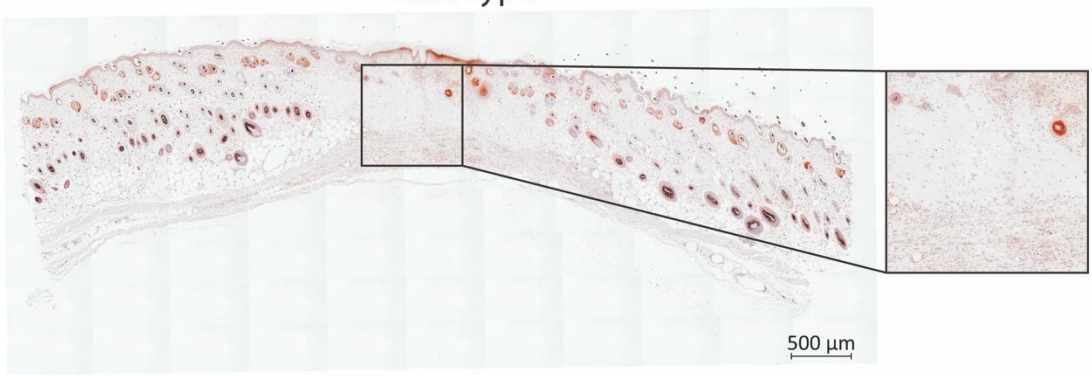
Appendix 4.7 - Murine kidney Hypoxyprobe Positive Control



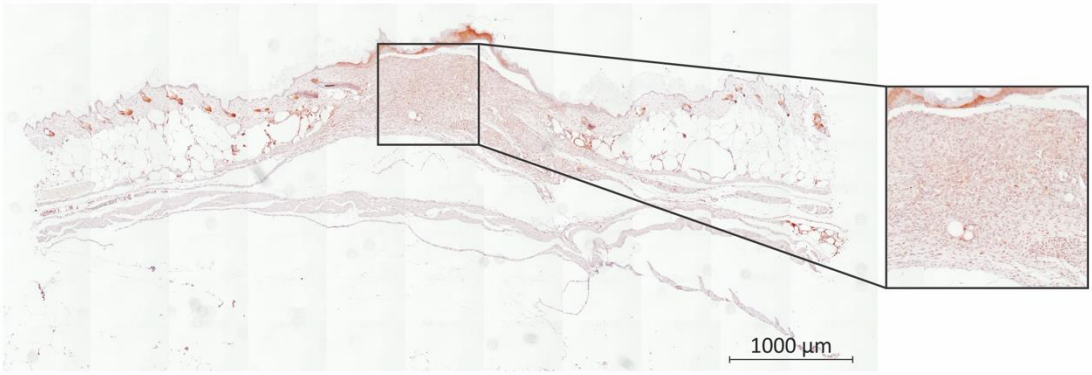
Appendix 4.8 – Figure showing diminished Hypoxyprobe™ staining at day 12 in the wild type. Note the wound core is still strongly staining for hypoxia in the db/db

