

MESENCHYMAL STROMAL CELLS IN ALGINATE DRESSINGS TO ENHANCE CHRONIC
WOUND HEALING

by

RENEA ALEXANDRINE FAULKNER

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ABSTRACT OF THE DISSERTATION

Mesenchymal stromal cells in alginate dressings to enhance chronic wound closure

By RENE ALEXANDRINE FAULKNER

Dissertation Director:

François Berthiaume

Chronic wounds are “stuck” in an inflammatory state characterized by hypoxia and high levels of inflammatory mediators such as inflammatory proteins, white blood cells and bacteria. The hostile environment of chronic wounds readily breaks down growth factors needed to promote wound healing. Recent studies have shown that mesenchymal stem/stromal cells (MSCs), which are non-hematopoietic stem cells, possess “anti-inflammatory” properties. These cells secrete factors that mitigate inflammation and promote wound healing of normal wounds. However, a major limitation with using MSCs is their ability to migrate away from the wound site. Therefore, we have generated a novel bioactive bandage made with alginate to immobilize the MSCs at the wound site. The goal of this project, unlike growth factor depots, is to have the MSCs provide sustained release of soluble factors to the wound and potentially modulate their secretion pattern depending on the wound environment. We hypothesize that MSCs can enhance wound healing by decreasing inflammation in the wound and increasing wound contraction to close the wound. This occurs through their secretion of different soluble factors lacking in chronic

wounds and through communication with wound healing cells such as macrophages and fibroblasts. Our results show that the alginate microenvironment does not alter MSC viability or secretion. Immobilized MSCs increased wound closure rate in a diabetic mouse model. We observed that this enhancement could in part be due to the MSCs' ability to promote differentiation of macrophages to the anti-inflammatory "M2" phenotype and differentiation of fibroblasts into α -smooth muscle actin myofibroblasts that deposit collagen and mediate wound contraction to close the wounds. In human chronic wounds, hypoxia may impair these responses; however, we found that MSCs were able to promote differentiation even in presence of hypoxic conditions.

DEDICATION

To my loving mother and father

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CHAPTER 1: INTRODUCTION

1.1 SKIN WOUND HEALING

Skin wounds can be classified as acute and chronic wounds. In acute wounds, normal healing takes place in a multistep process that includes three main phases: inflammation, proliferation and remodeling. After injury, platelets aggregate and form a fibrin clot to stop the bleeding caused by ruptured blood vessels in the wound [1, 2]. Damaged cells discharge signals, including damage-associated molecular pattern (DAMP) molecules, which attract immune cells to the wound to initiate the inflammatory phase[1]. The circulating immune cells from ruptured blood vessels bind to the provisional matrix and migrate into the wound bed. The main cell types involved in this phase are primarily neutrophils and macrophages. Neutrophils clear the wound from debris and bacteria and secrete proteases that breakdown damaged tissues [1, 2]. However, due to the different stimuli present in the wound, macrophages can take on different phenotypic properties that affect their behavior with respect to wound healing. The two distinct phenotypes that play a role in tissue repair are (1) the classically activated or M1 macrophages and (2) the alternatively activated or M2 macrophages. Granulocyte stimulating factor and interferon-gamma (IFN- γ) from blood serum [1, 3, 4] released into the wound as well as lipopolysaccharides (LPS) from invading bacteria activate macrophages towards the M1 phenotype [3, 4]. These CD86 positive M1 macrophages secrete high levels of TNF- α , work alongside neutrophils to clear the wound from debris and

bacteria and phagocytose spent neutrophils therefore becoming the predominant cells in the wound [1]. Once the wound is clear from pathogens, macrophages transition to the M2 phenotype due to interleukin (IL)-4 activation of anti-inflammatory genes [4]. These CD206 positive cells secrete essential growth factors such as IL-10 and transforming growth factor (TGF)- β 1, which attract fibroblasts and endothelial cells and regulate the proliferation phase of wound healing [4, 5].

Fibroblasts play a dominant role in this tissue repair process. They migrate from surrounding healthy tissues or are generated from circulating fibrocytes, pericytes or differentiated from stem cells surrounding hair follicles in the skin [1, 6]. These cells synthesize and deposit collagen to form the new extracellular matrix (ECM), which replaces the fibrin clot and become the new granulation tissue. This process aids in the migration of endothelial cells into the wound bed and stimulate angiogenesis, the formation of new blood vessels. Secreted factors by macrophages and fibroblasts stimulate endothelial cells to invade the ECM and form tube-like structures that become the lining of new blood vessels [7]. These new blood vessels carry oxygen and nutrients necessary to support cell metabolism for processes such as cell proliferation and migration. Epidermal cells such as keratinocytes then migrate into the wound bed due to the chemotactic effects of soluble factors such as IL-8. In a process called epithelialization, keratinocytes migrate and proliferate inwards to form a barrier between the wound and the environment [1]. Simultaneously, fibroblasts, in the presence of TGF- β 1, differentiate into α -smooth muscle actin (α -SMA)-expressing myofibroblasts. These cells form stress fibers to contract and close the wound [8, 9]. Following the proliferative phase is the

remodeling phase, which primarily involves the rearrangement and alignment of collagen fibers to form scar tissue.

Wound healing is complex and any disruption in any of the processes described above that cause dysfunction in cell growth, migration and contraction can lead to chronic or non-healing wounds.

1.2 CHRONIC WOUNDS

Chronic wounds are non-healing wounds that do not follow the normal wound healing cascade and are characterized by being in a prolonged inflammatory state. Advanced age, especially when combined with underlying diseases such as diabetes [6, 10, 11], increases the risk of slow healing wounds, such as venous, diabetic, and pressure ulcers [12]. A common problem in venous, pressure and diabetic ulcers is impaired blood flow to the tissue. Oxygen is an essential nutrient for wound healing. Several pre-clinical and clinical studies show that hypoxia delays wound healing [7]. Directly after injury, the wound becomes devoid of oxygen because cells rapidly consume oxygen to produce the energy needed for defense, cell migration, proliferation, collagen synthesis and contraction [7]. This acute hypoxia also elicits angiogenesis to increase oxygen tension in the wound but chronic wounds fail to elicit sufficient angiogenesis [13, 14] leading to persistent impaired nutrient, waste and oxygen transport [7, 15] and ultimately improper resolution of the inflammatory phase of wound healing.

Tissue hypoxia slows down mitochondrial oxidative phosphorylation, which results in a reduction in adenosine triphosphate (ATP) production and ATP

dependent mechanisms such as cell migration, cell proliferation and contraction. Ion pumps cannot function with the lack of ATP, which results in cell swelling, membrane disruption and ultimately in cell death [7]. The dying cells release DAMP molecules into the ECM, which attract and activate immune cells to produce elevated levels of pro-inflammatory mediators [1]. These immune cells are primarily neutrophils and macrophages. Neutrophils role in skin wound healing is to clean the wound and kill bacteria however, the high levels of pro-inflammatory mediators results in their prolonged persistence in chronic wounds. This results in an overabundance of secreted proteases to the point where they begin to breakdown surrounding healthy tissue. This bystander damage results in the formation of larger ulcers.

In chronic wounds, there is also an accumulation of M1 macrophages because the mechanism that normally transitions them to the M2 phenotype is impaired [16]. Together the neutrophils and M1 macrophages continuously produce reactive oxygen species (ROS). ROS impair angiogenesis by breaking down ECM components and essential growth factors and by activating pathways that result in elevated expression of matrix metalloproteinases (MMPs) and pro-inflammatory cytokines [7]. Detoxification of ROS is necessary to maintain cell survival. Detoxification maintains a balance between ROS production and clearance. In chronic wounds, detoxification is impaired so ROS is continuously produced therefore leading to no resolution of the inflammatory phase and ultimately non-healing and open wounds [2, 7].

1.3 CURRENT TREATMENTS

After an ulcer forms, one of the first points of treatment is surgical debridement to clear the wound of necrotic tissue and pro-inflammatory mediators in order to mimic an acute wound. Then dressings such as gauzes, films, alginates and other hydrogels are used to absorb and trap wound fluid inside the dressing fibers. Absorption of wound exudates is beneficial in removing some of the pro-inflammatory mediators found in chronic wounds. [17]. However, wound fluid travels along most of these dressing fibers and cause maceration of the surrounding healthy tissue. Current treatments also include high-tech treatments including vacuum assisted wound closure (VAC), platelet rich plasma (PRP) treatments and skin substitutes [17]. However, these treatments show variable clinical results due to the tremendous diversity in wound size and location, as well as failure to address underlying pathologies that impair healing: persistent inflammation; decreased cell migration and growth factor levels; impaired blood supply and inability to deposit a mechanically stable collagen matrix [18].

Methods tested in the clinic to reverse hypoxia in chronic wounds, depending on the wound size, location and the patients' prognosis, include the use of hyperbaric oxygen chambers and topical oxygen gas, and have led to variable outcomes. Hyperbaric oxygen treatment (HBO₂) temporarily provides pharmacological doses of oxygen to chronic wounds in order to induce neovascularization and ultimately wound closure [7, 19, 20]. Although HBO₂ works in some patients, others suffer from a range of side effects and complications, including near-sightedness, seizures, trauma to lungs, and in patients with congestive heart failure, further decline in

cardiac function [20]. Topical oxygen therapy is used in patients who cannot risk the systemic side effects of HBO₂. Some studies showed that topical oxygen increased vascular endothelial growth factor (VEGF) expression at the wound edges and decreased wound size [7, 19, 21]. Because oxygen needs to dissolve into biological fluids in order to become available to the cells, wound debridement to remove dead tissue and wound exudate is needed before each topical oxygen treatment. However, shortly after debridement, hypoxic wounds tend to develop a firm and gas impermeable layer [21]. Topical oxygen therapy has also resulted in oxygen toxicities that lead to patients having seizures and sometimes death [21]. Current treatments used in the clinic have shown to be at most 50% effective at entirely closing chronic wounds [22]. Current treatments need to shift the molecular balance in chronic wounds to that seen in acute wound healing in spite of the hypoxic environment.

1.4 MESENCHYMAL STROMAL CELLS (MSCs)

MSCs are non-hematopoietic, multipotent cells that can differentiate into a variety of different cell types and give rise to bones, cartilage and other mesenchymal tissues. MSCs have been studied extensively for different clinical applications due to their therapeutic ability. MSCs can migrate and home to different sites of injury depending on the systemic or local inflammatory site and can modulate the immune system through secreted factors or promote regeneration via differentiating into different cell types. MSCs are easily accessible since they are tissue culture plastic adherent cells as opposed to other cells from the bone marrow. This allows for easy

isolation of MSCs from the bone marrow and these cells can expand rapidly in a short period. MSCs can be biopreserved while maintaining multipotency and stored for point-of-care delivery. More importantly, human trials so far have shown no adverse reactions to allogeneic MSCs, allowing for large storage of third party donor MSCs for the increasing number of patients treated in clinical trials for cardiovascular, neurological and immunological diseases. [3, 23, 24].

MSCs have been shown to promote skin wound healing [28] by contributing to each phase of wound healing via secreted soluble products [24, 25], such as prostaglandin E2 (PGE2). PGE2 has been shown to promote the transition of macrophages from the M1 to the M2 phenotype [8]. MSCs and M2 macrophages can subsequently dampen inflammation in chronic wounds by secreting anti-inflammatory cytokines, growth factors and chemokines [3]. A subset of these factors such as IL-8 and TGF- β 1 helps to increase migration of fibroblasts and keratinocytes to the wound, pushing wound healing towards the proliferation phase [1, 6, 8]. The secretion of TGF- β 1 drives the differentiation of fibroblasts into α -SMA-expressing myofibroblasts, the cells that promote wound contraction [9]. Therefore, we hypothesize that MSCs can modulate prolonged inflammation and promote wound contraction and tissue remodeling in chronic wounds.

However, a major limitation of direct MSC implantation is their tendency to migrate away from the site of introduction [26] or die if not provided with a proper niche to engraft [27]. To overcome this limitation, MSCs can be immobilized in hydrogels, in which their viability and unique secretion pattern is preserved [28, 29].

1.5 ALGINATE AS A DELIVERY VEHICLE

A potential solution to decrease MSCs' tendency to migrate from the wound site is to immobilize the cells using hydrogels or porous scaffolds in order to increase cell survival and engraftment [30]. Biomaterials used for this purpose include fibrin, collagen and alginate. While fibrin and collagen emulate the native ECM, alginate is a more abundant, cost effective, biocompatible and a biodegradable wound covering, which is also approved for use as a wound dressing by the U.S. Food and Drug Administration (FDA). MSCs have been successfully immobilized in alginate microcapsules, which have been shown to preserve their secretion of anti-inflammatory molecules, thus creating a continuous release device that can modulate local immune responses and promote tissue repair [28]. One of the goals of this PhD dissertation research was to immobilize MSCs in alginate hydrogel bandages that can conform to wounds and promote chronic wound healing.

1.6 WOUND HEALING ANIMAL MODELS

Testing in a pre-clinical model is necessary to provide proof-of-concept data suggesting that the approach may work in an actual wound. Animals will be used for skin wound healing studies as in vivo models provide the only reliable way to evaluate the effect of treatments on wound healing. In vitro systems cannot take into account all of the factors involved in wound healing processes. Mice are commonly used for pre-clinical studies because they are easy to handle, require the use of small amount of reagents and so they are cost effective while transgenic animals are available to study different underlying diseases. Even though there are

differences between human and mouse skin, studies have shown that the altered biochemical and cellular properties in human diabetic wounds are similarly present in diabetic mouse wounds [11]. Genetically diabetic mice will be used in these studies. In addition, prior work has been done with these strains of mice showing delay in wound healing compared to normal mice.

1.7 DISSERTATION SUMMARY

The aim of this dissertation work is to continuously provide essential growth factors to the wounds using a cost effective and biocompatible approach. We immobilized MSCs in the FDA-approved alginate hydrogel in order to maintain MSC persistence at the wound site so they can continuously secrete essential growth factors into the chronic wound. We hypothesize that immobilized MSCs will enhance the healing of chronic wounds via paracrine signaling with macrophages and fibroblasts in the wounds. The following hypothesis was tested with the four specific aims:

1. To immobilize MSCs and assess the effect of the chronic wound environment on MSC function
3. To assess the effect of hypoxia on MSC-macrophage interaction
4. To assess the effect of hypoxia on MSC-fibroblast interaction
5. To show that immobilized MSCs enhance chronic wound healing in a diabetic mouse model

In chapter 2, we developed a method to immobilize MSCs in alginate sheets that can be applied to the open wounds on the dorsum of the genetically diabetic mice. The immobilized MSCs were cultured over time and remained over 90%

viable. There were also no significant differences in secretion of soluble factors when cells were cultured in a monolayer on tissue culture plastic or immobilized in alginate sheets. We also cultured MSCs in a chronic wound environment (hypoxia or chronic wound fluid) in vitro. We observed no significant differences in secretion of inflammatory mediators when cells were cultured in normoxia (21% v/v O₂) or hypoxia (1% v/v O₂). We also observed that chronic wound fluid from patients suffering from long-term diabetic foot ulcers suppressed MSC secretion of inflammatory mediators and increased their secretion of an anti-inflammatory mediator.

In chapter 3, we investigated the MSC immunomodulatory properties in hypoxia. Immobilized MSCs were co-cultured with macrophages in hypoxia and normoxia. Macrophages activated with LPS and co-cultured with MSCs produced significantly higher levels of IL-10 and significantly lower levels of TNF- α compared to cells cultured in medium with LPS alone. High levels of IL-10 production and TNF- α suppression are markers for M2 macrophages. However, in hypoxia, the levels of IL-10 produced by macrophages co-cultured with MSCs were significantly lower than cells cultured in normoxia. MSC-derived products also enhanced M2 surface marker expression, CD206. This data suggests that MSCs do help transition macrophages from the M1 to the M2 phenotype in hypoxia, but the hypoxic macrophage function is slightly impaired compared to normoxic macrophages.

In chapter 4, we created fibroblast-populated collagen lattices (FPCLs) to simulate the dermal layer of the skin and we investigated the impact of MSC-derived factors on the function of fibroblasts in hypoxia. There was no difference in

fibroblast viability when cultured in hypoxia compared to normoxia but there was a significant decrease in fibroblast contraction of collagen gels and a significant decrease in α -SMA expression. MSC-derived products restored function and α -SMA expression similar to those of normoxic FPCLs. We also treated FPCLs with an inhibitor to TGF- β 1 since TGF- β 1 is known to differentiate fibroblasts into α -SMA myofibroblasts, cells responsible for wound contraction. The inhibitor blocked most of the effect of MSCs, suggesting that TGF- β 1 is a major paracrine factor secreted by MSCs that can restore fibroblast function in hypoxia.

In chapter 5, we created large full-thickness excisional wounds on the dorsum of genetically diabetic mice. Wounds were then treated with immobilized MSCs in alginate sheets, MSCs in saline, alginate sheets or saline alone and wound closure was monitored over time. Immobilized MSCs closed wounds faster than all other conditions. In some studies the skin substitute AlloDerm® was placed on the wounds to slow down inherent contraction thus mimicking more closely wound healing found in human skin wounds, which require significant re-epithelialization. The AlloDerm® used had two different thicknesses (0.09mm and 0.41mm). The effect of immobilized MSCs was more prominent in wounds that received the 0.09 mm thick AlloDerm® compared to the 0.41mm thick AlloDerm®. Wounds that did not receive AlloDerm®, rapidly contracted but MSCs significantly enhanced this contraction. The 0.41mm AlloDerm® decreased angiogenesis in the wounds and required more wound closure time compared to the 0.09mm AlloDerm®. With the 0.41mm AlloDerm®, there was no significant difference in wound closure rate between the immobilized MSCs and alginate sheets. However, immobilized MSCs did

close the wounds faster and histological analysis showed more endothelium and α -SMA expression in those wounds compared to all other treatment conditions.

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CHAPTER 2: IMMOBILIZATION OF MESENCHYMAL STROMAL CELLS IN ALGINATE DRESSINGS

Note: Some data in this chapter was adapted from the following publication:

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2.1 INTRODUCTION

The hostile environment of chronic wounds consists of high levels of reactive oxygen species (ROS) and matrix metalloproteinases (MMPs) that readily breakdown essential growth factors for wound healing [1, 2]. Continuous release of growth factors to the wound is needed to overcome the activity of the MMPs and to regulate cellular behavior and promote wound healing [3, 4]. Literature evidence shows that growth factors that promote wound healing are down regulated in diabetic ulcers [4]. Applying exogenous transforming growth factor (TGF)- β 1 was effective in enhancing acute wound healing [5] but failed to increase wound closure in ischemic and chronic wounds [6]. Repeated dose may overcome the effects of MMPs but multiple dosing and administering multiple growth factors to the wounds are costly. Unlike growth factor depots, cells have the ability to provide sustained release of factors.

