DELIVERY OF PROLYL HYDROXYLASE INHIBITORS TO MSC SPHEROIDS FOR ENHANCED ANGIOGENIC FACTOR SECRETION

A Thesis
Presented to
The Academic Faculty

By

Katy Lassahn

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science in BioEngineering

Georgia Institute of Technology

August 2015

DELIVERY OF PROLYL HYDROXYLASE INHIBITORS TO MSC SPHEROIDS FOR ENHANCED ANGIOGENIC FACTOR SECRETION

Approved by:

Dr. Todd C. McDevitt, Advisor

School of Biomedical Engineering

Georgia Institute of Technology

Dr. Andrés J. García

School of Mechanical Engineering

Georgia Institute of Technology

Dr. Luke Brewster

School of Medicine

Emory University

Date Approved: June 03, 2015

ACKNOWLEDGEMENTS

I wish to thank first and foremost, my adviser, Todd McDevitt. Todd has been a mentor and adviser to me for almost seven years, and his research inspired me even before I came to Georgia Tech. In fact, it was the McDevitt lab's exhibit at Buzz on Biotechnology in which I saw beating cardiomyocytes in a petri dish as a high school junior and made up my mind to attend Georgia Tech for my bachelor's degree. Once I arrived at Tech I somehow found the confidence to reach out to the McDevitt lab and was fortunate enough to join the McDevitt lab my freshman year at Georgia Tech. I will always be grateful to Todd for giving me the opportunity to join his lab at such an early stage in my time at Georgia Tech, since my involvement in undergraduate research was one of the most shaping experiences of my career to date. Throughout the years Todd has been a huge proponent for my success, nominating me for various awards and writing me countless recommendations that I'm sure were major factors in my success. He's taught me many things, including the ability to detect the tiniest of abnormalities on powerpoint slides or posters and passed on a dis-taste for the words "show" or "these" in any writing. I have always felt very assured to have Todd in my corner in countless situations whether personal or professional and feel grateful knowing he will still be there after I graduate from the lab.

I next would like to thank a former McDevitt Lab member, Andres Bratt-Leal, who mentored me during my first three years in the lab as an undergraduate. He was an excellent mentor who encouraged my independence, while still running to the rescue when I inevitably messed something up. He taught me that sometimes some comic relief in stressful situations was exactly what was needed and his energy and kindness was

infectious in the lab. He made an effort to include me in the lab social activities and made me feel very at home in the lab. He's given me plenty of advice over the years and still stays in touch with me and gives me wise advice on life and career. I am very fortunate to have been molded by such an excellent researcher and person at such an important time in my life and am thankful for everything he has done for me.

I would like to acknowledge and thank Marta Costa, who was a visiting researcher in the McDevitt lab during my time there. She was instrumental in the early studies investigating the potential of various PHDi molecules on MSCs. I very much enjoyed working with her and wish her the best of luck in finishing her PhD in Portugual.

In the 7 years I have been in the McDevitt lab I have been helped and impacted by *many* people. I would like to particularly thank Carolyn Sargent, Alyssa Ngangan Kitchel, Barbara Nsiah, Ken Sutha, and Rich Carpenedo for their mentorship and support during my time as an undergraduate. I am also very thankful for the wisdom and advice of Carolyn and Alyssa whom I still reach out to for their insightful career and life advice. I would like to thank more recent McDevitt lab members, Melissa Kinney, Jenna Wilson, Anh Nguyen, Christian Mandrycky, Marissa Cooke, Yun Wang, Emily Jackson, Josh Zimmerman, Denise Sullivan, Olivia Burnsed, and Marian Heteriatchi, and countless others. It has been such an honor to get to know all of you and learn from you during this journey. I would like to particularly thank the post-doctoral fellows Lindsay Fitzpatrick and Tracy Hookway for their mentorship and for being such excellent role models for me to aspire to be like. Both are excellent researchers who I know will go on to do great things and be excellent professors. I would like to thank Jessica Butts, one of my closest friends from the McDevitt lab. Jessie is one of the hardest working graduate students I

know, and I know she will have a lot of success in San Francisco as she finishes her PhD. She is also quite the social butterfly, and I was very grateful for all of the social events she was always planning and inviting me to outside of lab. She was the best President.