Appendix 4.8 - Evidence of Hypoxia Resolution at Day 12 in the Wild Type

Wild Type

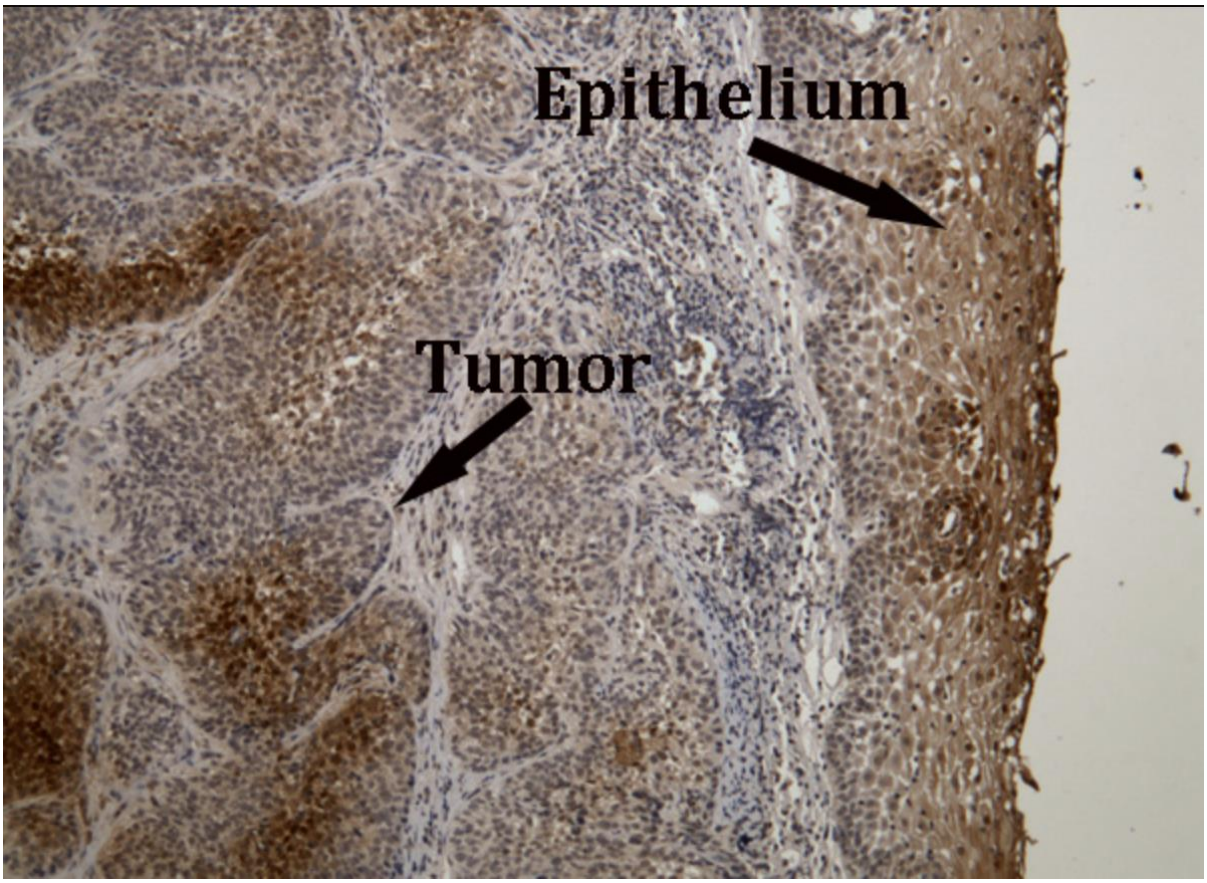


Diabetic



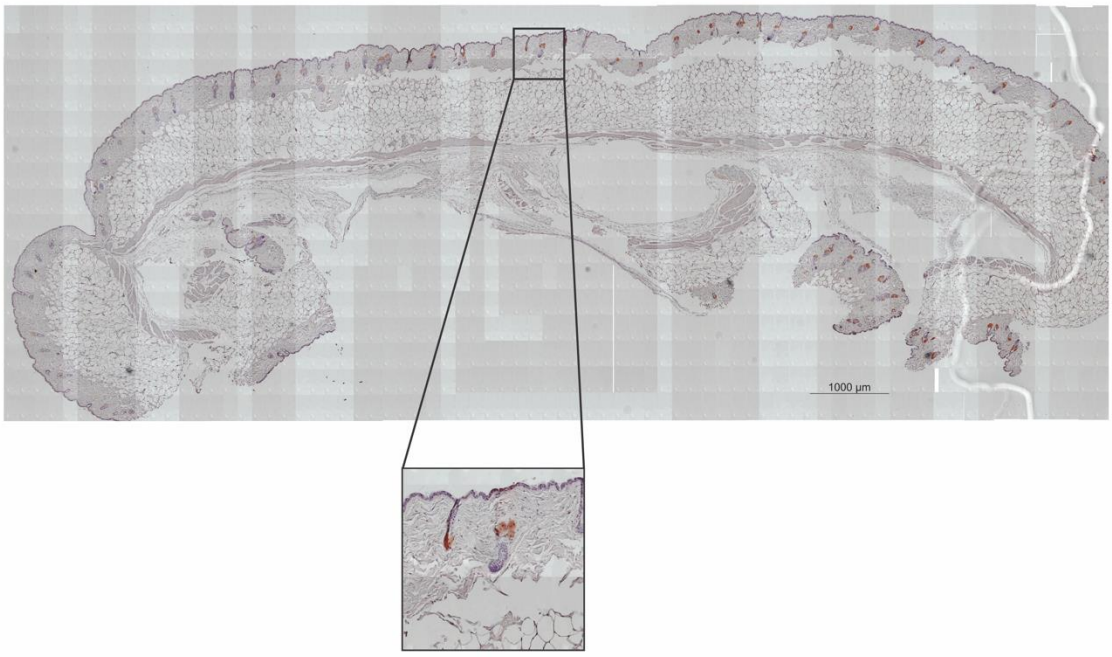
Appendix 4.9 – Figure showing profound Hypoxyprobe™ staining in a tumor, which is known to have extremely dysregulated vasculature.

Appendix 4.9



Appendix 4.10 – Unwounded Murine Hypoxyprobe™ staining

Appendix 4.10 - Unwounded Murine Hypoxyprobe Stain



Appendix 4.11 – Animal Protocol Approval Letter

Western

AUP Number: 2020-142
PI Name: Hamilton, Doug
AUP Title: Influence of biomaterials and material physiochemical properties on cell behaviour in vitro and in vivo.

Official Notification of ACC Approval: A modification to Animal Use Protocol **2020-142** has been approved.

Please at this time review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1. This Animal Use Protocol is in compliance with:
    - Western's Senate MAPP 7.12 [PDF]; and
    - Applicable Animal Care Committee policies and procedures.
2. Prior to initiating any study-related activities—as per institutional OH&S policies—all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have:
    - Completed the appropriate institutional OH&S training;
    - Completed the appropriate facility-level training; and