Mesenchymal stromal cells (MSCs) are a promising cellular therapeutic. These multipotent cells can differentiate into a variety of cell types and secrete a variety of beneficial molecules to modulate acute and chronic inflammation and to promote tissue repair [7, 8]. Pre-clinical models demonstrated MSCs' beneficial effects in cutaneous wound repair. However, while clinic trials demonstrated the safety of allogeneic MSC transplantation there was variable results proving significant efficacy [9]. Pre-clinical studies have shown that MSCs fail to home to the injury or tissue site and die due to improper niche or migrate away [10, 11]. In chronic wounds, the persistent inflammation can potentially return due to the decrease in therapeutic cells.

Studies currently focus on the use of hydrogels that emulate the extracellular matrix (ECM) as an external niche for MSCs [12-14]. We used alginate, a biocompatible and biodegradable biomaterial, to immobilize MSCs. Alginate is approved by the U.S. food and drug administration (FDA) as a material for wound dressing; these dressings can absorb and store wound fluid and maintain a moist wound environment [15]. Barminko et al. encapsulated MSCs in alginate microbeads and found that cell viability was maintained along with their secretory profile [16]. We developed a technique to immobilize MSCs in alginate sheets shaped like a bandage that conforms to the wounds. The immobilization of MSCs can potentially allow for long-term secretion of growth factors to chronic wounds.

In this chapter, we immobilized MSCs in alginate sheets and characterized their viability and secretion within the dressings. We also investigated the effect of chronic wound fluid and hypoxia on MSC secretion since besides the ischemic

environment, the wound experiences high levels of pro-inflammatory mediators. The immobilized MSCs in alginate sheets will be used in subsequent studies to investigate their effect on diabetic wound healing and on macrophages and fibroblasts function in the chronic wound environment.

2.2 MATERIALS AND METHODS

2.2.1 Development of PDMS mold

Fineline Imaging created a silicon wafer with the 2 x 2 x 0.05cm dimension of the alginate sheets. The silicon wafer was cleaned with sterile water and subsequently soaked in acetone then isopropanol (IPA) then sterile water for 10 min. The wafer was then dried using filtered air and baked at 150°C for 20 min to remove all water from the wafer. The wafer was coated with 250µm layer of SU-8 and spun at 1000 rpm for 30 sec at an acceleration of 300 rpm/second to allow even distribution of the SU-8 2100. Then, the wafer was baked at 65°C for 10 min followed by another 60 min at 95°C. The wafer was then exposed to UV light using soft contact mode at a dose of 100 mJ/cm². The wafer was then baked at 65°C for 10 min and then 95°C for 30 min and then developed for 25 min with heavy agitation and baked for 2 hrs at 95°C.

The wafer was placed in a vacuum desiccator with 5µL of Trichloro (1H, 1H, 2H, 2H-perfluorooctyl) silane on a glass next to the wafer. We applied a vacuum to allow exposure between the wafer and the silane for 20 min. Then we placed the wafer into a polystyrene petri dish, poured polydimethylsiloxane (PDMS) (10:1;

pre-polymer to curing agent) onto the wafer, degassed in the vacuum desiccator for 30 min and then baked at 65°C overnight. The PDMS was cut from the wafer and used to repeatedly create alginate sheets since the dimensions on the wafer were transferred to the PDMS.

2.2.2 Immobilization of MSCs in PDMS mold

MSCs (5×10^5 cells) were mixed with 200 μ L of 3% w/v sodium alginate (Sigma, St. Louis, MO). The cell suspension was poured into the 2 x 2 square, 0.05 cm deep, mold made of PDMS, followed by immersion in a 500mM calcium chloride solution for 10 min to cross-link the alginate thus forming a sheet. The sheets were then immersed in 0.05% w/v poly-L-lysine (PLL; 83.3 kDa; Sigma) solution for 2 min at room temperature to increase sheet stability as described elsewhere [16].

2.2.3 Viability determination

Viability of immobilized MSCs was measured weekly for 21 days while the cells were cultured in α -MEM with 10% v/v FBS, 1% w/v pen-strep, 4mM L-glutamine and 1ng/ml bFGF. The cells were incubated at 37°C with 3 μ M calcein-AM + 6 μ M ethidium homodimer-1 (Life Technologies) for 30 min in basal medium and the nuclei counterstained with Hoechst 33342 (Life Technologies). The cells were washed 5 times with medium before imaging on an Olympus IX81 spinning disc confocal microscope with a 10X objective. Five serial 500 μ m optical slices at 20 μ m intervals were taken per experimental condition. Slidebook software (Intelligent Imaging Innovations, Denver, CO) was used to quantify the number of live (green

fluorescence) and dead (red fluorescence) cells and calculate the percent viability as described elsewhere [17].

2.2.4 Cytokine secretion measurement

2.2.4.1 Alginate sheet

Human bone marrow-derived MSCs (2.5×10^5 cells/ml) at passage 3 were either immobilized in alginate sheets or plated as a monolayer on tissue culture plastic. The cells were cultured in α -MEM with 10% v/v FPS, 1% w/v pen-strep, 4mM L-glutamine and 1ng/ml bFGF, for 48 hrs under normoxia. The cell supernatant was collected and assayed for interleukin (IL)-8 (IL-8), TGF- β 1, IL-6 and prostaglandin E2 (PGE2) using commercial ELISA kits (BioLegend, San Diego, CA).

2.2.4.2 Chronic wound fluid (CWF)

CWFs were obtained with approval from the Rutgers University Institutional Review Board and after written consent from patients (n=3) with non-healing, diabetic foot ulcers attending Robert Wood Johnson Hospital. Only patients with diabetes and suffering from long-term non-healing diabetic foot ulcers were used in the study. The CWFs were collected from V.A.C. and centrifuged at 1000g for 10 min and filtered through a 0.22 μ m sterile filter. The CWF was then diluted with α -MEM with 10% v/v FBS, 1% w/v pen-strep, 4mM L-glutamine and 1ng/ml bFGF to obtain 10% and 50% CWF. Human bone marrow-derived MSCs (10×10^4 cells/ml) at passage 3 were plated as a monolayer in tissue culture plastic 96-well plates and cultured in 10%, 50% or 100% CWF for 72 hrs. The basal CWF (not placed on cells)

and the cell supernatant was collected and assayed for IL-6, IL-8, IL-1 β and IL-10 and using commercial ELISA kits (BioLegend, San Diego, CA).

2.2.4.3 Hypoxia

Human bone marrow-derived MSCs (2.5×10^5 cells/ml) at passage 3 were immobilized in alginate sheets and cultured at 37°C under normoxia (5% CO₂, 21% O₂, balance N₂) or hypoxia (5% CO₂, 1% O₂ balance N₂) for 48 hrs in a gas controlled chamber [18]. The cell supernatant was collected and assayed for IL-6, IL-8, PGE₂, vascular endothelial growth factor (VEGF) and TGF- β 1 using commercial ELISA kits (BioLegend, San Diego, CA).

2.3 STATISTICAL ANALYSIS

All numerical results are presented as means \pm standard error of the mean (SEM). Statistical analysis of three or more independent experiments were assessed using two-tail Student's t-test or one- way analysis of variance (ANOVA) followed by Fischer post hoc analysis where $p < 0.05$ represents statistical significance.

2.4 RESULTS

2.4.1 Immobilization of MSCs in alginate sheet

We immobilized MSCs in alginate flat sheets, the form in which the MSCs would ultimately be applied onto wounds (Figure 1). Using tweezers, the alginate sheets

were easy to handle, to manipulate, and to apply to wounds on the dorsum of genetically diabetic mice. Alginate sheets that were coated with 0.05% w/v PLL were more stable than alginate sheets without 0.05% PLL w/v PLL coating. The 0.05% w/v PLL coating maintained alginate sheet stability for over 21 days whereas alginate sheets without 0.05% w/v PLL coating were only stable for up to 72 hrs in culture in basal medium.

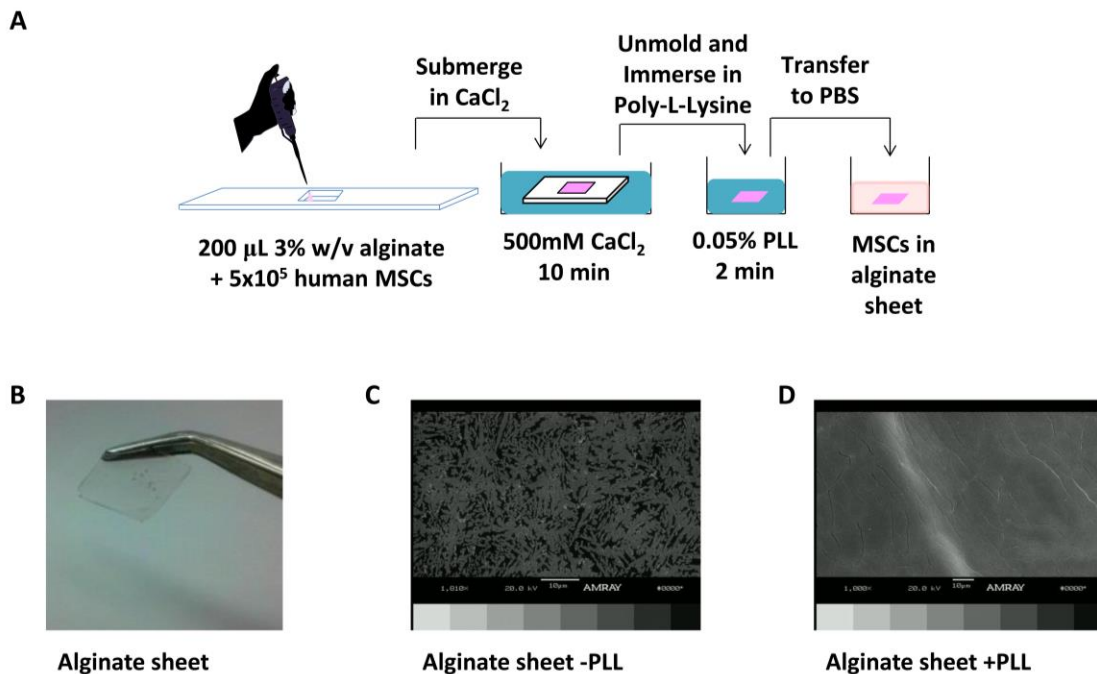


Figure 1. Immobilization of mesenchymal stromal cells (MSCs) in alginate sheets. (A) MSCs were mixed with soluble alginate and polymerized using calcium chloride to form alginate sheets containing MSCs. **(B)** Digital picture of the alginate sheet. **(C)** Outer layer of the porous alginate without the PLL coating. **(D)** PLL layer surrounding the alginate.

2.4.2 Alginate microenvironment does not affect MSC viability and secretion

MSCs in alginate sheets remained over 90% viable for up to 21 days (Figure 2A-B). The secretion pattern of immobilized MSCs was compared to that of MSCs cultured on standard tissue culture plastic, and there were no significant differences in secretion of IL-8, TGF- β 1, IL-6 or PGE2 (Figure 2C).

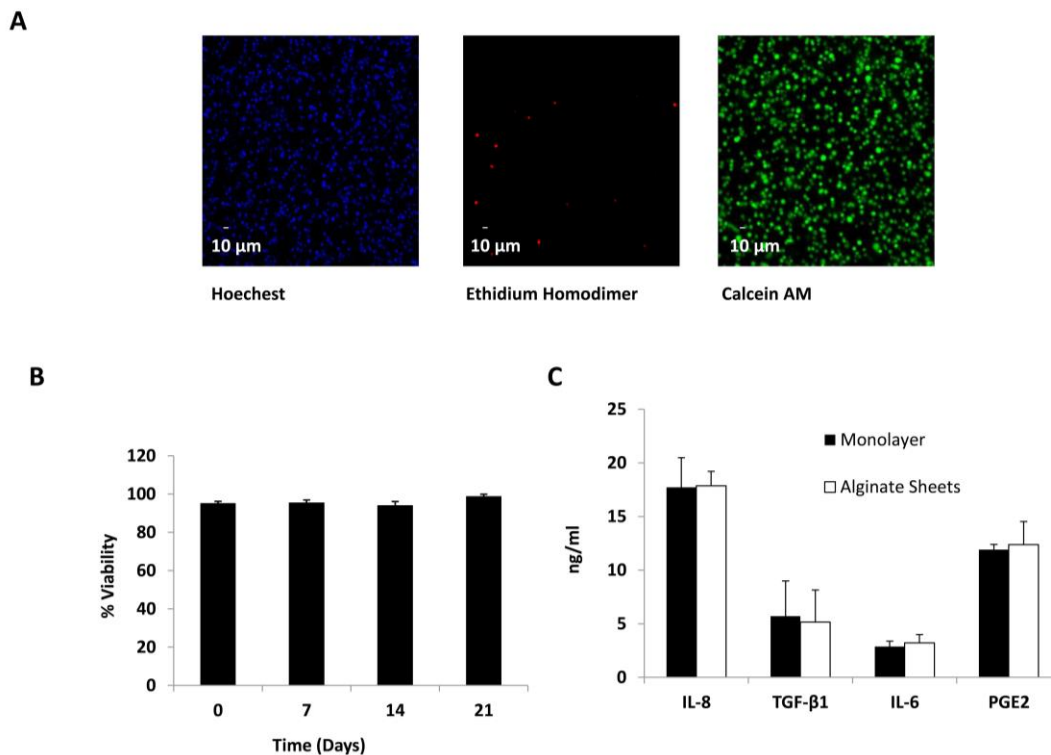


Figure 2. Immobilization of MSCs does not affect viability or secretion. (A, B) Immobilized MSCs were cultured for 21 days and percent viability was determined using live/dead fluorescent staining. Calcein AM⁺ and EthD-1⁻ MSCs in alginate sheets were considered viable. N=3. **(C)** Accumulated IL-8, TGF- β 1, IL-6 and PGE2 secreted by MSCs (2.5×10^5 cells/ml) after 48 hrs in

monolayer on tissue culture plastic vs. immobilized in alginate sheets for 48 h.
N=6.

2.4.3 CWF up-regulate MSCs secretion

High concentrations of CWF suppressed MSC secretion of pro-inflammatory mediators but increased MSC secretion of the anti-inflammatory mediator, IL-10. MSCs secreted significantly less IL-6 when cultured in 50% and 100% CWF compared to cells cultured in 10% CWF for 72 hrs (Figure 3A). Similarly, MSCs secreted significantly less IL-8 when cultured in 50% and 100% CWF compared to 10% CWF (Figure 3B). For IL-1 β , MSCs secreted non-detectable levels of IL-1 β when cultured in 10% CWF. There was a 70% increase in IL-1 β production in 50% CWF compared to 10% CWF but this production was significantly suppressed when the cells were cultured in 100% CWF (Figure 3C). Also, the levels of IL-1 β produced by MSCs were low compared to basal IL-1 β found in CWF. For the anti-inflammatory mediator, IL-10, MSCs produced significantly higher levels of IL-10 when cultured in 50% and 100% CWF compared to cells cultured in 10% CWF (Figure 3D). In fact, cells cultured in 10% CWF did not produce detectable levels of IL-10.

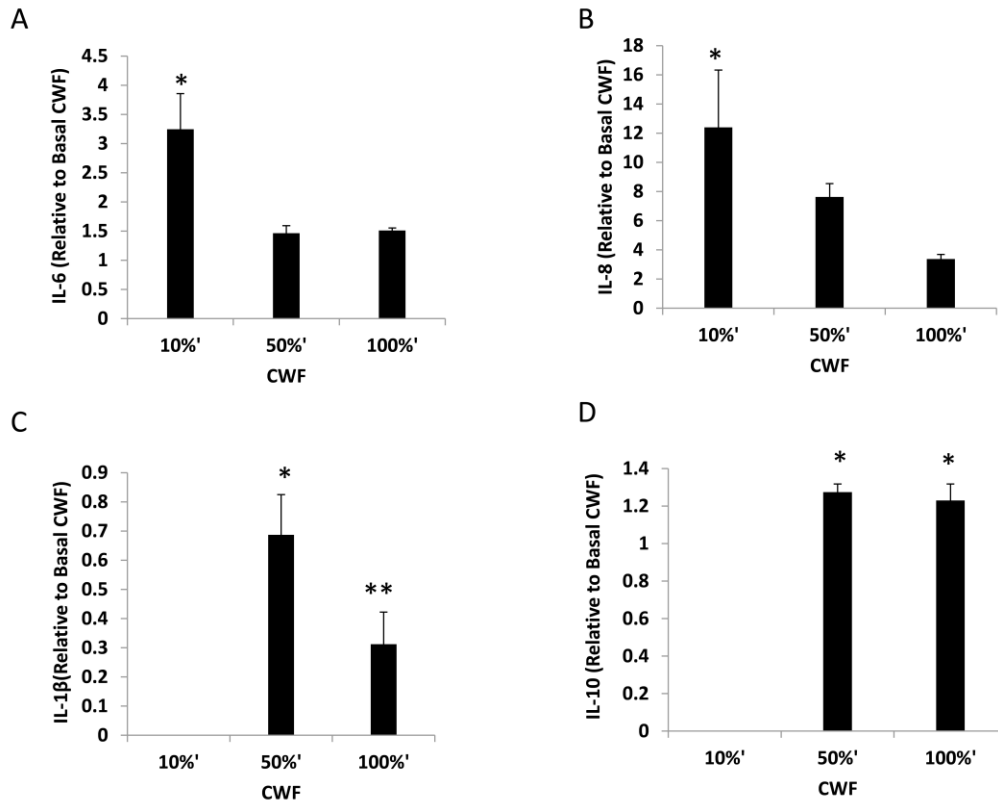


Figure 3. CWF up-regulate MSC secretion. Accumulated secreted factors by MSCs (10×10^3 cells/ml) after 72hrs in 10%, 50% or 100% CWF. (A) IL-6. N=6. *:p<0.01 compared to other conditions. (B) IL-8. N=6. *:p<0.05 compared to the 100% CWF. (C) IL-1 β . N=6. *:p<0.05 compared to the other conditions. **:p<0.05 compared to 10% CWF. (D) IL-10. N=6. *:p<0.0001 compared to 10% CWF.

2.4.4 Hypoxia differentially regulates MSC secretion

The culture conditions were designed to ensure that oxygen or the lack thereof was the only stimulus for MSCs. Evaluation of MSC-derived paracrine factors shows no significant differences in pro-inflammatory mediators when MSCs were cultured in normoxic vs. hypoxic conditions (Figure 4A-B). There were no significant

differences in secretion of anti-inflammatory mediators (Figure 5A-B) but there was a significant increase in secretion of the tissue repair TGF- β 1 (Figure 5C).