I would like to thank Andres Garcia and Luke Brewster for agreeing to be on my committee and for their insight and feedback on my research. I would particularly like to thank Dr. Garcia for his support as the Chair of the BioEngineering program at Georgia Tech. I have enjoyed working with him through the BioEngineering Graduate Association and immense respect for him as a leader at Georgia Tech. He has an excellent ability to connect with people and is a very sincere mentor who is always looking out for the best interest of the BioEngineering students. I was also fortunate to have Dr. Garcia as my professor for Cellular Engineering and learned a great deal from him during the course.

Finally, I would like to thank my family and friends. My parents have always been supportive of me in everything I do and I thank them for encouraging me to find a career that I love and that I feel will allow me to make a difference in the lives of others. I would like to thank my big sister, Joanna, for being my best friend and second mother. She is one of the most caring people I know and gives so much of herself to help others and never expects anything in return. I am very fortunate to have a person like her in my life. Lastly, I would like to thank my husband, Jamie Lassahn. My journey in graduate school has been a very bumpy one, with many twists and turns along the way, but I am so grateful to have a supportive husband like you making sure I stay upright and never get lost. Long-distance marriage coupled with military deployments has been very difficult, but it has made me even more excited for our future life we are building together.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

PHD	Prolyl Hydroxylase
PHDi	Prolyl Hydroxylase inhibitors
MSCs	Mesenchymal Stromal/Stem Cells
VEGF	Vascular Endothelial Growth Factor
HUVEC	Human Umbilical Vein Endothelial Cells
PLGA	Poly Lactic-co-Glycolic Acid
IOX2	N-[[1,2-dihydro-4-hydroxy-2-oxo-1-(phenylmethyl)-3-quinolinyl]carbonyl]-glycine
DMOG	Dimethyloxalyglycine
3,4-DHB	ethyl 3,4-dihydroxybenzoate
HIF	Hypoxia Inducible Factor
ECs	Endothelial Cells
ECM	Extracellular Matrix
TGF-β	Transforming Growth Factor-β
HGF	Hepatocyte Growth Factor
IL	Interleukin
MCP-1	Monocyte Chemotactic Protein-1

Summary

Approximately 6.5 million Americans suffer from chronic non-healing wounds each year with the average cost of treatment estimated to be $3,927 per incidence[1]. This pathological wound healing is typically indicative of a chronically inflamed environment that is unable to promote successful angiogenesis and collagen production to heal the wound. An approach for improving angiogenesis in chronic non-healing wounds is through the use of mesenchymal stromal/stem cells (MSCs). It has been shown that MSCs enhance wound healing due to their ability to both modulate the immune response to prevent a chronically inflamed environment and their ability to promote angiogenesis. MSCs promote angiogenesis through secretion of growth factors such as VEGF that recruit endothelial progenitor cells that are critical for rebuilding the damaged vasculature [2], [3]. In hypoxic environments, the pro-angiogenic effects of MSCs are enhanced through stimulation of the HIF-1α pathway[4]. Small molecules termed prolyl hydroxylase inhibitors (PHDi) stimulate the HIF-1α pathway through inhibition of prolyl hydroxylase (PHD) the protein involved in HIF-1α degradation. PHDi have been shown to chemically induce a hypoxic response from MSCs[5] and may allow for greater control over the pro-angiogenic secretory response of transplanted cells by increasing the duration and dosage of exposure. In addition to hypoxia the culture format can affect the angiogenic properties of MSCs. The culture of MSCs as three dimensional spheroids has been shown to promote secretion of angiogenic growth factors such as VEGF[6] as well as immunomodulatory factors[7]. The ***objective*** of this project was to investigate methods to enhance the pro-angiogenic factor secretion of MSCs, which would lead to improved strategies for treatment of chronic non-healing wounds. The ***overall hypothesis*** was that sustained PHDi treatment and three-dimensional culture would lead to an enhancement of pro-angiogenic factor secretion from MSCs.

Treatment of MSCs with PHDi has been shown to enhance cell survival[8], improve bone regeneration[9], and increase new vessel formation *in vivo*[9]. Dimethyloxalyglycine (DMOG), and ethyl 3,4-dihydroxybenzoate(3,4-DHB) are two of several PHDi that have been investigated in MSCs and shown to activate the HIF-1α pathway. However, a potentially more promising candidate is N-[[1,2-dihydro-4-hydroxy-2-oxo-1-(phenylmethyl)-3-quinolinyl]carbonyl]-glycine (IOX2)[10] due to its higher specificity for PHD than other PHDi. However, the potency of IOX2 in modulating the angiogenic secretion of MSCs has not been studied. To compare the potency of DMOG, IOX2, and 3,4 DHB on the pro-angiogenic factor secretion of MSCs, a systematic screen was performed of each PHDi at a range of concentrations previously reported to be effective in other cell types. Vascular endothelial growth factor (VEGF) was used as the primary indicator of a pro-angiogenic response due to the well established link between VEGF and angiogenesis. Cell number was used as an indicator of cell growth. The results demonstrated that DMOG and IOX2 were the most potent inhibitors and optimized dosages were identified that produced the highest VEGF secretion without adversely affecting cell growth.