    - Reviewed related (M)SDS Sheets.

5.0 Curriculum Vitae

Name: Michael Roman Grynyshyn

Post-secondary Education and Degrees: Western University, London
Ontario, Canada
2014-2020 B.A. Health Sciences

Honours and Awards: Western Admission Scholarship, 2014
Ontario Graduate Scholarship, 2020

Related Work Experience Research Assistant
Western University
2019-2020

Publications

- Hamilton DW, Walker JT, Tinney D, Grynyshyn M, El-Warrak A, Truscott E, Flynn LE. The pig as a model system for investigating the recruitment and contribution of myofibroblasts in skin healing. Wound Repair Regen. 2022 Jan;30(1):45-63. doi: 10.1111/wrr.12981. Epub 2021 Nov 6. PMID: 34708478.
    - Published January 2022, Wound Repair and Regeneration, Volume 30, Issue 1, pages 45-63
- Wang J, Tinney D, Grynyshyn M, Pickering JG, Power A, Dubois L, Hamilton DW. Microcirculation surrounding end-stage human chronic skin wounds is associated with endoglin/CD146/ALK-1 expression, endothelial cell proliferation and an absence of $p16^{Ink4a}$. Wound Repair Regen. 2023 May-Jun;31(3):321-337. doi: 10.1111/wrr.13081. Epub 2023 Apr 13. PMID: 37017097.
    - Published April 2023, Wound Repair and Regeneration, Volume 31, Issue 3. Pages 321-337