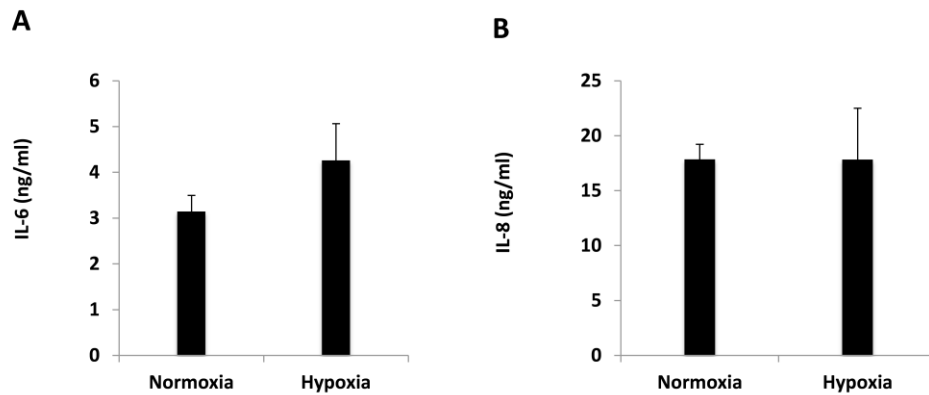


Figure 4. Hypoxia does not affect MSC secretion of pro-inflammatory mediators. Accumulated secreted factor by MSCs (2.5 x 10⁵ cells/ml) after 48hrs in normoxia or hypoxia. (A) IL-4. N=5. (B) IL-8. N=8.

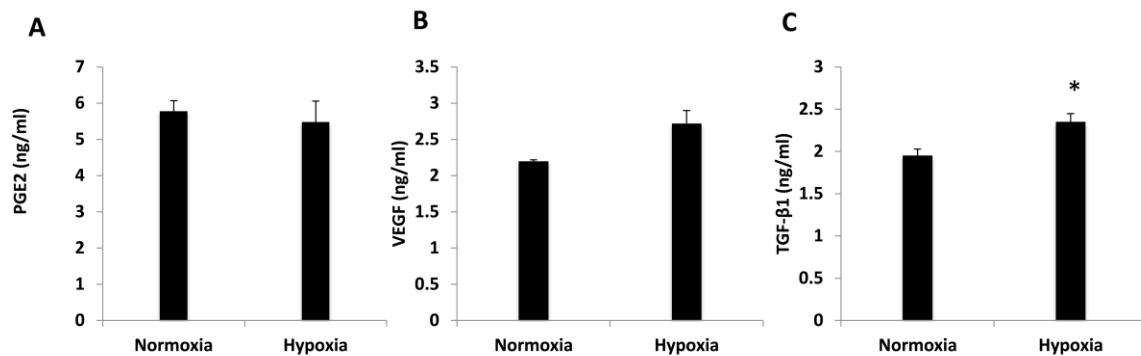


Figure 5. Hypoxia differentially regulates MSCs secretion of tissue repair growth factors. Accumulated secreted factor by MSCs (2.5 x 10⁵ cells/ml) after 48hrs in normoxia or hypoxia. (A) PGE2. N=4. (B) VEGF. N=3. (C) TGF- β 1. N=6.

*:p<0.05 compared to normoxia based on Student's t-test.

2.5 CONCLUSION

We designed a technique to immobilize MSCs in alginate-flat sheets. We found that the alginate microenvironment maintained over 90% cell viability and there was no difference in cell secretion when the cells were immobilized in alginate sheets compared to cells grown in monolayers on tissue culture plastic. As they can conform to the wound, these dressings will subsequently be used in in vivo studies to determine the efficacy of MSCs as a cellular therapeutic for chronic wounds. However, as an initial assessment of the effect of the chronic wound environment on MSC function, we recapitulated this hostile environment in vitro using chronic wound fluid (CWF) and hypoxia.

MSCs were cultured in CWF obtained from patients suffering from long-term, non-healing diabetic foot ulcers. We measured high levels of pro-inflammatory mediators in the CWF but when MSCs were cultured in concentrated CWF their production of these pro-inflammatory factors was suppressed. MSCs produced less IL-6, IL-8 and IL-1 β in 100% CWF but the CWF stimulated the same cells to produce significantly higher levels of IL-10. The data suggest that MSCs can modulate chronic inflammation by up-regulating their production of anti-inflammatory factors, which also act to modulate chronic inflammation.

We cultured MSCs in a hypoxic environment at low oxygen tensions found in chronic wounds. Compared to cells cultured in normoxia, hypoxia did not alter MSC secretion of pro-inflammatory mediators, anti-inflammatory mediators or angiogenic mediators but significantly increased TGF- β 1 production. TGF- β 1 promotes tissue repair and plays a role in all phases of wound healing. TGF- β 1

regulates fibroblast activity, facilitates collagen deposition, collagen remodeling and wound contraction. Overall, the data suggest that the hostile environment of chronic wounds does not have a deleterious effect on MSCs but in fact stimulate the cells to decrease inflammation.

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CHAPTER 3: MESENCHYMAL STROMAL CELLS PROMOTE ANTI-INFLAMMATORY PROPERTIES IN HYPOXIC MACROPHAGES

Note: This chapter will be reproduced from the thesis for the following publication:

Faulknor R, Przyborowski Olekson M, Berthiaume F. Mesenchymal stromal cells promote anti-inflammatory properties in hypoxic macrophages. *To be submitted to Journal of Cellular Physiology*.

3.1 INTRODUCTION

Skin heals by a series of events including an inflammatory phase to recruit immune cells that kill bacteria and remove dead cells, followed by a proliferation phase where the cells that reform the damaged skin components proliferate and migrate [1]. The mechanisms that control the transition from the inflammatory to the proliferative phase are complex and multi-faceted, and recent evidence suggests that macrophages play a key role in coordinating this process. For example, macrophages present at the wound site switch from a classically activated M1 to an alternatively activated M2 phenotype to resolve the inflammation [1-3]. M1 macrophages secrete high levels of proteases and pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α and interleukin (IL)-12 [2, 3]. In contrast, M2 macrophages secrete low levels of TNF- α , high levels of the anti-inflammatory cytokine interleukin (IL)-10, and other growth factors and chemokines that regulate fibroblast, endothelial cell and keratinocyte migration into the wounds. These cells play a role in the proliferation phase of wound healing [2, 3].

Disruption in the wound healing process can lead to non-healing chronic wounds. Although chronic wounds may have different etiologies, a common denominator is impaired blood flow that causes tissue hypoxia, impaired cellular functions, and ultimately cell death [1, 4]. As a result, chronic wounds appear to be “stuck” at the inflammatory stage [1, 4]. Several studies have shown that macrophages isolated from chronic venous ulcers or diabetic ulcers exhibit prolonged M1 phenotype [3]. In vitro studies suggest that hypoxic macrophages fail to differentiate into the M2 phenotype to initiate the tissue repair process [3]. Furthermore, ischemia impairs some of the bacterial killing mechanisms, such that ischemic chronic wounds are often infected [5]. The continual presence of bacterial-derived products also promotes persistent secretion of pro-inflammatory factors by macrophages [3, 5].

Recent studies have shown that adult bone marrow-derived mesenchymal stromal cells (MSCs) secrete soluble factors that promote macrophage differentiation from the M1 to the M2 phenotype in vitro, including prostaglandin E2 (PGE2) [2, 6]. Furthermore, there is evidence that MSCs may promote wound healing in experimental animals, albeit these studies were carried out in wounds that are expected to experience normoxic conditions [6-8]. Here we asked the question whether the effect of MSCs on macrophage M1 to M2 transition is affected under conditions of hypoxia, as would be expected to occur in human chronic wounds.

3.2 MATERIALS AND METHODS

3.2.1 Cell culture maintenance

Human bone-marrow derived MSCs were purchased from the Institute of Regenerative Medicine at Texas A&M at passage 1 and cultured as previously described [9]. Briefly, MSCs were cultured in Roswell Park Memorial Institute (RPMI; Life Technologies, Grand Island, NY) 1640 medium, supplemented with 10% v/v fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA), 1% v/v penicillin-streptomycin (pen-strep; Life Technologies) and 4mM L-glutamine (Life Technologies). Passage 3 cells were cultured until 70% confluency and immobilized in alginate sheets as previously described in chapter 2. MSC-conditioned medium (MSC-CM) to be used in the subsequent studies was made by culturing the immobilized MSCs in RPMI 1640 medium supplemented with 10% v/v FBS, 1% v/v pen-strep and 4mM L-glutamine, at a concentration of 2.5×10^5 cells/ml for 48 hours at 37°C in 5% CO₂ atmosphere balanced with air.

Primary peripheral blood-derived macrophages were isolated as described previously [10]. Briefly, human whole blood was purchased from the Blood Center of New Jersey (East Orange, NJ) and mononuclear cells were isolated from the peripheral blood by density gradient centrifugation. Once the mononuclear cells were isolated using 1.077 g/ml ficoll paque (GE Healthcare, Rahway, NJ), CD14⁺ monocytes were isolated using magnetic activated cell sorting. 10 million CD14⁺ monocytes were cultured in a 175 cm² tissue culture flask in advanced RPMI 1640 medium supplemented with 10% v/v FBS, 1% v/v pen-strep and 4mM L-glutamine for 2 hrs to allow for cell attachment. Then, cells were washed with phosphate

buffered saline (PBS) to remove non-adherent cells. CD14⁺ monocytes were then cultured in advanced RPMI 1640 medium with 10% v/v FBS, 1% v/v pen-strep and 4mM L-glutamine, supplemented with 5ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF; R&D, Minneapolis, MN) for 7 days. CD14⁺ monocytes stimulated with GM-CSF produce a subset of macrophages expressing M1 phenotype [11]. After 7 days in culture, cells were detached from the flasks using trypsin-ethylene diamine tetraacetic acid (EDTA; Life Technologies) and 1×10^6 cells/cryovial were frozen in 1ml advanced RPMI medium with 40% v/v FBS, 1% v/v pen-strep and 4mM L-glutamine, supplemented with 10% dimethyl sulfoxide (DMSO) for future experiments.

3.2.2 Macrophage dose response

Human peripheral blood-derived macrophages (5×10^4 cells/ml) were cultured in advanced-RMPI medium with 10% v/v FBS, 1% v/v pen-strep and 4mM L-glutamine, with increasing concentrations of exogenous PGE2 (Cayman Chemicals; Ann Arbor, MI) or lipopolysaccharide (LPS; Sigma) in normoxia or hypoxia for 48 hrs. Then, medium was collected from the cells and assayed for TNF- α and IL-10 using commercially available enzyme-linked immunosorbent assay (ELISA) kits (BioLegend, San Diego, CA).

3.2.3 Macrophage co-culture assay

Human peripheral blood-derived macrophages (5×10^4 cells/ml) were plated onto 3 μ m pore size, 24mm Transwell® membrane (Corning; Corning, NY) in RPMI 1640

medium with 10% v/v FBS, 1% v/v pen-strep and 4mM L-glutamine, and allowed to attach overnight. Then, the medium on the macrophages was changed to medium with LPS and immobilized MSCs or alginate sheets without cells were placed at the bottom of the Transwell® in a 6cm² well at a concentration of 2.5x10⁵ cells/ml. The cells were cultured together for 48 hrs at 37°C under normoxia (5% CO₂, 21% O₂, balance N₂) or hypoxia (5% CO₂, 1% O₂ balance N₂) [12] in a gas controlled chamber. Media were collected from the Transwell® and bottom well and assayed for anti-inflammatory and pro-inflammatory cytokines.

3.2.4 Cytokine secretion measurement

Immobilized MSCs were cultured in RPMI 1640 medium with 10% v/v FPS, 1% v/v pen-strep and 4mM L-glutamine for 48 hrs under normoxic or hypoxic conditions at 37°C. The cell supernatant was collected and assayed for PGE₂, TNF-α, IL-6, IL-10, TGF-β₁, and VEGF using commercial ELISA kits (BioLegend).

3.2.5 Cellular viability measurement

Macrophages were plated in 6-well (5 x 10⁴cells/transwell) plates and cultured in normoxia or hypoxia for 48 hrs. The cells were incubated at 37°C with 3 μM calcein-AM + 6 μM ethidium homodimer-1 and the nucleus counterstained with Hoechst 33342 (Life Technologies) for 15 min in advanced RPMI 1640 with 10% v/v FBS, 1% v/v pen-strep and 4mM L-glutamine. The cells were washed briefly with medium and imaged on an Olympus fluorescent microscope using 10X objective. Slidebook software (Intelligent Imaging Innovations, Denver, CO) was used to quantify the

number of live (green fluorescence), dead (red fluorescence) cells and total (blue fluorescence) cells to calculate the percent viability as described elsewhere [12, 13].

3.2.6 Western blotting analysis

Human peripheral blood-derived macrophages (2×10^5) were treated with 1X RIPA Buffer (Thermo Scientific, Waltham, MA), in the presence of 1% protease inhibitor, 1% EDTA and 1% phosphatase inhibitor and then detached from the well using a cell scraper. Total protein content was determined using a bicinchoninic acid protein assay (Thermo Fisher). Equal amounts of total protein were separated by 10% SDS-PAGE gel (Bio-Rad, Hercules, CA) followed by blotting to nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% w/v non-fat milk (Bio-Rad) or 5% w/v bovine serum albumin (BSA; Sigma) in Tris-buffered saline-Tween 20 for 2 hrs and then incubated with a polyclonal anti-hypoxia inducible factor-1 α (HIF-1 α) antibody (Abcam, Cambridge, MA) or anti-phospho-p42/44 MAPK (Cell Signaling Technologies; Danvers, MA) at 4°C overnight. After washing, the membrane was incubated with goat to rabbit secondary antibody for 1 hour at room temperature. Signals were detected by staining the membrane with SuperSignal™ west pico chemiluminescent (Thermo Fisher) for 5 min and bands were digitized with a scanner.

3.2.7 Immunocytochemistry

Human peripheral blood-derived macrophages (5×10^4 cells) were cultured on glass bottom 24-well plates for 48 hrs in MSC-CM or medium with LPS alone in normoxia

or hypoxia. Cells were washed with PBS and fixed with 4% w/v paraformaldehyde for 15 min. Cells were washed 3x and incubated in 1% w/v BSA (Sigma) + 10% v/v goat serum (Sigma) + 0.3M w/v glycine (Sigma) + 0.1% v/v Tween-20 (Sigma) in PBS for 1 hr to permeabilize the cell membranes and to block non-specific protein-protein binding. Cells were washed 3x with 5 min incubation in between washes and stained for CD206 using anti-mannose receptor antibody (1 μ g/ml; Abcam) overnight at 4°C. Following primary antibody staining, cells were washed 3x for 5 min and incubated with the secondary antibody. The cells were stained with Alexa fluor 488 goat anti-rabbit IgG (green; Abcam) at 1/1000 dilution and the nucleus counterstained using Hoechst 33342 (blue; Life Technologies) at 1/5000 dilution for 1 hr. Cells were imaged on an Olympus fluorescent microscope using 10X objective. Slidebook software (Intelligent Imaging Innovations, Denver, CO) was used to quantify the total number of cells (blue fluorescence) and CD206⁺ (green fluorescence) cells and calculate the percent of CD206⁺ cells.

3.3 STATISTICAL ANALYSIS

All numerical results are presented as means \pm standard error of the mean (SEM). Statistical analysis of three or more independent experiments were assessed using two-tail Student's t-test or one-way analysis of variance (ANOVA) followed by Fischer post hoc analysis where $p < 0.05$ represents statistical significance.

3.4 RESULTS

3.4.1 Hypoxia and LPS regulate macrophage secretion

Primary macrophages were differentiated from monocytes isolated from human whole blood and treated with GM-CSF to acquire the M1 phenotype [10]. These cells were cultured under hypoxia, at a level similar to that found in chronic wounds (1% v/v oxygen), to assess any changes in function and ability to differentiate into the M2 phenotype. After 48 hrs under hypoxia there was a significant increase in HIF-1 α expression (Figure 1A), a marker for cellular adaptation to low oxygen tension. We also observed that after 48 hrs under normoxia or hypoxia, macrophages remained over 90% viable with no significant difference in viability between the two groups (Figure 1B). Macrophages are activated by microbial stimuli to produce factors involved in the inflammatory phase of wound healing. LPS is known to activate macrophages to produce cytokines and growth factors [2]. We performed a dose response to determine the concentration of LPS that significantly increases macrophage secretion of TNF- α and IL-10 (Figure 1C-D). In normoxia, 1 μ g/ml of LPS induced a 167-fold increase in TNF- α production, and a similar 164-fold increase in hypoxia, compared to cells cultured without LPS (Figure 1C). For IL-10 secretion, 1 μ g/ml of LPS induced a 341-fold increase under normoxia, and a 66-fold increase under hypoxia (Figure 1D). Therefore, an LPS concentration of 1 μ g/ml was used to activate macrophages for subsequent studies.