The culture of MSCs as three-dimensional spheroids rather than monolayer culture has been shown to increase paracrine factor secretion[7][11], [12]. Thus, the effects of PHDi treatment on MSC spheroids was hypothesized to further enhance VEGF secretion compared to monolayer cultures. MSC spheroids were formed by forced aggregation and cultured in rotary culture for 3 days while being treated with either DMOG or IOX2. As expected, spheroid culture alone increased VEGF secretion by 6 fold. Addition of PHDi increased secretion of VEGF by 2-fold in both monolayer and spheroid cultures. This demonstrates that the combined strategy of PHDi treatment and spheroid culture is a viable option to enhance the pro-angiogenic factor secretion of MSCs.

PHDi stabilization of HIF-1α is a transient effect, thus sustained exposure to PHDi could promote sustained secretion of pro-angiogenic factors. Sustained delivery of PHDi

could be achieved within spheroids via biomaterial based microparticle incorporation[13]. The delivery of PHDi from microparticles within spheroids allows for controlled PHDi presentation from within the spheroids, rather than requiring treatment with soluble PHDi in the culture media. This is particularly important for clinical translation, in which the spheroids would be treated beforehand and transplanted into the body. Thus the encapsulation of DMOG and IOX2 into PLGA microparticles was investigated. The encapsulation of DMOG into PLGA MPs proved challenging due to the amphiphillic nature of DMOG. More success was found with IOX2 which is hydrophobic and allowed for successful encapsulation into PLGA MPs. It was hypothesized that sustained delivery of IOX2 in MSC spheroids via MP incorporation would further enhance the angiogenic potential of the MSC spheroids compared to spheroid culture alone. The effect of PHDi delivery on angiogenesis was assessed by measuring secretion of VEGF in the conditioned media and HUVEC migration assays. The delivery of IOX2 via first generation microparticles was as effective as soluble treatment, however, the formation of IOX2 crystal debris during formation necessitated the formulation of a second generation IOX2 MP that was both smaller and did not have crystallized IOX2 debris. These microparticles, however, had approximately 10 times less IOX2 encapsulated and ultimately were unable to have an appreciable effect on the VEGF secretion of MSC spheroids. This could be optimized in the future through further tuning of IOX2 concentration and co-solvent percentage used in the emulsion process to allow for improved delivery and a more controlled exposure of IOX2 to MSC spheroids. Additional biomaterials could also be investigated to increase encapsulation of IOX2. The ability to modulate the hypoxia response of MSC spheroids through IOX2 delivery will prolong and enhance the pro-angiogenic secretory response of hypoxic environments on MSCs for improved angiogenesis and wound healing after transplantation.

CHAPTER 1 INTRODUCTION

Chronic non-healing wounds are a major healthcare concern in the U.S, especially due to the growing diabetic and elderly population. Wound healing is a complex biological process that is dependent on cells at the site of injury to signal and recruit immune cells, fibroblasts, and endothelial cells in order to rebuild damaged vasculature via angiogenesis. MSCs aid in wound healing and revascularization of damaged tissue because of their ability to secrete pro-angiogenic cytokines such as VEGF, Interleukin-6, hepatocyte growth factor (HGF) monocyte chemotactic protein-1 (MCP-1) and transforming growth factor-β1 (TGF-β1) [14] which recruit immune cells and endothelial cells to the site of injury to form new blood vessels. Hypoxia is known to be a key regulator in the angiogenic response of many cells, including MSCs. Small molecule drugs termed prolyl hydroxylase inhibitors (PHDi) are able to cause a hypoxic response in cells and can enhance MSCs ability to facilitate angiogenesis. The objective of this project was to investigate and compare the effects of commercially available PHDi on pro-angiogenic factor secretion of MSCs. Additionally, since the response to the drugs is likely short-lived, a method for prolonged delivery or exposure of PHDi to MSCs was investigated.