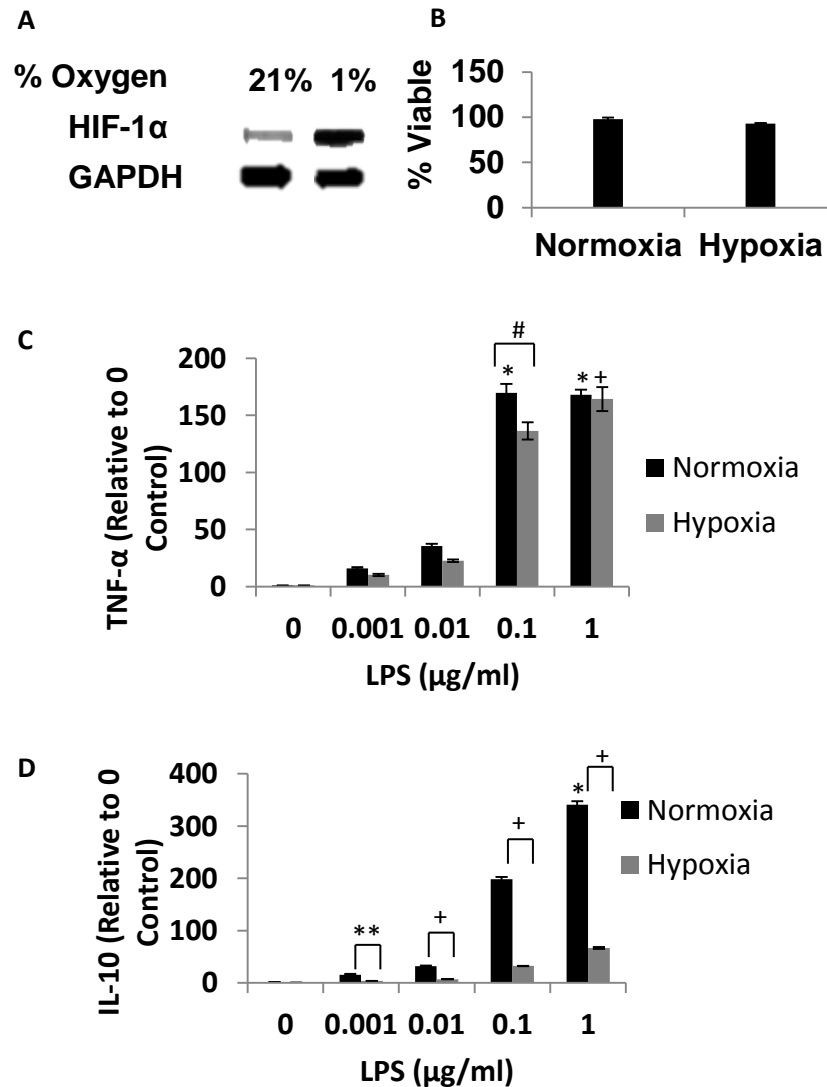


Figure 1. Hypoxia and LPS regulate macrophage secretion. Macrophages derived from human whole blood were incubated for 48 hrs under hypoxia (1% v/v O₂) or normoxia (21% v/v O₂). (A) HIF-1α protein levels as determined by Western blot analysis. GAPDH was used as loading control. (B) Percent cell viability as determined by fluorescent live/dead staining. Calcein AM⁺ and EthD-1⁻ cells were deemed viable. N=3. (C) TNF-α secreted by macrophages (50,000 cells/ml) to increasing concentrations of LPS. N=4. *:p<0.0001 compared to all LPS concentrations < 0.1 μg/ml in normoxia.

+:p<0.001 compared to all LPS concentrations < 1 µg/ml in hypoxia. #:p<0.0001. (D) IL-10 secreted by macrophages (50,000 cells/ml) to increasing concentrations of LPS. N=4. *:p<0.0001 compared to all LPS concentrations < 0.1 µg/mL. **:p<0.001. +:p<0.0001.

3.4.2 LPS regulates MSC secretion

While LPS is a stimulus for immune cells, we also investigated its effect on MSC secretion. MSCs were cultured in normoxia for 48 hrs with or without LPS (1ug/ml). MSCs secreted very little TNF- α or IL-10 as noted by the low picogram levels in Figure 2A-B. MSCs have not been reported to secrete those factors, which are primarily secreted by immune cells [2]. In addition, LPS did not affect MSC secretion of TGF- β 1 (Figure 2C) or VEGF (Figure 2E). In contrast, LPS significantly increased IL-6 and PGE₂ secretion by MSCs (Figure 2D; 2F). These data are consistent with evidence that LPS binding to toll-like receptors (TLR) on MSCs upregulate their secretion of inflammatory mediators such as IL-6 and IL-8 but fail to induce IL-12 or TNF- α production [2].

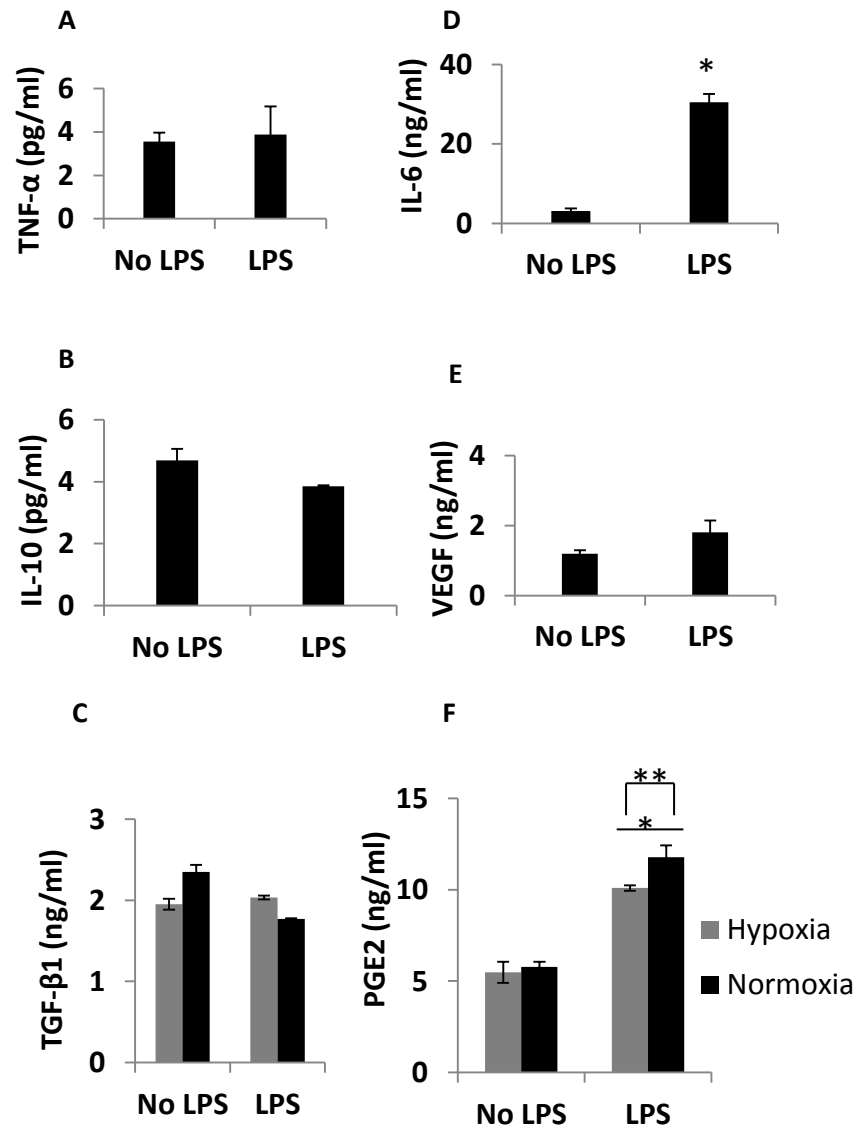


Figure 2. LPS regulates MSC secretion. Accumulated paracrine factors secreted by MSCs (2.5×10^5 cells/ml) after 48 hrs in culture with $1 \mu\text{g/ml}$ LPS or without LPS. All measurements were done under normoxic conditions, except otherwise noted. (A) TNF- α . N=6. (B) IL-6. N=3. *:p<0.003. (C) IL-10. N=6. (D) VEGF. N=6. (E) TGF- β 1. N=3. (F) PGE₂. N=4. *:p<0.0001 compared to no LPS in normoxia or hypoxia. **:p<0.05.

3.4.3 MSCs promote macrophage anti-inflammatory phenotype

Macrophages were co-cultured with MSCs in normoxia or hypoxia to assess MSCs' ability to modulate inflammation in hypoxia. Activated macrophages co-cultured with MSCs in a Transwell® under normoxia, secreted significantly less TNF- α than cells cultured without MSCs. There was an 81% decrease in secretion of TNF- α in the presence of MSCs and a 71% decrease in the presence of MSCs stimulated with LPS (Figure 3A). Conversely, these same cells secreted significantly more IL-10 than in the absence of MSCs. Activated macrophages secreted 138% more IL-10 in the presence of MSCs and 115% more in the presence of MSCs stimulated with LPS (Figure 3B).

MSCs were cultured with LPS (MSC-CM+LPS) or without LPS (MSC-CM) for 48 hrs and this conditioned medium was transferred onto activated macrophages that were then cultured under normoxia or hypoxia for 48 hrs. There was a significant increase in CD206⁺ cells when cultured in MSC-conditioned medium compared to cells cultured in medium with LPS alone (Figure 3C). There was a 1.7-fold increase in CD206⁺ macrophage population when cells were cultured in MSC-CM and a 1.8-fold increase when cells were cultured in MSC-CM+LPS (Figure 3C) compared to cells cultured in medium with LPS alone. There was no significant difference in CD206 expression when cells were cultured in MSC-CM or MSC-CM+LPS.

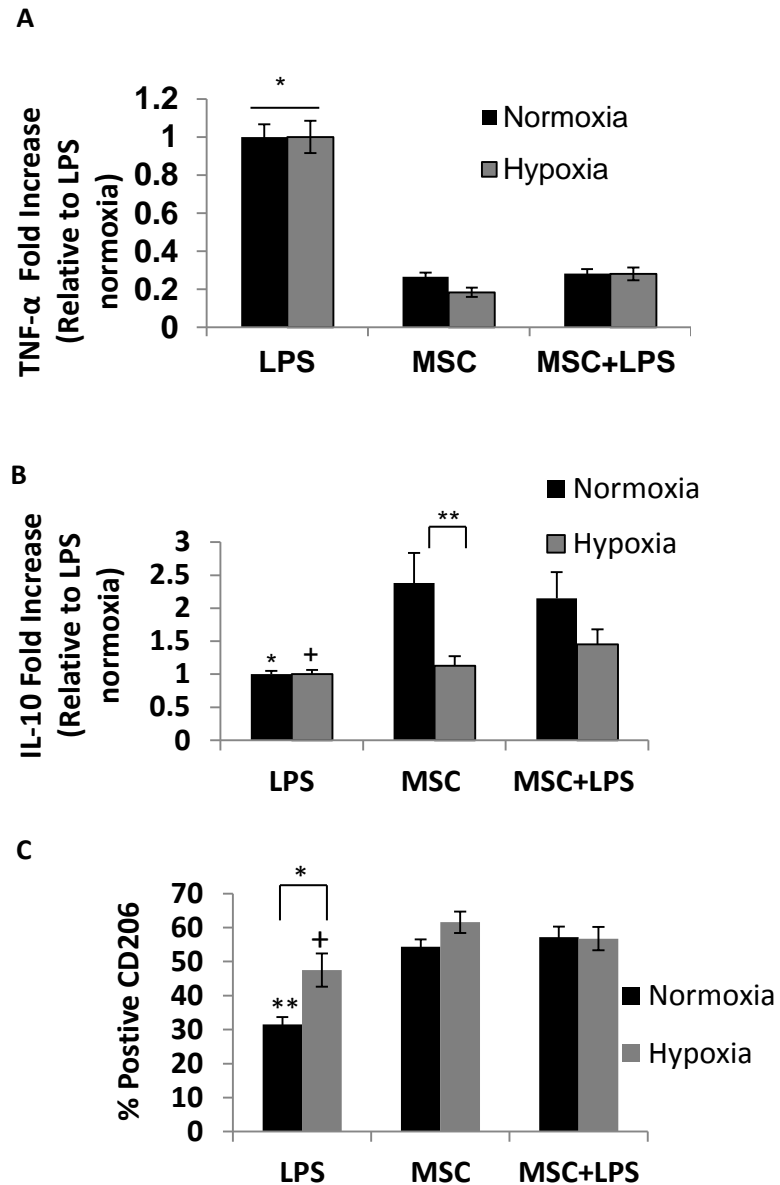


Figure 3. The ability of MSC secreted factors to promote M2 phenotype in macrophages is altered under hypoxia. Monocytes isolated from human whole blood were cultured with GM-CSF for 7 days, followed by culture in MSC-CM or co-cultured with MSCs for 48 hrs in normoxia or hypoxia. Medium was collected and assayed for TNF- α and IL-10 by ELISA. (A) TNF- α . N=6.

***:p<0.0001 compared to all MSC groups. (B) IL-10. N=6. *:p<0.01 compared to all MSC groups. +p<0.05 compared to MSC+LPS in hypoxia group. **:p<0.01. (C) CD206 expression on macrophages cultured in normoxia or hypoxia in medium with LPS alone (control) or MSC-CM from MSCs cultured with or without LPS. N=6. **:p<0.0001 compared to MSC groups in normoxia. +:p<0.01 compared to MSC no LPS group (MSC-CM) in hypoxia. *:p<0.001. No significant difference between the MSC and MSC+LPS groups.**

3.4.3 Hypoxia downregulates M2 macrophage secretion of IL-10

Activated macrophages co-cultured with MSCs in Transwell® secreted significantly less TNF- α and significantly higher levels of IL-10 (Figure 3A-B). There was also a significant increase in CD206⁺ cells in the presence of MSC-conditioned medium in hypoxia (Figure 3C). The data suggest that MSCs promoted macrophage differentiation from the M1 to the M2 phenotype needed to resolve inflammation and promote wound healing. However, IL-10 production, which increased by 115% when co-cultured with MSCs stimulated with LPS in the normoxic group, only increased by 45% in the hypoxic group as shown in Fig 3B. Similarly, there was a 138% increase in IL-10 production in normoxia when macrophages were co-cultured with unstimulated MSCs but only a 12% increase in hypoxia when compared to cells cultured in medium with LPS alone.

Macrophages produce IL-10 through the activation of the Erk signaling cascade. PGE2 binds to the EP4 receptor on macrophages and activate the MARK/Erk signaling pathway [14, 15]. We investigated if hypoxia downregulated

the Erk signaling pathway due to the decrease in IL-10 production by macrophages in hypoxia. Activated macrophages were cultured with different concentrations of exogenous PGE₂. By increasing the concentrations of PGE₂, macrophage production of TNF- α was significantly suppressed in a dose dependent manner in hypoxia or normoxia (figure 4A). In normoxia, 5ng/ml of PGE₂ significantly decreased macrophage TNF- α production but in hypoxia 10ng/ml was needed to significantly decrease TNF- α secretion. In normoxia, PGE₂ presence did significantly increase macrophage production of IL-10 but not in a dose dependent manner (figure 4B). There was no significant difference in macrophage secretion of IL-10 when treated with PGE₂ concentrations between 5 – 100ng/ml. However, under hypoxia there was only a significant difference in macrophage secretion of IL-10 with PGE₂ concentrations of 10 and 100ng/ml (figure 4B). MSC secretion of PGE₂ is between 5-10ng/ml (Figure 2F) so the cells are secreting enough PGE₂ to elicit IL-10 production from the macrophages but in hypoxia this production is still less than the normoxic cells. Interestingly, activated macrophages cultured in hypoxia for 1 hour upregulated phosphorylated MAPK/Erk 1/Erk 2 expression compared to normoxic cultures (figure 4C). Even though there was the expected activation of the Erk signaling cascade, PGE₂ did not increase IL-10 production in hypoxia as seen in normoxia.

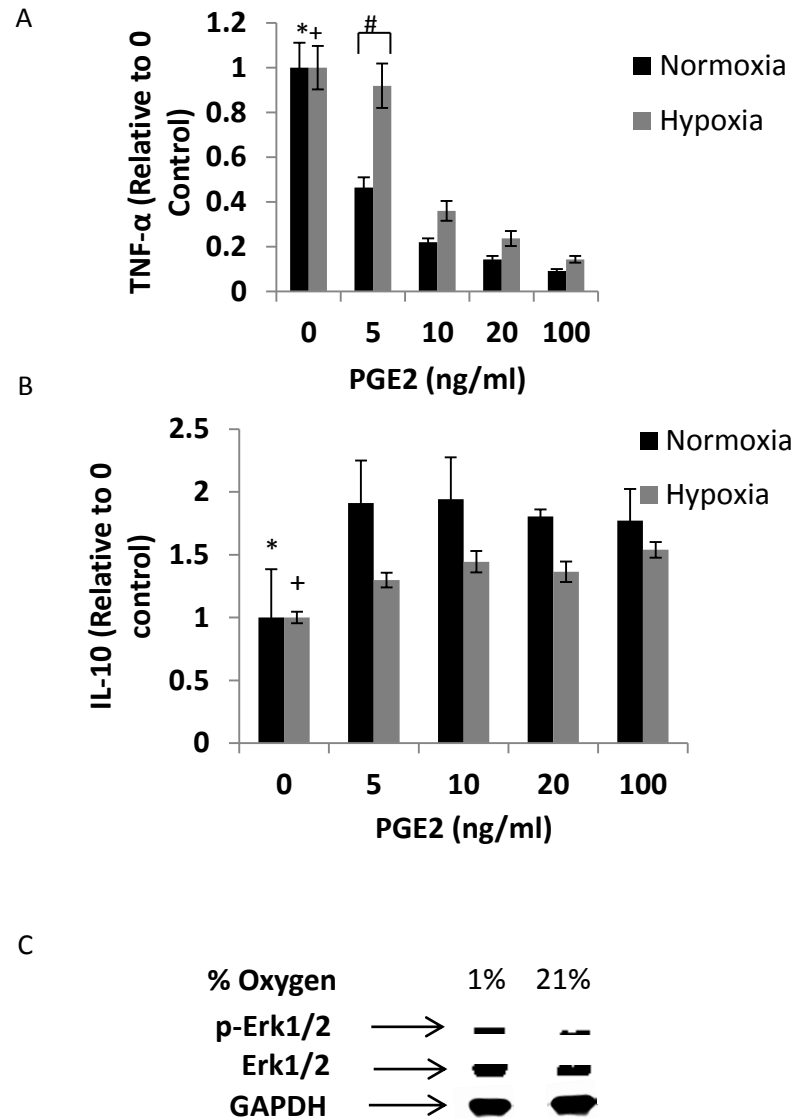


Figure 4. Hypoxia upregulates MAPK/Erk 1/Erk 2 phosphorylation and increases PGE₂-induced macrophage TNF- α production but decreases PGE₂-induced macrophage IL-10 production. Monocytes isolated from human whole blood were cultured with GM-CSF for 7 days, followed by culture in PGE₂ doses ranging from 0 to 100 ng/mL for 48 hrs under normoxia or hypoxia. Medium was collected and assayed for TNF- α and IL-10 by ELISA. (A) TNF- α . N=4. *:p<0.0001 compared to all other PGE₂ concentrations >0ng/ml in normoxia.

+:p<0.0001 compared to all other PGE2 concentrations >5ng/ml in hypoxia. #:p<0.0001. (B) IL-10. N=4. *:p<0.05 compared to all other PGE2 concentrations >0ng/ml. (C) Macrophages were harvested after 1 hr under normoxia or hypoxia. ERK 1/2 levels were measured by Western blot analysis. GAPDH was used as a loading control.

3.5 CONCLUSION

Macrophages transitioning from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype play a major role in the resolution of the inflammatory phase in wound healing. In chronic wounds, there is a delay in wound healing because the wound is in a prolonged inflammatory state. Impaired blood flow is a common pathology in pressure, venous and diabetic foot ulcers, which leads to tissue hypoxia and impaired cell function. We investigated how hypoxia affects M1 macrophage transition to the M2 phenotype needed to resolve inflammation.

In order to drive macrophage transition, M1 macrophages were co-cultured with MSCs in hypoxia or normoxia. Macrophages co-cultured with MSCs in normoxia or hypoxia had a significant suppression in TNF- α production but a significant increase in IL-10 production compared to cells cultured without MSCs. These macrophages co-cultured also had more CD206 expression, a surface marker for M2 macrophages. However, macrophages co-cultured with MSCs in normoxia secreted 115% more IL-10 than cells cultured without MSCs, while in hypoxia, there was only

a 45% increase in IL-10 production by macrophages co-cultured with MSCs compared to macrophages cultured without MSCs.

PGE2 is known to be the MSC-derived product that drives macrophage transition from the M1 to the M2 phenotype [6]. Treating the macrophages with varying concentrations of exogenous PGE2 showed no difference in IL-10 production in hypoxia. PGE2 was unable to elicit a significant increase in IL-10 production when the cells were cultured in hypoxia. These data suggested a possible impairment in the MAPK/Erk signaling pathway because upon activation there should have been an increase in IL-10 production. However, our data showed that the upregulation in MAPK/Erk phosphorylation was unaffected by hypoxia, suggesting that hypoxia does not affect the activation of the MAPK/Erk signaling pathway. Next, we plan to assess the effect of hypoxia on IL-10 macrophage gene expression to determine whether the impairment may be at the level of IL-10 transcription.

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CHAPTER 4: MSCS REVERSE HYPOXIA-MEDIATED SUPPRESSION OF ALPHA-SMOOTH MUSCLE ACTIN EXPRESSION IN HUMAN DERMAL FIBROBLASTS

Note: This chapter is adapted from the following publication:

R.A. Faulknor, et al., Mesenchymal stromal cells reverse hypoxia-mediated suppression of α -smooth muscle actin expression in human dermal fibroblasts, *Biochemical and Biophysical Research Communications* 458 (2015) 8-13. PMID: 25625213

4.1 INTRODUCTION

Cell proliferation and migration are impaired in chronic wounds due, at least partially, to the prevailing hypoxic conditions, which are especially exacerbated on the lower extremities [1-3]. In skin, fibroblasts play a dominant role in the tissue repair process by synthesizing collagen as well as by promoting wound contraction and closure via differentiation into α -smooth muscle actin (SMA)-expressing myofibroblasts [2]. However, under hypoxic conditions, fibroblasts exhibit decreased proliferation and migration, impaired differentiation into α -SMA-expressing myofibroblasts, increased senescence, and impaired collagen secretion [1, 3, 4]. This ultimately leads to delayed wound contraction and closure [5-7].

Recent preclinical studies have shown that mesenchymal stromal cells (MSCs) enhance acute skin wound healing [8-11] in small animal models. In the context of chronic wounds, there are currently five clinical trials registered with clinicaltrials.gov involving the use of MSCs as a treatment for diabetic foot ulcers. So

far published data from one of the studies showed that transplantation of MSCs temporarily improved microcirculation and wound healing in 18 out of 22 diabetic foot ulcer patients with critical limb ischemia [12]. Reports suggest that MSCs cultured in hypoxia quickly adapt to the low oxygen environment [13-15]. MSCs cultured in hypoxia exhibit an increase in proliferation and an increase in secretion of growth factors (e.g. VEGF and TGF- β) that play an important role in wound healing [13, 15] as seen in chapter 2. Studies also suggested that conditioned medium from MSCs cultured in hypoxia significantly increased the migration of dermal fibroblasts in vitro and wound healing in vivo [13, 15]. However, there are no reports on how MSC-secreted factors affect target cells exposed to prolonged hypoxia. In this study, we investigated the impact of soluble factors released by MSCs on the function of hypoxic fibroblasts cultured in collagen gels, used as models of skin dermis.

MSCs in suspension applied in vivo die or migrate away from the wound site within 7-14 days [10]. Application of MSC therapy requires consistent MSC numbers to be retained at the wound site, and treatment durations are likely to be on a time scale of weeks. MSCs have been successfully immobilized in alginate microcapsules, which have been shown to preserve their secretion of anti-inflammatory molecules, thus creating a continuous release device that can modulate local immune responses and promote tissue repair [16]. In this study, we modified the original immobilization technique to a flat sheet that would be more easily secured onto actual wounds [refer to chapter 2]. Using immobilized MSCs, we report that TGF- β_1 released by MSCs is a key factor that reverses many of the hypoxia-induced

impairments in fibroblast function such as decreased differentiation into α -SMA-expressing myofibroblasts and ultimately wound contraction.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture maintenance

Human bone-marrow derived MSCs were purchased from the Institute of Regenerative Medicine at Texas A&M at passage 1 and cultured as previously described [16]. Briefly, MSCs were cultured in α -minimal essential medium (α -MEM; Life Technologies, Grand Island, NY), containing no deoxyribo- or ribo- nucleotides, supplemented with 10% v/v fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA), 1% w/v penicillin-streptomycin (pen-strep; Life Technologies), 4mM L-glutamine (Life Technologies) and 1ng/ml basic fibroblast growth factor (bFGF; Life Technologies). Passage 3 cells were cultured until 70% confluency and immobilized in alginate sheets as described in chapter 2. MSC-conditioned medium (MSC-CM) to be used in the subsequent studies was made by culturing the alginate-embedded MSCs in low serum DMEM (0.1% v/v FBS and 1% w/v pen-strep) at a concentration of 5×10^5 cells/ml for 48 hrs at 37°C in 5% CO₂ atmosphere balanced with air.

Human dermal fibroblasts were donated from the W.M. Keck Center (Rutgers University, Piscataway, NJ) at passage 4. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% v/v FBS and 1% w/v pen-strep until confluence.

4.2.2 Fibroblast contraction assay

Human dermal fibroblasts (1×10^5 cells) were mixed with 600 μ L type I collagen (BD Biosciences, San Jose, CA) at pH 7 and a concentration of 1.5mg/mL and added to each well of a 24-well plate. Plates were incubated for 30 min at 37°C to induce collagen gelation, thus forming fibroblast-populated collagen lattices (FPCLs) [17], and then cultured overnight in 1ml of low serum DMEM. The FPCLs were then switched to treatment conditions, which consisted of 1ml of one of the following: MSC-CM, low serum DMEM + 1ng/mL of TGF- β 1 (R&D, Minneapolis, MN), or low serum DMEM with no other supplements. The FPCLs were then cultured at 37°C under normoxia (5% CO₂, 21% O₂, balance N₂) or hypoxia (5% CO₂, 1% O₂ balance N₂) for 48 hours [18] in a gas controlled chamber. After 48 hrs and without changing the medium on the cells, the FPCLs were shortly removed from the chamber, manually detached from the well edges in order to initiate contraction, and returned to the chamber to continue hypoxia or normoxia. Top down images of the FPCLs were taken after 24 and 48 hrs to reveal their cross sectional areas using a digital camera, which were later quantified to calculate the extent of contraction.

For inhibition studies, FPCLs were treated for 1 hr with 10 μ M SB431542 (Sigma), a potent and specific inhibitor of TGF- β 1 activin receptor-like kinase, or 0.38% dimethyl sulfoxide (DMSO) vehicle, before switching the cells to treatment conditions (MSC-CM, low serum DMEM + 1ng/mL of TGF- β 1, or low serum DMEM supplemented with DMSO or SB431542) for 48 hrs. Contraction was induced and measured for 48 hrs as described above.

4.2.3 Cell viability determination

Viability of fibroblasts in the FPCLs was determined after 96 hrs of culture in normoxia or hypoxia. The cells were incubated at 37°C with 3 μ M calcein-AM + 6 μ M ethidium homodimer-1 (Life Technologies) for 30 min in basal medium and the nuclei counterstained with Hoechst 33342 (Life Technologies). The cells were washed 5 times with medium before imaging on an Olympus IX81 spinning disc confocal microscope with a 10X objective. Five serial 500 μ m optical slices at 20 μ m intervals were taken per experimental condition. Slidebook software (Intelligent Imaging Innovations, Denver, CO) was used to quantify the number of live (green fluorescence) and dead (red fluorescence) cells and calculate the percent viability as described elsewhere [19].

4.2.4 Western immunoblotting analysis

FPCLs were homogenized for cell extraction using a 2ml Dounce tissue homogenizer (Sigma) and 1X RIPA Buffer (Thermo Scientific, Waltham, MA), in the presence of 1% protease inhibitor and 1% ethylene diamine tetraacetic acid (EDTA). Total protein content was determined using a bicinchoninic acid protein assay (Thermo Fisher). Equal amounts of total protein were separated by 10% SDS-PAGE gel (Bio-Rad, Hercules, CA) followed by blotting to nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% w/v non-fat milk (Bio-Rad) in Tris-buffered saline-Tween 20 for 2 hrs and then incubated with a polyclonal anti- α -SMA (Abcam, Cambridge, MA) and anti-hypoxia inducible factor (HIF)-1 α (Abcam) at 4°C

overnight. After washing, the membrane was incubated with goat to rabbit secondary antibody for 1 hr at room temperature. Signals were detected by staining the membrane with SuperSignal™ west pico chemiluminescent (Thermo Fisher) for 5 min and bands were digitized with a scanner.

4.3 STATISTICAL ANALYSIS

All numerical results are presented as means \pm standard error of the mean (SEM). Statistical analysis of three or more independent experiments were assessed using two-tail Student's t-test or one-way analysis of variance (ANOVA) followed by Fischer post hoc analysis where $p < 0.05$ represents statistical significance.

4.4 RESULTS

4.4.1 Hypoxia decreases fibroblast differentiation

Hypoxia has been shown to decrease the expression of α -SMA in dermal fibroblasts (7). We embedded fibroblasts in type I collagen to create FPCLs that simulate the dermal layer of the skin. Ensuing FPCL contraction was used as an in vitro wound contraction model. FPCLs were switched to 1% O₂ tension to replicate the hypoxic environment typical of chronic wounds. HIF-1 α expression increased after 4 hrs in the hypoxia chamber compared to normoxic controls, which confirms that the cells were responding to the hypoxic stress (Figure 1A). Hypoxic culture did not affect viability, as the cells in the FPCL remained over 90% viable after 96 hrs in culture

(data not shown). However, FPCL gel area, which decreased to 70% initial area in the normoxic group (-hypoxia/-TGF- β 1/-MSC-CM), only decreased to 90% initial area in the hypoxic group (+hypoxia/-TGF- β 1/-MSC-CM) as shown in Figure 1B. Consistent with this observation, the expression of α -SMA, a marker of differentiation into myofibroblasts, which are important mediators of ECM contraction, decreased compared to the normoxic control (Figure 1C).

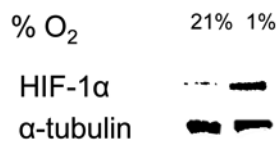
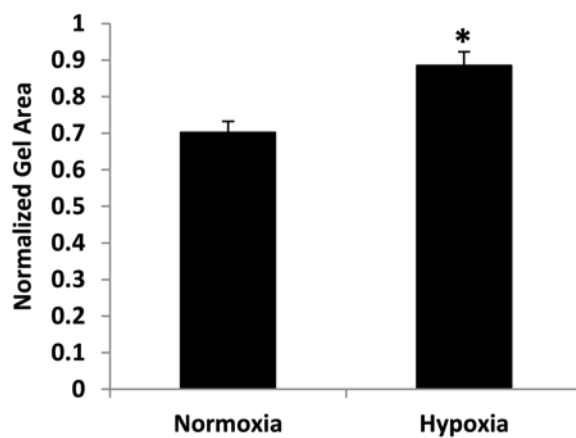
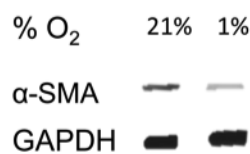
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Figure 1: Hypoxia induces HIF-1 α while inhibiting α -SMA expression and FPCL contraction. Fibroblast-populated collagen lattices (FPCLs), each containing 1×10^5 normal human dermal fibroblasts seeded in 200 μ L collagen type I (1.5 mg/ml), were cultured in low serum medium and exposed to normoxia (21% v/v O₂) or hypoxia (1% v/v O₂). (A) FPCLs were harvested after 4 hours of incubation and analyzed by Western blot for HIF-1 α and α -tubulin (B) FPCL area was measured after 48 hrs of incubation and normalized to initial area. N=6. *:p<0.01 based on Student's t-test. (C) FPCLs were harvested after 96 hrs of incubation and levels of α -SMA and GAPDH measured by Western blotting.

4.4.2 MSCs reverse hypoxia-mediated impairments in FPCL contraction via TGF- β 1

FPCLs exposed to MSC-CM (-hypoxia/-TGF- β 1/+MSC-CM) exhibited a significant increase in contraction compared to standard culture (-hypoxia/-TGF- β 1/-MSC-CM), as the remaining gel area decreased to 52% initial area in the former, compared to 70% in the latter (Figure 2A). Furthermore, this effect persisted under hypoxic conditions where FPCLs in hypoxia alone (+hypoxia/-TGF- β 1/-MSC-CM) increased gel area to 90% initial area, and in the presence of MSC-CM, this area decreased to 65% (+hypoxia/-TGF- β 1/+MSC-CM). It is noteworthy that the latter value is similar to what was seen in the normal culture conditions (-hypoxia/-TGF- β 1/-MSC-CM); thus MSC-CM restored contraction of hypoxic gels to levels similar to normal culture conditions.

Among products known to be secreted by MSCs, TGF- β 1 was thought to be a plausible candidate to mediate this response since TGF- β 1 is known to promote

fibroblast differentiation into myofibroblasts [17, 20]. Addition of exogenous TGF- β 1 alone to FPCLs caused a dose-dependent increase in contraction rate, and doses ranging from 0.1 to 1 ng/mL led to contraction rates similar to that seen with MSC-CM (Figure 2B). We measured TGF- β 1 secretion by the MSCs cultured in low serum (0.1% FBS) medium, and found concentrations in the range of 0.6-1ng/ml (data not shown); which is consistent with the contraction response induced by the MSC-CM (Figure 4B).

To verify whether TGF- β 1 released by MSCs promotes FPCL contraction, FPCLs were incubated with MSC-CM in the presence of the TGF- β 1 inhibitor SB431542. In the presence of the inhibitor, FPCL remaining gel area increased from 62% in the MSC-CM + vehicle group to 83% initial in the MSC-CM + SB431542 group. Similarly, the inhibitor caused the gel area to remain at 95% initial with 1 ng/mL TGF- β 1, while 1 ng/mL TGF- β 1 alone decreased gel area to 60% (Figure 2C). Taken together, these data suggest that TGF- β 1 is a major MSC-derived factor that promotes FPCL contraction.

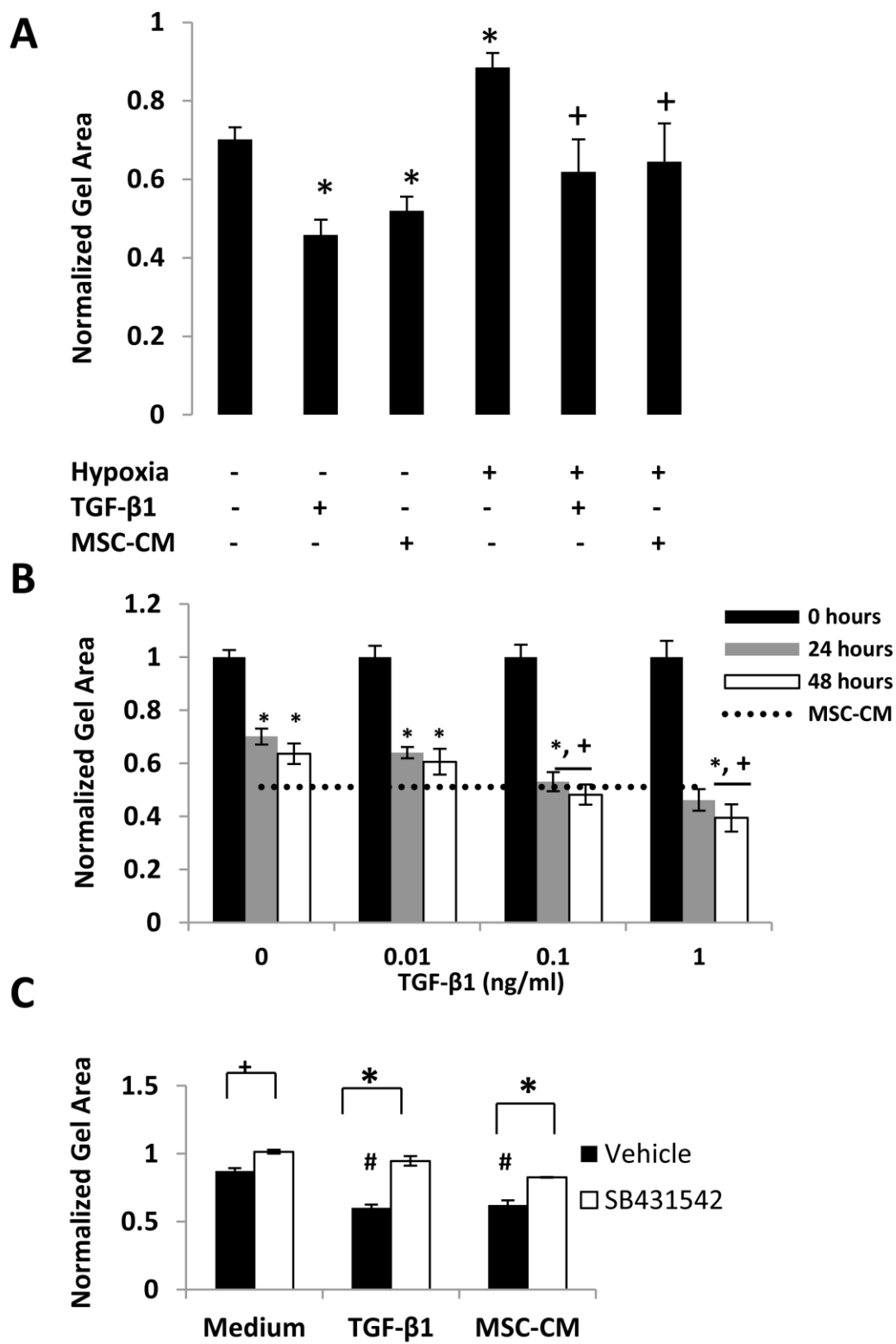


Figure 2: MSC-CM and recombinant TGF- β 1 promote fibroblast-mediated contraction. (A) FPCLs were placed in low serum medium, or the same supplemented with 1ng/ml TGF- β 1, or MSC-CM for 48 hrs and gel area was measured after 24 hrs of incubation. FPCL area was normalized to initial area. N=6. *:p<0.01 compared -hypoxia/-TGF- β 1/-MSC. +:p<0.05 compared to +hypoxia/-TGF- β 1/-MSC. Statistical significance determined by ANOVA. (B) FPCLs were treated with increasing concentrations of recombinant TGF- β 1 for 48 hrs and the extent of contraction was measured after 24 and 48 hrs of incubation. FPCL area at each time point was normalized to initial area. N=5. *:p<0.0001 compared to 0 hour time point. +:p<0.01 compared to the group receiving 0ng/ml TGF- β 1. Statistical significance determined by ANOVA. (C) FPCLs were treated with 10 μ M SB431542 or vehicle for 1 hr, and then switched to low serum medium, or the same supplemented with 1ng/ml recombinant TGF- β 1, or MSC-CM. After 48 hrs incubation, contraction was induced and measured after 24 hrs. FPCL areas at that time were normalized to initial area. N=4. *:p<0.0001. +:p<0.01. #:p<0.0001 compared to group receiving medium with vehicle. Statistical significance determined by ANOVA.