Specific Aim 1: Evaluate the pro-angiogenic factor secretion of Mesenchymal Stromal/Stem Cells (MSCs) in response to prolyl hydroxylase inhibitors and culture format. Hypoxia has been shown to enhance MSCs wound healing and angiogenic potential. Multiple small molecule drugs are commercially available that stabilize HIF-1α through inhibition of prolyl hydroxylase, the enzyme responsible for hydroxylation of HIF-1α leading to its degradation in the presence of oxygen. The three small molecules investigated here are DMOG, IOX2, and 3,4-DHB. Thus, a side-by-side comparison of the three drugs was performed to determine optimal concentrations that provide the highest VEGF secretion, without adversely effecting cell growth. The lasting effects of

PHDi on MSCs after PHDi removal was assessed. Additionally, the two best conditions were investigated in a 3D culture format to further study the effect of soluble delivery on MSCs. *It is hypothesized that treatment of MSC spheroids with PHDi will have a greater effect than treatment of PHDi on monolayer cultured MSCs due to a known effect of spheroid culture on the secretion of paracrine factors*[11].

Specific Aim 2: Encapsulate DMOG and IOX2 in PLGA Microparticles for delivery to MSC Spheroids for enhancement of pro-angiogenic factor secretion. Due to the short-term effects of soluble treatment with DMOG and IOX2, a method to prolong delivery of the drug when transplanting MSC spheroids in a clinical setting would be beneficial. Incorporation of microparticles containing PHDi into MSC spheroids would allow for the sustained delivery of PHDi from within the spheroid as opposed to a soluble pre-treatment in culture media. The objective of this study is to encapsulate DMOG and IOX2 in PLGA microparticles and assess effects on angiogenic factor secretion when incorporated into the MSC spheroids. *It is hypothesized that PHDi microparticle incorporation will cause similar pro-angiogenic responses as soluble treatments as determined by VEGF secretion, HUVEC migration, and endothelial scratch assays.*

Motivation and Significance: MSCs have shown promise in animal models of wound healing and angiogenesis largely through modulation of the immune response and promotion of angiogenesis leading to improved vascularization of the wound bed. However, there are still challenges for their successful clinical translation. First, engraftment and persistence of single cell MSCs is poor which limits the time that MSCs can secrete paracrine factors at the site of injury. Transplantation of spheroids, however, appears to be a promising method for enhancing engraftment and improving therapeutic efficacy through higher paracrine factor secretion. Enhancement of the paracrine factor secretion of MSC spheroids through PHDi treatment may further improve MSC spheroid therapeutic efficacy during the course of treatment.

CHAPTER 2 Wound healing

2.1 Wound healing

2.1.1 Challenges and Unmet Clinical Needs of Wound healing

Approximately 6.5 million people in the U.S suffer from chronic non-healing wounds each year. These wounds are most common in diabetics and the elderly and are largely pressure ulcers or diabetic foot ulcers. The elderly accounts for approximately 72% of pressure ulcers noted in hospitals[15]. Pressure ulcers are caused by pressure or shearing forces being applied to the skin for prolonged period of times leading to a decrease in oxygen tension, tissue necrosis and ischemic reperfusion injury[16]. Diabetic foot ulcers are the other main cause of chronic wounds. Approximately 25% of diabetic patients will develop foot ulcers, and 12% of these cases will lead to foot amputation. To further exacerbate this issue, approximately 50% of amputees will develop a foot ulcer in the surviving foot within 5 years[17]. Typical treatments for non-healing wounds are treated using the TIME methodology (Tissue removal, Infection prevention, Moisture rebalance, Epithelialization promotion)[18]. However, many wounds are not responsive to this therapy and necessitate more advanced therapies in order to successfully revascularize the wound bed. This is thought to be due to phenotypic abnormalities in the cells of diabetic or elderly patients that include a decrease in responsiveness to growth factors, reduced migration, and lack of response to hypoxia[19], [20]. These abnormalities impede ECM deposition and formulation of granulation tissue ultimately impairing the wound healing process. Thus more advanced therapies are necessary to treat non-healing wounds.

2.1.2 Wound Healing Process

The wound healing process is a complex series of events broken into three main stages: inflammation, proliferation, and remodeling. When an injury occurs it disrupts blood

flow to the local vasculature, causing low oxygen tension in those tissues and triggering a hypoxic cellular response to begin the wound healing process. A blood clot forms through platelet adhesion to the damaged vasculature and acts as a scaffold for growth factors and cells migrating in response to the damage.