4.4.3 MSCs promote myofibroblast differentiation in FPCLs

Next we investigated whether MSC-CM promoted fibroblast expression of α -SMA, since exogenous TGF- β 1 is known to increase α -SMA expression in FPCLs [4]. α -SMA protein levels in FPCLs were indeed increased by both MSC-CM and TGF- β 1 (Figure 3A). Furthermore, fibroblasts rich in cytoskeletal F-actin also use the F-actin stress

fibers for wound contraction [17]. Fibroblasts in the collagen lattices were stained for F-actin using FITC-conjugated phalloidin. Fibroblasts cultured under normoxic conditions have an elaborate cytoskeletal network (Figure 3B-D). Under normoxia and in plain medium, we observed well-developed stress fibers (Figure 3B); however, under hypoxia with no other treatment (+hypoxia/-TGF- β 1/-MSC-CM), fibroblasts were spindle shaped and did not have an elaborate F-actin network (Figure 3E). Under normoxic conditions, there were no differences in the F-actin cytoskeleton when fibroblasts were treated with 1ng/ml TGF- β 1 (Figure 3C) or MSC-CM (Figure 3D). The stress fibers were prominent and elongated. In contrast, fibroblasts cultured under hypoxia and treated with MSC-CM had more compact linear bundles that are aligned and coursing one direction (Figure 3G) whereas cells treated with 1ng/ml TGF- β 1 had more loosely organized stress fibers that were less compact (Figure 3F).

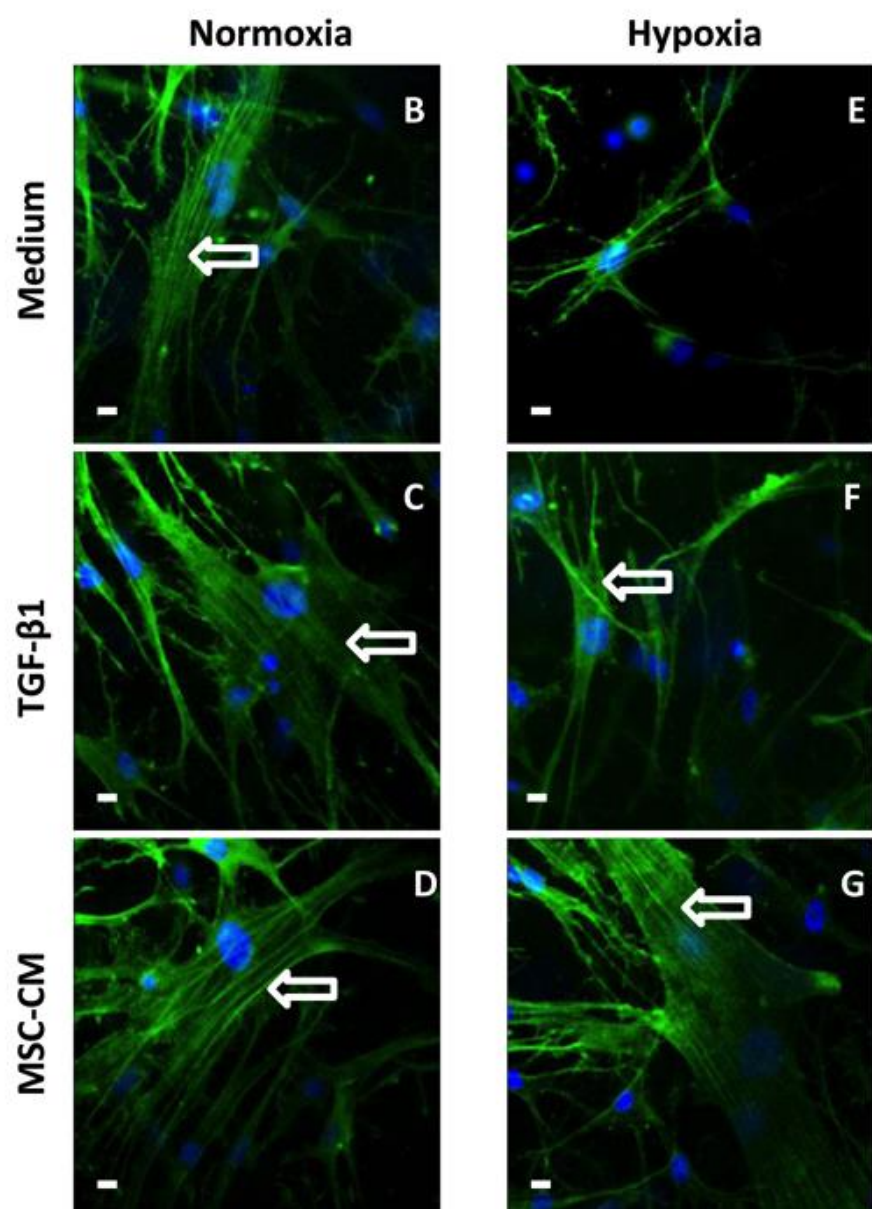
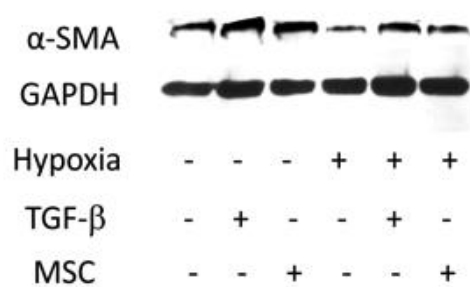
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Figure 3: MSC-CM and recombinant TGF- β 1 promote the differentiation of hypoxic fibroblasts. FPCLs were placed in low serum medium, or the same supplemented with TGF- β 1 (1 ng/mL), or MSC-CM, and incubated for 96 hrs under normoxia or hypoxia for 96 hrs. (A) Levels of α -SMA and GAPDH protein as determined by Western blotting. (B-G) The morphology of F-actin stress fibers in the fibroblasts was assessed by imaging FPCLs stained with FITC-conjugated phalloidin. Arrows indicate F-actin cytoskeleton. (B-D) Fibroblasts cultured under normoxic conditions have an elaborate F-actin network that is elongated and prominent. (E) Fibroblasts cultured in hypoxia with no other treatment are spindled shape without the elaborate F-actin network (F) Fibroblasts cultured in hypoxia and treated with 1ng/ml TGF- β 1 have larger stress fibers that are loosely organized (G) Fibroblasts cultured in hypoxia and treated with MSC-CM have large compact linear bundles that are aligned. Scale bar = 10 μ m.

4.5 DISCUSSION

While MSCs are multipotent cells [8-11], some of their therapeutic properties that appear most promising involve their secretion of immunomodulatory molecules with beneficial effects in cases of acute inflammation (e.g. spinal cord injury) as well as chronic inflammation (e.g. inflammatory bowel disease, diabetic foot ulcers) [12, 21]. Several studies have suggested that MSCs may promote skin wound healing also via their secreted products [22]. Since skin wounds are associated with hypoxic

conditions, we asked the question whether hypoxia alters the response of the wound cells targeted by the MSC-derived products. Among the soluble factors secreted by MSCs that promote wound healing, we focused on TGF- β 1, a potent regulator of fibroblast activity and all phases of wound healing [6, 17, 20, 23]. TGF- β 1 enhances the proliferation and migration of fibroblasts into the wound bed and induces the secretion of collagen and other components of the new ECM [17, 24]. Furthermore, it has been suggested that TGF- β 1 induces α -SMA expression in myofibroblasts, which enables them to generate the strong contractile forces needed to cause wound contraction [17, 20].

While hypoxia alone decreased both α -SMA expression in human dermal fibroblasts, as well as FPCL contraction, we report for the first time that human bone marrow-derived MSCs could overcome these changes. MSCs enhanced FPCL contraction by roughly the same magnitude under normoxia vs. hypoxia. This is interesting in as much as hypoxia could deplete ATP stores and in turn slow down the rate of any energy-requiring process [25], such as cell-mediated contraction of the FPCLs. Since MSC-CM, as well as TGF- β 1, could increase FPCL contraction in spite of the hypoxia, this suggests that cellular energy production was not limiting this process. Thus, local administration of MSCs or their secreted products may be a suitable alternative to reverse the deleterious effects of hypoxia. Furthermore, TGF- β 1 secretion by MSCs was increased under hypoxic conditions; (data shown in chapter 2), it is conceivable that this could also increase the potency of the MSCs in the chronic wound environment.

Much of the effects of MSCs could be reproduced when using recombinant TGF- β 1 at levels secreted by MSCs. A specific inhibitor of the TGF- β 1 pathway, SB431542, when used in presence of MSC-CM, significantly decreased FPCL contraction. Taken together, these data suggest that MSCs affect fibroblast differentiation to myofibroblasts via a TGF- β 1-mediated mechanism. It has been reported that TGF- β 1 induces α -SMA-expression in part by the SMAD signaling pathway [20, 26, 27]. When active TGF- β 1 binds to the TGF- β RII, it phosphorylates and forms a heteromeric receptor complex with TGF β RI. The activated complex then phosphorylates SMAD2 and SMAD3, which binds to SMAD4, followed by translocation into the nucleus where the complex increases gene transcription via DNA transcription factors [23, 26-28]. Another member of the SMAD family, SMAD7, is on the other hand an inhibitor of TGF- β 1 signaling [27, 28] which is induced by hypoxia via HIF-1 α expression. SMAD7 interacts with E3 ubiquitin ligases and recruits them to the TGF- β receptor (TGF- β R) complexes, which then degrade the phosphorylated TGF- β RI and subsequently decrease SMAD2 and SMAD3 phosphorylation. SB431542 prevents TGF- β RI phosphorylation, and thus effectively mimics the effect of hypoxia-induced increase SMAD7. Therefore, it is plausible that hypoxia may increase SMAD7, thus TGF- β 1 signaling, as shown by a decrease in FPCL contraction α -SMA expression under hypoxic conditions.

Other than activated HIF-1 α , inflammatory mediators such as tumor necrosis factor- α (TNF- α) have been reported to upregulate SMAD7 expression [26]. Chronic wounds exhibit prolonged high levels of inflammatory proteins [4-6]. MSCs are known to be immunomodulatory and through paracrine signaling decrease TNF- α

secretion by inflammatory cells [11, 29]. While MSCs cannot change local oxygen tension in tissue, they may benefit the wound healing process by secreting TGF- β 1 on the one hand, and decreasing the levels of pro-inflammatory mediators that inhibit the TGF- β 1 signaling pathway on the other hand.

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CHAPTER 5: IMMOBILIZED MESENCHYMAL STROMAL CELLS ENHANCE CHRONIC WOUND HEALING IN VIVO

Note: This chapter will be reproduced from the thesis for the following publication:

Faulknor R, Przyborowski Olekson M, Berthiaume F. Immobilized mesenchymal stromal cells enhance chronic wound healing in vivo. *To be submitted to Wound Repair and Regeneration*.

5.1 INTRODUCTION

Chronic wounds burden the US healthcare at a cost of \$25B per year, affecting 8 million Americans with 1.8 million new incidences annually [1, 2]. Advanced age, especially when combined with diabetes [3, 4], increases the risk of slow healing wounds, such as venous, diabetic, and pressure ulcers [5]. Such wounds are a major cause of amputation in diabetes, with age-adjusted amputation rates for diabetics at 5.5 per 1,000 vs. 0.2 per 1,000 for nondiabetic individuals [2]. The situation is worsening due to the rising prevalence of obesity and type II diabetes in our aging population.

Current treatments show variable clinical results due to the diversity in wound size and location, as well as failure to address underlying pathologies that impair healing: persistent inflammation; decreased cell migration and growth factor levels; impaired blood supply; inability to deposit a mechanically stable collagen matrix [6]. We hypothesize that these deleterious mechanisms can be ameliorated using exogenously applied mesenchymal stromal cells (MSCs), which are non-

hematopoietic stem cells that have been shown to modulate inflammation and promote regeneration. Human clinical trials have also shown no adverse reactions to allogeneic MSCs [7-10].

Although MSCs have the ability to differentiate into various cell types, the bulk of the evidence suggests that secreted products account for the majority of their beneficial effects on skin wound healing as well as in other injury and inflammation models [8, 11-13]. Recent studies have shown that adult bone marrow-derived MSCs secrete factors that promote macrophage transition from the M1 to the M2 phenotype *in vitro*, including prostaglandin E2 (PGE2) [13]. MSCs, as well as M2 macrophages, secrete transforming growth factor- β 1 (TGF- β 1), which is known to promote fibroblast proliferation and differentiation to myofibroblasts. Interestingly, *in vitro* studies also indicate that inflammatory mediators present in the wound environment stimulate MSCs to enhance their secretion of PGE2, which favors macrophage differentiation towards the pro-resolution M2 phenotype as seen in chapter 3. The subsequent decrease in inflammation causes MSCs to decrease PGE2 and increase TGF- β 1 secretion, which further drives the differentiation of fibroblasts into myofibroblasts [14]. Therefore, MSCs can alter their secretion profile in a coordinated manner as the wound transitions from the inflammatory to the proliferative phase (**Figure 1**).

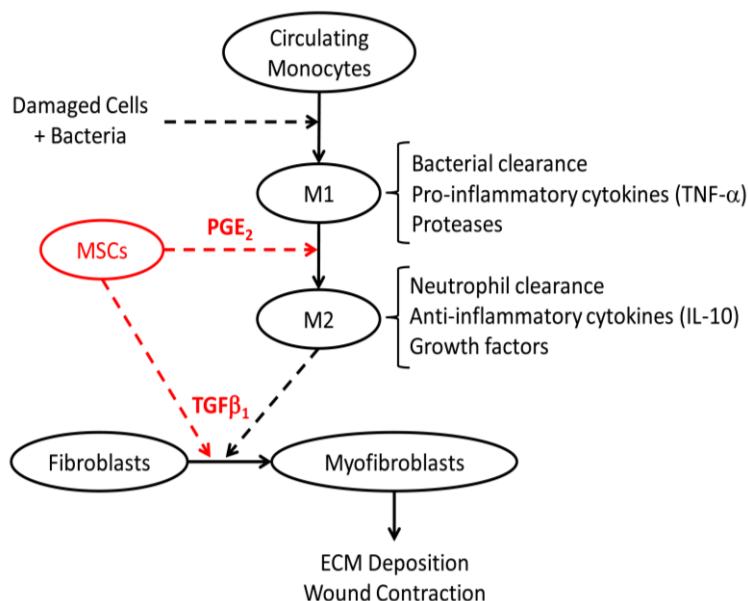


Figure 1. Effect of MSC-derived PGE₂ and TGF-β₁ on the phenotype of macrophages and fibroblasts in the wound. PGE₂ promotes the transition of macrophages from a pro-inflammatory M1 to a pro-resolution M2 phenotype [13]. MSCs and M2 macrophages can subsequently dampen inflammation by secreting anti-inflammatory cytokines, growth factors and chemokines [15]. MSCs also secrete TGF-β₁, which drives the differentiation of fibroblasts into α-smooth muscle actin (α-SMA)-expressing myofibroblasts, which are very active in extracellular matrix (ECM) deposition and are responsible for wound contraction [14].

5.2 MATERIALS AND METHODS

5.2.1 Excisional wounding

Male C57Bl/6 lepr db/db genetically diabetic mice were purchased from the Jackson Laboratory (Bar Harbor, ME) between 7-9 weeks of age. These mice have a defect in

the leptin receptor, are obese early on and experience the onset of diabetes between 4-8 weeks. Wounding surgeries were performed at 10 weeks of age and were approved by the Rutgers Animal Use and Care Committee. The day prior to the surgery, mice were anesthetized with a mixture of 5% isoflurane and 500mL/min of oxygen and the hair was removed from their dorsum using clippers and Nair® (Church & Dwight Co., Princeton NJ). On the day of the surgery, the mice were anesthetized and their toes were pinched to ensure no reflex before their shaved dorsum was sterilized three times using betadine surgical scrub and 70% ethanol. A 1 cm x 1 cm sterile template was then placed on the dorsum and the perimeter traced using a black marker. The skin was gently raised using sterile tweezers and cut along the traced perimeter using sterile surgical scissors therefore creating a full-thickness skin defect removing the epidermis, dermis and subcutaneous layers of the skin. The excised skin measured at a thickness of 0.54mm. In some studies, wounds were covered with two different thicknesses of the skin substitute, Alloderm®, before the wounds were covered with the different treatments found in **table 1** followed by suturing a semi- occlusive adherent silicone dressing (Tegaderm™) onto the dorsum. Digital photos were taken on day 0 to determine starting wound size.

Table 1. Experimental groups for in vivo studies.

Animal Groups	Comments
Saline (200 μ l)	Blank control, equivalent to no treatment
Free human MSCs in saline (27 μ l)	MSCs not expected to remain at site
Cell free alginate sheets (2 cm x 2 cm)	Control for effect of alginate alone
Immobilized human MSCs in alginate sheets (2 cm x 2 cm)	Therapeutic group

5.2.2 Wound closure and contraction analysis

Digital photos were taken during the course of healing to record wound closure time (day at which there is full epithelialization) and wound contraction dynamics. Wound area at each time point and at closure was normalized to initial wound area and subsequently quantified by image analysis (NIH ImageJ software).

5.2.3 Histological analysis

Wounds were excised at day 49, fixed with 10% formalin and processed for hematoxylin and eosin (H&E), antigen Ki-67 (Ki67), cluster of differentiation 31 (CD31) and α -smooth muscle actin (α -SMA) staining. Images were taken on a Zeiss AXIO inverted microscope.