2.1.2.1 Inflammation

The acute inflammatory stage of wound healing typically lasts three days[21]. Growth factors secreted by the local damaged tissue in response to hypoxia, recruit inflammatory cells to the site of injury. Neutrophils are a major immune cell recruited at the early stages and are critical for protease secretion and phagocytosis for debridement of the wound and to kill bacteria. Neutrophils are recruited by IL-8 and MCP-1[22] and after approximately two days, the neutrophils are phagocytosed by macrophages recruited to the wound site by molecules such as RANTES, MCP-1, and MIP-1α[3][23]. Sources of the chemotactic factors responsible for macrophage recruitment include platelets trapped in the blood clot, keratinocytes, fibroblasts, and leukocytes[22]. Macrophages become activated and secrete potent growth factors such as VEGF, TGF-β, bFGF, and PDGF which are involved in ECM deposition and cell proliferation[24].

2.1.2.2 Proliferation and Angiogenesis

The proliferative phase occurs approximately three to ten days after wounding. Local keratinocytes and epithelial stem cells are involved in the re-epithelialization process. In order to facilitate full re-epithelialization, angiogenesis must occur to restore blood flow and oxygen to the proliferating cell populations. Secretion of growth factors such as VEGF, PDGF, and bFGF activate endothelial cells (ECs) in existing vascular networks. ECs dissolve the basal lamina to allow for migration into the wound site via a process known as sprouting. As the ECs migrate, cells at the leading edge secrete MMPs that breakdown tissue to allow for continued migration and proliferation. The resulting network of sprouts eventually interconnect and form vessels. Pericytes are recruited to

provide further maturation and support to the vessel walls for arterial or venule formation. Once blood flow is restored the process of angiogenesis is considered complete.

2.1.2.3 Remodeling

The final stage of wound healing is remodeling and scar formation and can last up to two years. In this phase fibroblasts will gradually replace ECM components such as fibronectin and hayaluronic acid with a collagen matrix. MMPs are secreted by fibroblasts in addition to macrophages and endothelial cells and aid in the remodeling process. Myofibroblasts at the wound edge contribute to wound contraction allowing for wound closure. The collagen matrix is disorganized at first, but will eventually become oriented. This process if highly regulated by PDGF, TGF-β, and FGF[25].

2.1.3 Pathological Wound Healing

While most wounds are typically healed within two weeks, diabetic and elderly patients often suffer from chronic non-healing wounds that are unable to repair themselves for months even with treatment from hospitals. This is typically due to an excessive and chronic inflammatory phase, infections, or a loss of response to reparative stimuli such as hypoxia that results in insufficient revascularization and wound closure[18][26].

2.1.4 Current Advanced Treatments for Wound Healing

Autologous or engineered skin grafts and growth factor treatments are two of the more common current advanced treatments. A full-thickness portion of patient's skin can be removed from a non-wounded area and transplanted to the site of the wound. It is critical that there is no infection present, sufficient hemostasis, and removal of pressure at the wound site for the weeks following the grafting procedure. Tissue-engineered human skin equivalents can also be used such as Integra or Dermagraft[27][28]. These skin substitutes consist of cross-linked collagen and glycosaminoglycans that mostly serve to

transiently close the wound to allow for the patients cells to migrate and subsequently reject, degrade, and rebuild its own collagen matrix for successful wound healing.

Many growth factors are known to play critical roles in wound healing although successful clinical translation has been limited[19]. Vascular Endothelial Growth Factor (VEGF) is critical for endothelial cell recruitment and angiogenesis and has been proven in animal and in vitro models to improve wound healing[29]. However, clinical trials have shown limited efficacy. This could be due to multiple reasons, including the need to deliver supraphysiological doses due to the low residency time at the wound site. Additionally, short half-life of growth factors and high costs can be an issue. PDGF-bb is the only clinically proven growth factor to improve wound healing in clinical trials[30], [31]. However, recent studies have also shown concern of an increased cancer risk in cases of recurrent treatments with PDGF-bb, since many patients with chronic non-healing wounds may have multiple in the span of one year[32].

2.1.5 Mesenchymal Stem Cells and Wound Healing

MSCs are known to be involved in wound healing and are currently being investigated for treatment of chronic wounds in diabetic patients[33], [34]. The use of MSCs is a promising clinical therapy due to evidence that allogeneic MSCs are well tolerated by the body. This is thought to be due to the lack of expression of co-stimulatory molecules B7-1, B7-2, CD40, and CD40 Ligand which may prevent the activation of alloreactive T cells [35]. The mechanism of action of MSCs in wound healing is thought to be three fold. First, MSCs secrete anti-microbial factors such as LL-37 that help to reduce infection[36]. Second, MSCs are able to modulate the immune response to prevent a chronically inflamed environment which is known to inhibit wound healing. Third, MSCs

are capable of promoting angiogenesis through secretion of paracrine factors such as VEGF and inducing cell migration and proliferation to the site of the wound.

2.1.5.1 Antimicrobial

Bacterial infection can be a major barrier in successful wound healing. MSCs aid in bacterial clearance not only through their effects on immune cells responsible for clearing bacteria, but also through secretion of the anti-microbial peptide LL-37[36] which is known to directly kill microorganisms. This is important since persistence of infection in the wound bed leads to the prolonged presence of neutrophils and may further exacerbate a chronic non-healing wound.

2.1.5.2 Modulation of Immune Response

MSCs have been shown to modulate the immune response in a chronic inflammatory environment and are currently being investigated for treatment of diseases such as Crohn's disease[37] and multiple sclerosis[38] that are characterized by chronic inflammation. MSCs modulate the immune response by affecting multiple immune cell types. IFN-gamma and TNF-α at the wound site stimulate the MSCs to secrete PGE2 which effects the cytokine secretion of dendritic cells, T-cells, and natural killer cells causing the immune cells to increase secretion of anti-inflammatory molecules and decrease secretion of pro-inflammatory molecules[39]. The resulting change in cytokine environment is critical for wound healing to progress from the inflammation phase to the proliferative phase. Prolonged inflammatory phases are a major cause of chronic non-healing wounds[18].

2.1.5.3 Angiogenesis

Angiogenesis is critical for successful wound healing so that blood flow and oxygen can be returned to the tissue. Damaged keratinocytyes at the site of the wound secrete the cytokine CCL21 that recruit MSCs to the site of injury to aid in the repair of blood

vessels[40]. Additionally, MSCs at the site of a wound have been shown to secrete paracrine factors such as VEGF and FGF that recruit endothelial cells for growth of new blood vessel walls[41]. MSCs improve functional hemodynamics and functional vascular regeneration in a chronic ischemic skin flap model through pro-angiogenic paracrine factor secretion[42]. Grafting of MSC spheroids into ischemic tissue has been shown to increase new blood vessel formation and improve limb survival[11]. Direct cell contact with endothelial progenitor cells and MSCs has also been shown to increase differentiation to endothelial cells from both cell types in an *in vitro* tube forming assay[43]. Thus, the mechanisms of MSCs involvement are through both paracrine factor secretion, but also as support cells to regenerate the vasculature.

2.2 Hypoxia and Angiogenesis

2.2.1 Hypoxia

The hypoxia response of cells controls many biological processes including revascularization of damaged tissue, tumor growth, wound healing, and cell metabolism. Additionally, hypoxic culture of MSCs contribute to maintenance of stem cell potency *in vitro* which is thought to be due to a better recapitulation of the bone marrow niche in which MSCs can reside in the body.[44] which. Cells are able to quickly respond to hypoxia by transcription of hypoxia responsive genes by Hypoxia Inducible Factor(HIF).

2.2.2 Hypoxia Inducible Factor Signaling Pathway

The HIF signaling pathway is the main regulator of effects due to lack of oxygen. The pathway consists of HIF-1,2,and 3 with HIF-1 having the most prominent effect and thus is discussed in more detail below. HIF-1 is a heterodimer consisting of an oxygen responsive HIF-1α subunit and an oxygen independent HIF-1β subunit. When oxygen is present, HIF-1α is hydroxylated by prolyl hydroxylase 2 (PHD2), which requires oxygen, Fe^{2+}, and 2-oxoglutarate as co-substrates for successful hydroxylation. The hydroxylation

of proline residues 402 and 564 marks HIF-1α for degradation by the 26s proteasome through its interaction with the β domain of the Von Hippel-Lindau tumor suppressor protein (pVHL) causing it's ubiquitination by the pVHL-E3 ligase complex.

In hypoxic conditions, PHD2 lacks oxygen as a co-substrate and cannot hydroxylate HIF-1α for degradation, leading to the accumulation of HIF-1α in the nucleus where it dimerizes with HIF-1β. There, a complex is formed with p300/Creb binding protein which binds to Hypoxia Response Elements (HREs) and promotes the transcription of hypoxia response genes such as vascular endothelial growth factor (VEGF), heme oxygenase-1 (HO-1), erythropoietin (EPO), inducible nitric oxide synthase (iNOS), glucose transporter protein-1 (Glut-1), insulin-like growth factor 2 (IGF-2), endothelin 1, and transferrin, among others. These genes are involved in the regulation of angiogenesis, proliferation, survival, and glucose transport.