5.3 STATISTICAL ANALYSIS

All numerical results are presented as means \pm standard error of the mean (SEM). Statistical analysis of experiments were assessed using one-way analysis of variance (ANOVA) followed by Fischer post hoc analysis where $p < 0.05$ represents statistical significance.

5.4 RESULTS

In the first in vivo study, large defects (1 cm x 1 cm) were created on the dorsum of C57Bl/6 lepr db/db genetically diabetic mice and 5×10^5 human MSCs were seeded. MSCs were either in saline suspension or immobilized in 3% w/v alginate sheets coated with 0.05% w/v PLL. Controls included saline and PLL-alginate sheets with no cells. Wound closure was quantified and although up to day 7 little difference was seen among the groups, by day 14 the immobilized MSCs group significantly increased closure rate ($p < 0.05$) compared to the saline and sheet groups. The immobilized MSCs group was the first to have complete wound closure by day 21 while the other three groups (hMSCs, sheets and saline) caught up 2 weeks later on day 35. However, we noticed rapid wound contraction and little re-epithelialization. Mouse skin has a panniculus carnosus layer, which is human is only found in the neck area and result in rapid contraction after a defect to the skin [16].

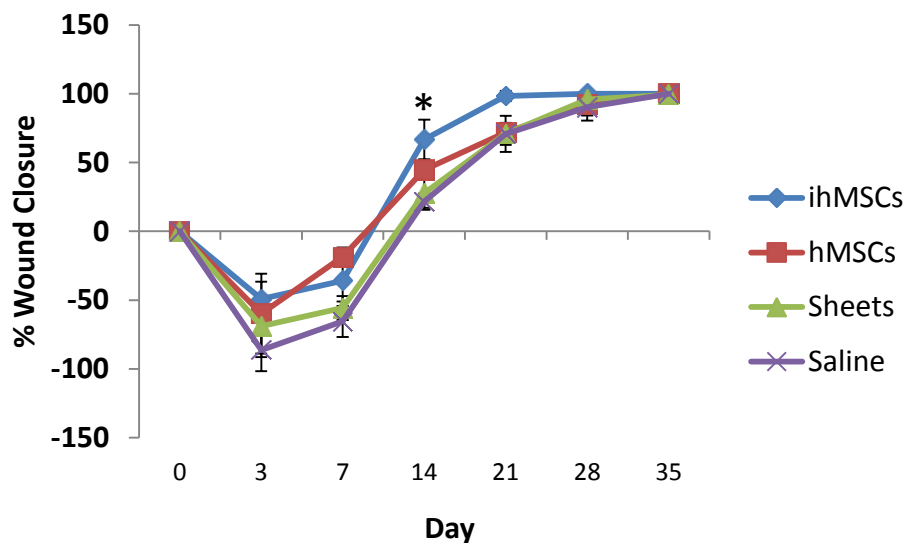


Figure 2. Effect of MSCs on wound closure as a function of time. Dorsal 1 x 1 cm wounds were covered with PLL-alginate sheets seeded with MSCs. Controls include similar wounds covered with PLL-alginate sheets with no cells, MSCs in free suspension in PBS, or PBS alone. Data shown are calculated as 1 - fraction of remaining open wound area at each time point normalized to initial wound area *:p<0.05 compared to Sheets and Saline groups. N=5.

In the second in vivo study, the wounds were covered with Alloderm®, a 0.09mm thick decellularized dermis used to inhibit contraction thus increasing the role of cell migration and proliferation so that the wound healing mimics more closely what is seen in human wounds. In this study, we noticed a decrease in wound closure rate for all treatment groups; however, only the wounds treated with immobilized MSCs were closed by day 35 (Figure 3). All wounds in the other treatment groups were still open at the end of the study. The wound closure rate of the wounds treated with immobilized MSCs was 27% faster than the wound closure rate of wounds treated with MSCs in suspension in saline.

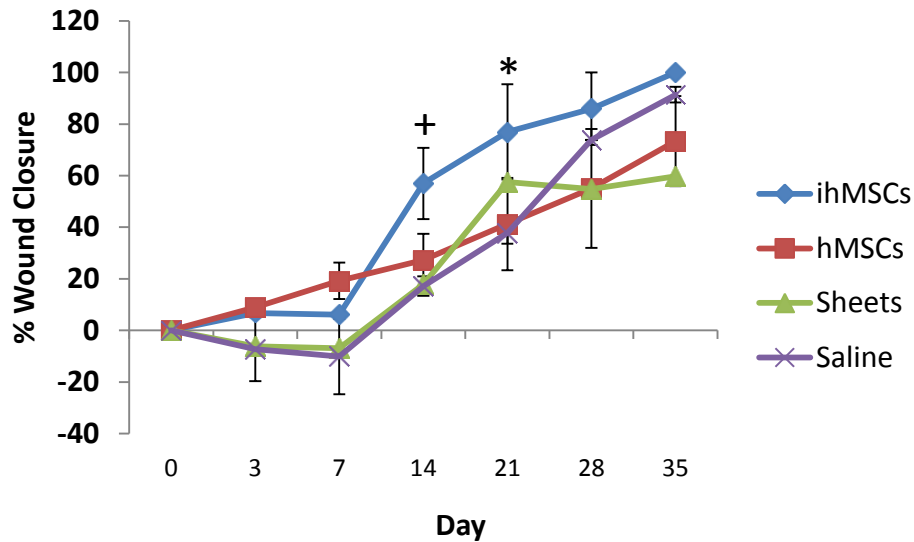


Figure 3. Effect of MSCs in presence of thin Alloderm on wound closure as a function of time. Dorsal 1 x 1 cm wounds were covered with 0.09 mm Alloderm and then PLL-alginate sheets seeded with MSCs. Controls include similar wounds covered with Alloderm and then PLL-alginate sheets with no cells, MSCs in free suspension in PBS, or PBS alone. Data shown are calculated as 1 - fraction of remaining open wound area at each time point normalized to initial wound area. +:p<0.05 compared to saline group. *:p<0.05 compared to hMSCs and Saline groups. N=2.

In the third in vivo study, the thickness of the Alloderm® was 0.41mm, just 0.13mm thinner than the excised skin removed to create the full-thickness excisional wound. Wound closure rate was significantly delayed resulting in this study lasting 49 days. Again, only wounds treated with immobilized MSCs were closed by day 49 (Figure 4). The wound closure rate with immobilized MSCs was 37% faster than wounds treated with MSCs in suspension in saline.

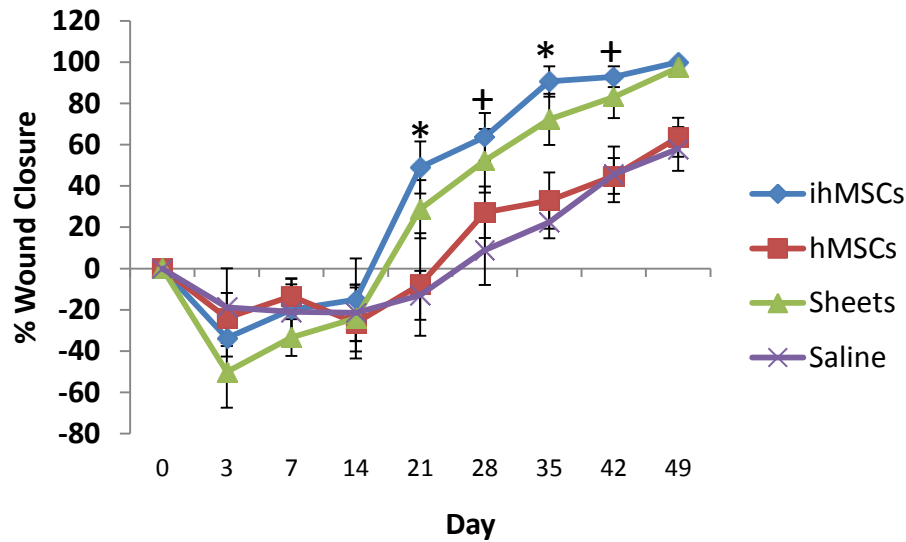


Figure 4. Effect of MSCs in presence of thick Alloderm on wound closure as a function of time. Dorsal 1 x 1 cm wounds were covered with 0.41 mm Alloderm and then PLL-alginate sheets seeded with MSCs. Controls include similar wounds covered with Alloderm and then PLL-alginate sheets with no cells, MSCs in free suspension in PBS, or PBS alone. Data shown are calculated as 1 - fraction of remaining open wound area at each time point normalized to initial wound area. *:p<0.01 compared to hMSCs and Saline groups. +:p<0.05 compared to hMSCs and Saline groups. N=4.

Wounds from the third in vivo study were prepped for histological analysis. H&E stain showed that the stratum corneum, dermal layer and subcutaneous fatty layer of the scar tissue in the immobilized MSCs and the alginate sheet groups (Figures 5C-D). The saline and MSCs in suspension groups still had the intact Alloderm® present in the wounds and only a barely noticeable dermal layer (Figures 5A-B). We did observe migrated cells within the Alloderm®, indicating that cells were

migrating into the skin substitute. We also noted that there were more endothelial cells present in both groups treated with MSCs (either in free suspension or immobilized), as indicated by the CD31 expression (Figures 6C, D). In addition, there was a lot more CD31 expression in the immobilized MSCs group than the alginate sheet or saline groups (Figures 6A, B, D).

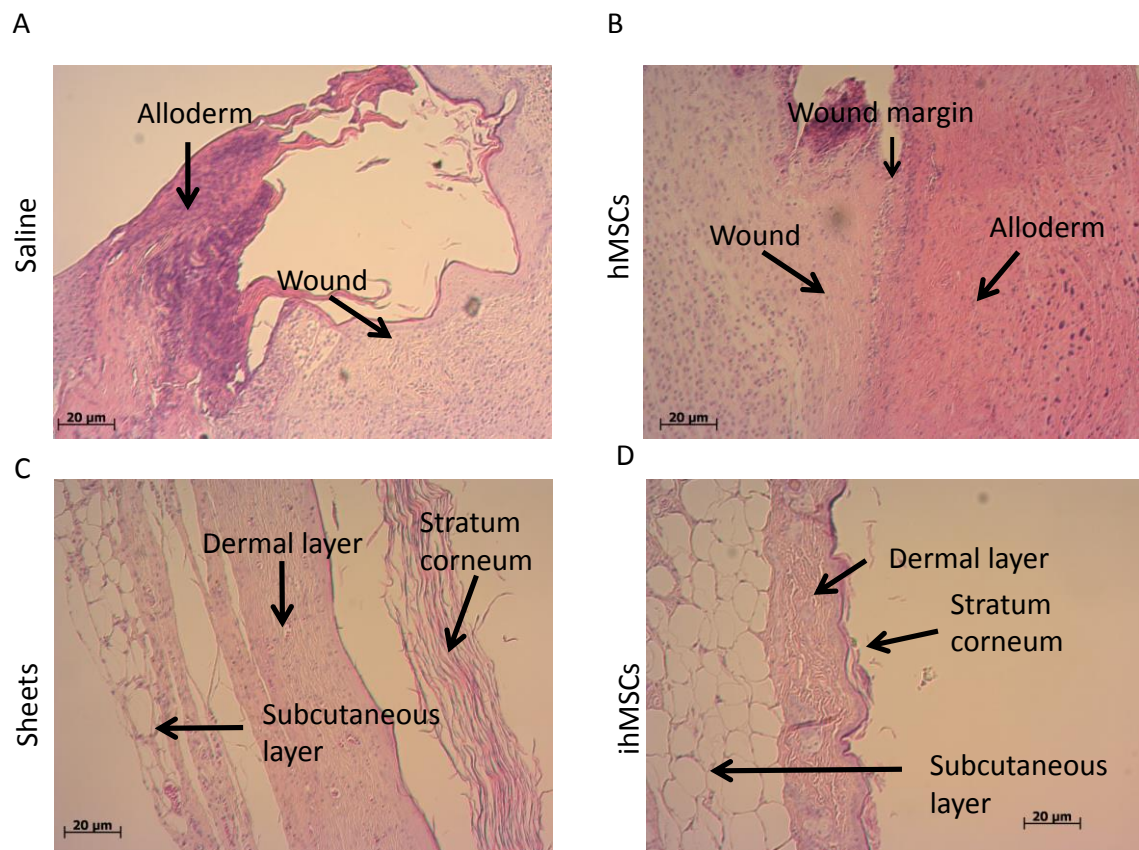


Figure 5. Histology of wounds on day 49 after wounding. Pictures show H&E staining of the center of the wound at a 10X magnification. (A) Wounds treated with saline show an intact Alloderm® present next to a dermal layer. There is some indication of a stratum corneum forming adjacent to the dermal layer. (B) Wounds treated with MSCs in suspension show an intact Alloderm® present next to a dermal layer. There is no indication of a stratum corneum

layer present. (C) Wounds treated with alginate sheets show a stratum corneum layer, a dermal layer and a subcutaneous fatty layer of the new skin. (D) Wounds treated with MSCs immobilized in alginate sheets show an intact stratum corneum layer, a dermal layer and a subcutaneous fatty layer of the new skin.

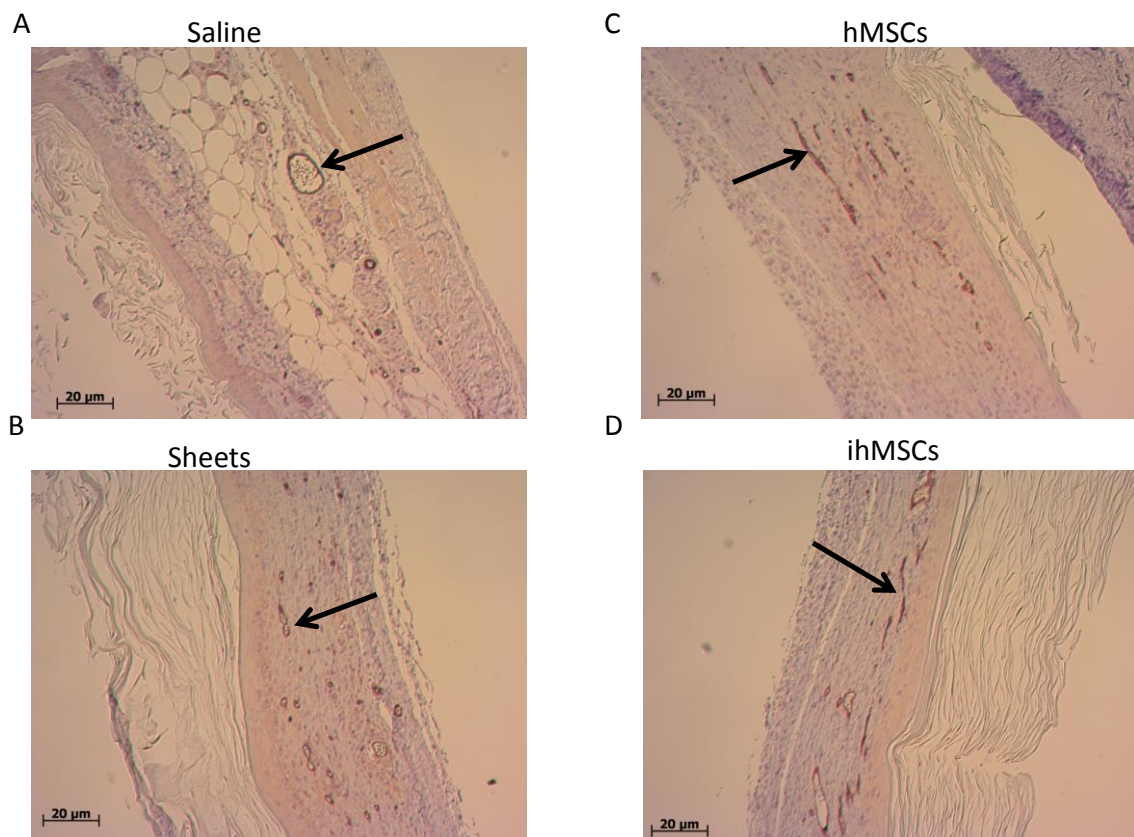


Figure 6. CD31 expression in wounds on day 49 after wounding. Pictures show CD31 staining of the center of the wound. (A) Wounds treated with saline show the endothelium (B) Wounds treated with MSCs in suspension show thin lines of endothelium. (C) Wounds treated with alginate sheets show large endothelium. (D) Wounds treated with immobilized MSCs in alginate sheets show several large endothelium. Arrows indicate endothelium.

The expression of Ki67, a cellular marker for proliferation, was more prevalent in the saline- and MSC suspension-treated groups (figures 7A-B). The wounds in these groups were still significantly open at day 49 indicating that the proliferating cells may be macrophages or endothelial cells. The wounds treated with alginate sheets or immobilized MSCs had proliferating cells primarily localized near the stratum corneum (figures 7C-D).

Lastly, we stained for α -SMA in the wound, a marker for myofibroblasts, cells that are primarily responsible for wound contraction. There were similar levels of α -SMA expression in the wounds treated with alginate sheets with no cells or immobilized MSCs (figures 8C, D). The α -SMA expression in the group treated with alginate sheet with no cells was localized near the stratum corneum (figure 8C) while in the group treated with the immobilized MSCs, it was within the dermal layer and near the stratum corneum (figure 8D). Alpha-SMA levels were much lower in the groups treated with saline and MSCs in suspension (figures 8A, B).