2.2.3 Methods to perturb the HIF-1α System

Prolyl Hydroxylase can be inhibited through multiple methods in normoxic conditions. One of the oldest methods used is to disrupt the balance of Fe^{2+} either by use of an iron chelator such as Deferoxamine (DFO) or Cobalt Chloride, which competitively inhibits iron. As mentioned previously, iron is a co-substrate needed for PHD to become activated, thus iron effectors are quite effective at inhibiting PHD. However, iron is a necessary cofactor in many biological processes so the lack of specificity and the risk of off-target effects is not desirable.

Another method for inhibiting PHDs is through the use of 2-oxoglutarate analogs such as L-Mimosine (L-Mim), Dimethyloxalyl-glycine (DMOG), 3,4-Dihydroxybenzoate (3,4-DHB), and N-[[1,2-dihydro-4-hydroxy-2-oxo-1-(phenylmethyl)-3-quinolinyl]carbonyl]-glycine (IOX2). However, 2-oxoglutarate analogs also have drawbacks because they may inhibit other 2-oxoglutarate oxygenases. Importantly, histone demethylases are 2-oxoglutarate dependent, which could cause unintended effects

on the epigenetics of the cells treated. DMOG is one of the most commonly used 2-oxoglutarate analogs. However, Chowdhury *et al* found that DMOG was less potent than IOX2 with relative IC50 values of 5μM and 0.022μM respectively. Also, increase in HIF-1α appeared to be due to both inhibition of PHD2 and Factor inhibiting HIF (FIH) which can be distinguished by asparingyl hydroxylation of HIF-1α rather than prolyl hydroxylation of HIF-1α. Additionally, it was determined that IOX2 was 2-5000 times more selective for PHD2 than histone demethylases assayed. The authors concluded that IOX2 was the ideal PHD inhibitor since it was commercially available, highly potent, and most importantly more selective towards PHD2 than the histone demethylases tested[10].

2.2.4 Effects of hypoxia on MSCs

The effects of hypoxia on the differentiation potential of MSCs *in vitro* has been well studied. Yang *et al* found that MSCs cultured in hypoxia or treated with the iron chelator DFO exhibited decreased osteogenic potential[45]. A more recent report studied the effect of hypoxia and DFO or DMOG on human primary MSCs and found that hypoxia and both PHDi promoted osteogenesis but suppressed adipogenesis[46]. The discrepancies in the osteogenic potential results may be due to differences in the systems used for hypoxic culture, or variations in donor MSCs. Hypoxia has also been found to aid in the maintenance of potency and prevention of senescence of MSCs during long term culture[44].

Hypoxic pre-conditioning of MSCs has been shown to affect the migration and engraftment of MSCs. Hung *et al* found that pre-conditioning of MSCs in hypoxia led to increased expression of CXC3RI and CXCR4, both of which are known to be important for modulating MSC migration to sites of injury[47]. The exposure to DFO was also able to increase expression of the receptors. Hypoxic pre-conditioned MSCs were mixed with normoxic MSCs and competitively engrafted into a chick embryo. Hypoxic MSCs engrafted preferentially and incorporated into the tissues of the developing embryo

demonstrating that hypoxic pre-conditioning aids in the homing and engraftment of MSCs[47]. Rosova *et al* also found that hypoxic preconditioning improved MSC cell migration and increased Akt activation. Additionally, hypoxia pre-treated MSCs accelerated restoration of blood flow to the hind limbs in a hind-limb ischemia model[4].

Changes in paracrine factor secretion from MSCs in response to hypoxia have been observed. Kinnaird *et al* found that VEGF, FGF-2, IL-6, PlGF, and MCP were secreted at higher levels under hypoxic culture at 1% O_2 than normoxic culture[41]. Although there have been multiple studies investigating the effects of various PHDi on MSCs and many other cell types, the use of PHDi as an engineering tool for potential therapies has not been thoroughly investigated. Pre-conditioning of MSCs in hypoxia before transplantation has been shown to have lasting effects on cell migration up to 14 hours after treatment[4]. While this may be useful for clinical applications in which the MSCs are needed to home to the site of injury- wound healing applications would likely involve direct application of the MSCs for longer than 14hours and thus may require methods to allow for longer term exposure to the PHDi to maintain effects.