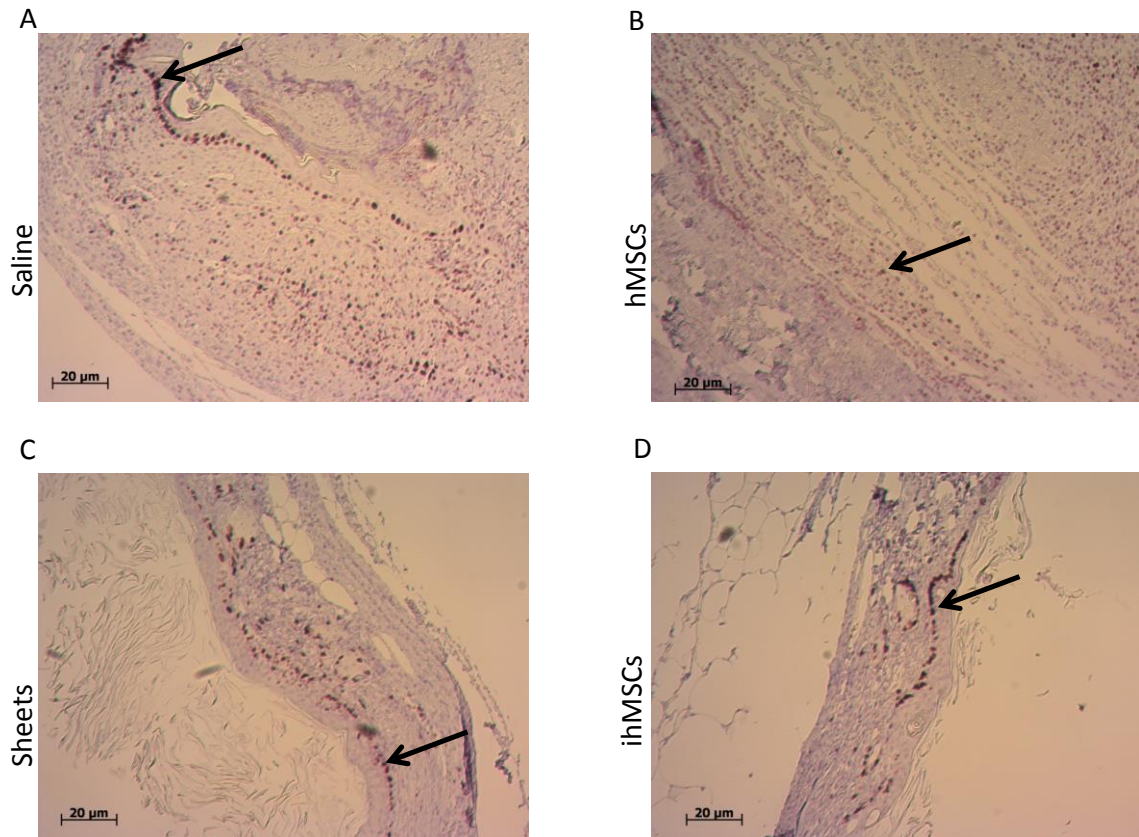


Figure 7. Ki67 expression in wounds on day 49 after wounding. Pictures show Ki67 staining of the center of the wound. (A) Wounds treated with saline show several proliferating cells within the dermal layer. (B) Wounds treated with MSCs in suspension show several proliferating cells within the dermal layer. (C) Wounds treated with alginate sheets show several proliferating cells within the dermal layer. These cells are localized adjacent to the stratum corneum. (D) Wounds treated with MSCs immobilized in alginate sheets show several proliferating cells within the dermal layer. These cells are localized adjacent to the stratum corneum. Arrows indicate proliferating cells.

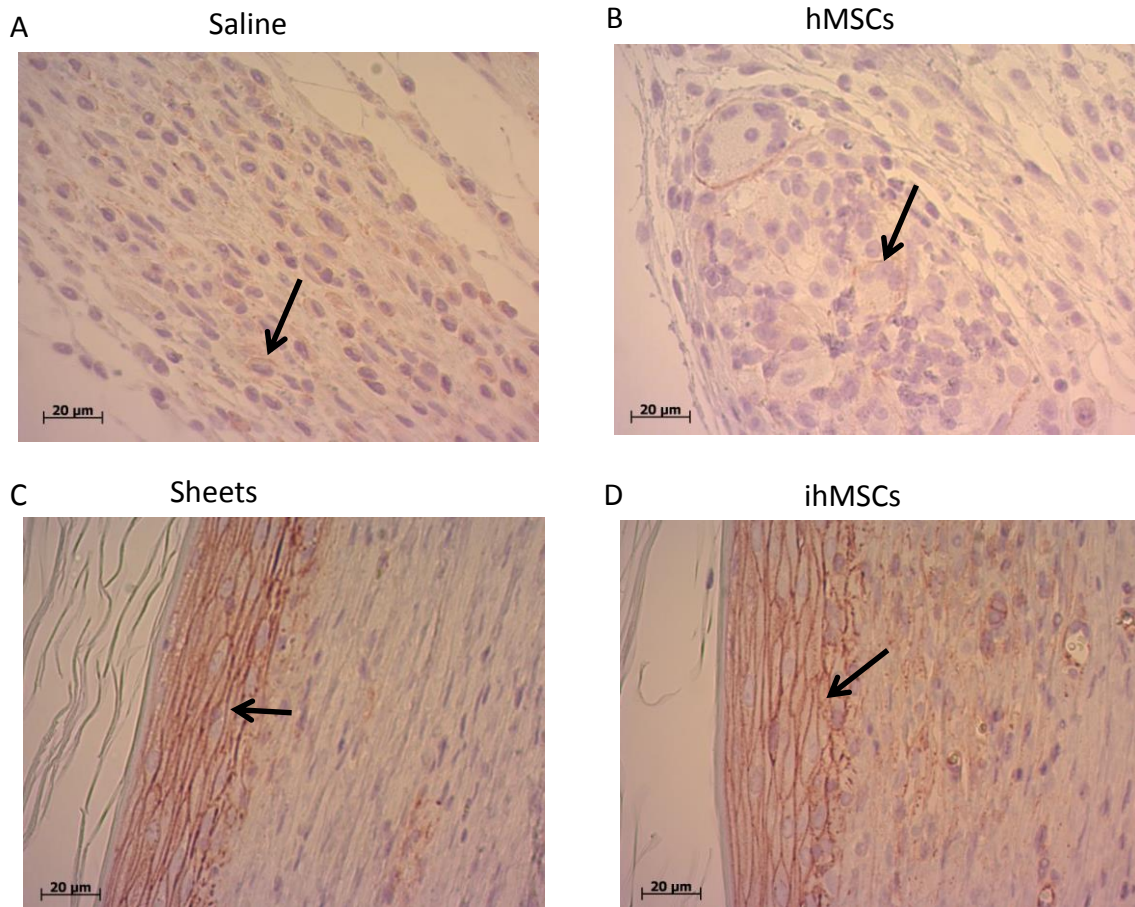


Figure 8. Alpha-smooth muscle actin expression in wounds on day 49 after wounding. Pictures show α -SMA staining of the center of the wound. (A) Wounds treated with saline show some α -SMA expression. (B) Wounds treated with MSCs in suspension show some α -SMA expression. (C) Wounds treated with alginate sheets show high α -SMA expression in the dermal layer near the stratum corneum. (D) Wounds treated with immobilized MSCs in alginate sheets show high α -SMA expression within the dermal layer and near the stratum corneum. Arrows indicate α -SMA.

5.5 CONCLUSION

MSCs are known to modulate inflammation and promote tissue repair [7, 13, 15, 16]. MSCs secrete factors such as interleukin-10, vascular endothelial growth factor and TGF- β 1, which are essential for decreasing inflammation, increasing vascularization, and promoting tissue repair [17]. In this chapter, full thickness excisional wounds were created on the back of genetically diabetic mice. These mice are known to have delayed wound closure and decreased levels of growth factors essential for wound healing [9, 18]. These wounds were treated with MSCs either immobilized in alginate sheets or in a saline suspension. We immobilized MSCs to increase their persistence in the wound and we observed that, in this mode of delivery, they significantly enhanced wound closure. Mouse wounds heal primarily by wound contraction, and the data suggest that immobilized MSCs stimulated the wound contraction process. To mimic wound closure that is similar in human skin wound healing, a skin substitute was applied to the wounds to increase cell migration and re-epithelialization. Even though the skin substitute delayed wound closure rate compared to wounds without a skin substitute, immobilized MSCs still accelerated wound healing. Wounds treated with immobilized MSCs were the only group completely healed by the end of the studies that included the skin substitute.

Skin substitutes have been used as a treatment for acute and chronic wounds however, their effectiveness has only been 50-55% in clinical trials [19]. We noticed a similar trend with mice wounds treated with Alloderm[®] and saline. Even though, we observed migrated cells with the Alloderm[®] there was a significant delay in wound closure rate (figure 4). The skin substitute provides the template for

regeneration but evidently, the growth factors are needed to stimulate cells. Therefore, there is a need to improve skin substitute effectiveness by continuously delivering essential factors to the wound to enhance cell migration and re-epithelialization.

5.6 ACKNOWLEDGEMENTS

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CHAPTER 6: CONCLUSION

6.1 KEY FINDINGS

The overall goal of this dissertation work is to accelerate chronic wound healing. The hostile environment in chronic wounds readily breaks down essential growth factors that promote cell migration, angiogenesis and wound contraction. Without these essential factors, together with the lack of oxygen due to hypoxia, cells within the wounds experience decreased proliferation, function, and increased apoptosis. Current treatments used in the clinic are only 50% effective due to the variability in size, location and pathological state of the wounds. We immobilized MSCs in alginate sheets to enhance their persistence at the wound site so they can continuously provide factors to chronic wounds. Immobilizing MSCs at the wound site enhanced wound closure. In vitro, we observed that MSCs modulate inflammation by differentiating macrophages to the anti-inflammatory phenotype under hypoxia. In vitro and in vivo studies showed that MSCs also promote tissue repair via fibroblast differentiation into α -SMA-expressing myofibroblasts.

6.1.1 Immobilization of mesenchymal stromal cells in alginate dressings

We successfully immobilized MSCs in alginate sheets and found that immobilization of MSCs in alginate sheets does not alter viability or secretion compared to cells plated on tissue culture plastic. We also cultured MSCs in a chronic wound environment (hypoxia or chronic wound fluid) and found that this environment

positively altered their secretion pattern. More specifically, hypoxia did not alter MSC production of pro-inflammatory mediators compared to cells cultured in normoxia but did increase their secretion of TGF- β 1, which is needed to promote fibroblast differentiation into α -SMA-expressing myofibroblasts. Also, when MSCs were cultured in chronic wound fluid, their secretion of pro-inflammatory mediators was suppressed but their secretion of the anti-inflammatory mediator, IL-10, was increased.

6.1.2 Mesenchymal stromal cells promote anti-inflammatory properties in hypoxic macrophages

MSC immunomodulation properties were observed via their effect on macrophage phenotype. MSCs were co-cultured with M1 macrophages in normoxia or hypoxia for 48 hours. Protein analysis of the supernatant from macrophages co-cultured with MSCs showed suppressed TNF- α production and enhanced production of IL-10. Measurement of CD206 expression on macrophages cultured in MSC-conditioned medium for 48 hours showed significantly higher levels of CD206 expression compared to controls cultured in basal medium. High levels of CD206 expression coupled with enhanced production of IL-10 and a suppression of TNF- α production are properties of the M2 macrophage phenotype. In hypoxia, MSCs were able to promote macrophage transition from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype. However, we noticed that the M2 macrophages produced significantly less IL-10 in hypoxia compared to the M2 macrophages

cultured in normoxia. Therefore, even acute hypoxia alters some properties of M2 macrophages.

6.1.3 Mesenchymal stromal cells reverse hypoxia-mediated suppression of alpha-smooth muscle actin in human dermal fibroblasts

Hypoxia significantly decreased fibroblast differentiation to α -SMA-expressing myofibroblasts, the cells responsible for wound contraction. Fibroblasts embedded in type I collagen and cultured in hypoxia, contracted collagen significantly less and expressed significantly less α -SMA expression compared to cells cultured in normoxia. When these same cells were treated with MSC-conditioned medium and cultured in hypoxia, α -SMA expression and collagen contraction returned to the levels seen in the controls cultured in normoxia and in basal medium alone. The data showed that MSC-secreted factors were able to reverse impairments caused by hypoxia.

6.1.4 Immobilized mesenchymal stromal cells enhance chronic wound healing in vivo

MSCs immobilized in alginate sheets significantly enhanced wound healing in our genetically diabetic mouse model of chronic wound healing. MSCs in alginate sheets were always the first group of wounds to completely close. In studies where a skin substitute was incorporated to increase cell migration and mimic human skin wound healing, wounds treated with MSCs in alginate sheets were the only wounds completely closed by the end of the study. Histological analysis of one of the studies showed higher levels of CD31 and α -SMA expression compared to other treatment

groups (MSCs in PBS, alginate sheets without MSCs or PBS alone). The histological analysis showed that MSCs enhanced wound healing by increasing angiogenesis and wound contraction.

6.2 LIMITATIONS

6.2.1 Choice of MSCs

Several pre-clinical and clinical trials show the promise of MSCs as a therapy for chronic wound healing [1, 2]. MSCs secrete a multitude of soluble factors that reduce inflammation and promote tissue repair [3, 4]. These studies show that MSCs intravenously injected into the body, migrate to the wound environment. However, these studies do not show long-term engraftment at the injury site. A major limitation of MSC use as a therapeutic is the lack of homing [5]. Therefore, MSCs studies have also focused on increasing homing and efficacy of MSCs. Our use of MSCs required that we developed a method to immobilize MSCs at the injury site. MSC immobilization added a new element to the dissertation project, their need for immobilization.

6.2.2 Choice of alginate as a cellular carrier

Alginate is a polysaccharide extracted from seaweed and widely used in the food industry, applied in the pharmaceutical industry and used as a polymer for wound dressings [6, 7]. The manufacture of alginate wound dressings began in the late

1800s and has been shown to promote hemostasis, absorb wound exudate and provide a moist wound environment optimal for cell migration and healing [7].

As a biomaterial, alginate has been used as a delivery vehicle for proteins and a carrier for cells. Alginate is biocompatible and non-immunogenic. Also, cells do not attach to the alginate so they cannot migrate out of the carrier and can continue to secrete factors into the wound. However, we are unable to control alginate degradation therefore, we used poly-L-lysine (PLL) as a coating to increase the strength of the alginate. In vitro, we noticed that non-PLL coated alginate sheets started to degrade after 72 hours. PLL-coated alginate sheet maintained its integrity for over 21 days in vitro. However, in vivo we noticed that the PLL-coated alginate sheets containing MSCs started to degrade once the moisture within the wound receded, normally around day 21. Literature studies also showed that PLL-coated alginate microbeads activate macrophages to produce higher levels of pro-inflammatory factors than non-PLL-coated microbeads in vitro [8]. Therefore, there is a possibility that the PLL coating on the MSC-alginate dressings is interfering with the effect of the MSCs.

6.2.3 Excisional wounding studies

The genetically diabetic mice used in the wounding studies showed delayed wound closure but lacked the ischemic component in human chronic wounds. Therefore, we have focused heavily on in vitro studies to investigate the effect of hypoxia on MSCs, macrophages and fibroblasts function. Mice wounds also heal primarily by wound contraction [9, 10] therefore, we have had to rely on the use of skin

substitutes to increase re-epithelialization and mimic human skin wound healing. We used commercially available Alloderm® purchased from LifeCell. However, we rely on what the manufacturer can provide us with, therefore we cannot control the thickness of the product. Due to this, we had to separate our animal studies based on the thickness of the Alloderm®. This also increased the cost of the studies and altered the time course of wound healing.

Overall, mouse models for wounding studies are cost effective and allow us to test multiple treatment strategies. The most effective treatment strategy can then be used in wounding studies using pig models. Pig skin is more similar to human skin and therefore, wounding studies using pig models would mimic human wound healing [11]. However, pig models are very costly and require the use of more reagents.

6.3 FUTURE DIRECTIONS

Future studies include performing histological analysis and measuring protein and gene expression on the wounds over time. Performing these analyses on the wounds on day 7, 14, 21 and 28 would show us the progression of wound healing. We could observe the phenotype of macrophages in the wound overtime, the peak of cell migration and the type of cells migrating into the wound bed. The data from these studies could tell us if we should change the dressings at different times during the study instead of using a single treatment applied on the day of the surgery.

We could also retrieve the alginate dressing before it degrades in order to monitor MSC behavior. We could then culture the cells in vitro and measure cell viability and secretion. Overall, we could see if the MSCs retrieved from the dressings behave similarly to MSCs cultured in chronic wound fluid in vitro. In addition, we could also isolate the cells from the alginate dressings and observe their phenotype, whether the cells differentiated because of the wound environment or maintained their stemness.

Furthermore, the thickness of the Alloderm® significantly affected the course of the wound studies and effectiveness of the treatments. The thicker Alloderm® from all three studies delayed wound closure because of a significant lack of angiogenesis observed in the histological analysis. Therefore, we investigated the use of polyheme, an oxygen carrying blood substitute, on chronic wound healing. In the pilot study, we applied multiple dose of polyheme on the wound with the thick Alloderm® and found that the polyheme closed the wounds on day 35 compared to day 49 with the immobilized MSCs in alginate sheets (figure 1). Next, we plan on combining the polyheme with the immobilized MSCs so that the polyheme can provide the wound with oxygen while the MSCs provide factors to the wound to decrease inflammation and increase tissue repair. We want to further enhance wound closure so that no matter the thickness of the Alloderm®, our treatment will be effective.

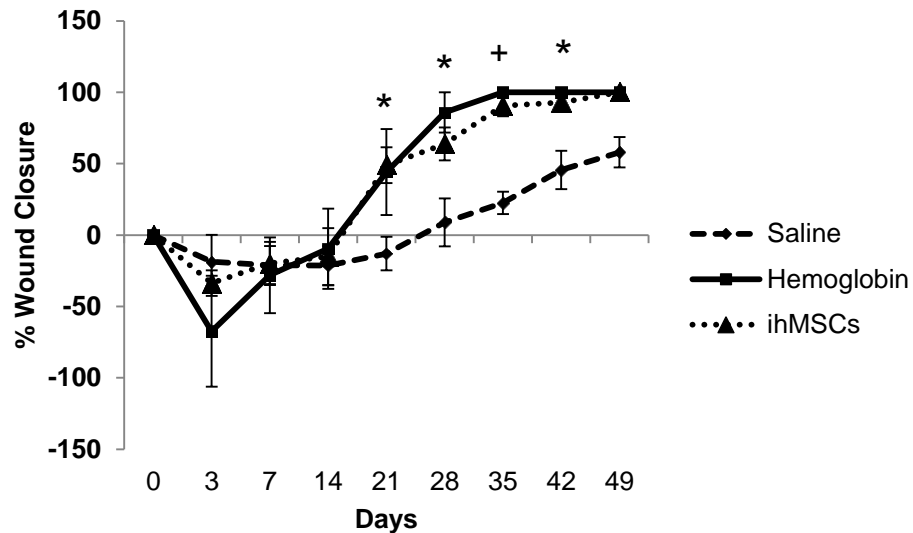


Figure 1. Effect of polyheme in the presence of thick Alloderm on wound closure as a function of time. Dorsal 1 x 1 cm wounds were covered with 0.41mm Alloderm and then polyheme or PLL-alginate sheets seeded with MSCs. The control include similar wound covered with Alloderm and then PBS alone. Data shown are calculated as 1 – fraction of remaining open wound area at each time point normalized to initial wound area. N=5 (saline). N=2 (hemoglobin) N=4 (ihMSCs) *:p<0.05 vs. saline (days 21, 35 and 42). +:p<0.01 vs. saline (days 35).

6.4 REFERENCES

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