CHAPTER 3 Effect of Prolyl Hydroxylase Inhibitors on the Secretion of Angiogenic Factors by Mesenchymal Stromal/Stem Cells

3.1 Introduction

Prolyl Hydroxylase inhibitors are commonly used to increase HIF-1α expression in many cell types. Commonly used PHD inhibitors are DFO, an iron chelator, and the 2-oxoglutarate analogs, DMOG and 3,4-DHB. Pharmaceutical companies, however, have moved away from these molecules due to their lack of specificity and have developed drugs that claim to be highly specific for PHDs. The pharmaceutical company Glaxo Smith Kline, currently has clinical trials ongoing with a PHDi molecule GSK1278863, for treatment of anemia, chronic kidney disease, and peripheral artery disease[48]. The use of more specific PHD inhibitors is important to decrease risks of off-target effects such as inhibition of histone demethylases which are in the same family of enzymes as PHD[10]. A recent study found that the PHDi, IOX2, was more potent than DMOG and is commercially available. While IOX2 has been characterized in other cell lines, it has not been thoroughly investigated in MSCs. It has been shown to affect MSCs in a similar manner as hypoxia in terms of autophagy of MSCs, however, paracrine factor secretion was not assessed, nor effects on wound healing or angiogenesis[49]. Due to the potential advantages IOX2 may have due to its increased potency and high selectivity, IOX2 was chosen as a candidate to investigate further in this study.

The culture of MSCs as three dimensional spheroids may have multiple advantages to single cell culture. First, spheroid culture increases the immunomodulatory and angiogenic paracrine factor secretion of MSCs compared to monolayer[7][12]. Additionally, MSC aggregates have been shown to have improved cell retention and survival when delivered *in vivo*[50]. This is thought to be due to a pre-conditioning to local hypoxia by cells in the interior of the spheroid, thus conditioning the spheroids to better tolerate ischemic environments *in vivo*[11]. The maintenance of native ECM and

cell-cell contacts are also contributing mechanisms in increased cell survival and enhanced paracrine factor secretion of MSC spheroids compared to single cells[51].

The first objective of this study was to examine the effects of two common PHD inhibitors DMOG and 3,4 –DHB and the more specific PHD inhibitor, IOX2, on the angiogenic factor secretion of MSCs. Second, the effect of PHDi treatment on MSC spheroids formed by forced aggregation will be assessed. It is hypothesized that this will further enhance the pro-angiogenic factor secretion of MSCs due to previous literature that both spheroid formation and hypoxic culture enhance MSC angiogenic factor secretion.

3.2 Methods

3.2.1 Mesenchymal Stromal/Stem Cell Culture

Human bone marrow derived Mesenchymal Stem Cells were obtained from the Texas A&M College of Medicine Institute for Regenerative Medicine and cultured according to established protocol[52]. Approximately 1 x 10^6 cryopreserved MSCs of a passage number no greater than four, were plated onto a 150mm tissue culture dish in 20 mL of MSC complete media (Minimum Essential Media, Alpha, [Mediatech, Inc., Manassas, Va, USA], 16.5% Fetal Bovine Serum [HyClone, Logan, UT, USA], 2 mmol/L L-glutamine [Mediatech, Manassas, VA, USA], 100 U/mL penicillin and 100 μg/mL streptomycin [Mediatech]) and incubated overnight(37°C, 5%CO_2). The day after thawing, cells were rinsed with PBS and detached from the plate using 0.25% Trypsin and 2.21mM ethylenediamine tetraacetic acid in Hank's Buffered Salt Solution (Mediatech). Equal volumes of MSC complete media were added and the dissociated cells were counted using a hemacytometer. Cells were plated onto 150mm tissue culture dishes at a density of 60 cells/cm^2 in 20mL of MSC complete media. Cells were fed by complete media exchange every three days until cells reached approximately 70%

confluency. Cells were then passaged using trypsin and used for subsequent experiments. Monolayer experiments were plated at a density of 5,500cells/cm^2 in 24-well plates.

3.2.2 Spheroid Formation and Culture

MSC spheroids were formed using forced aggregation into agarose micro-wells to allow for the high-throughput generation of homogeneously sized spheroids. Briefly, 6 x 10^5 human MSCs were added to 24-well micro-well inserts consisting of approximately 1200 wells that are 400μm in size to form ~500 cell spheroids. The plates were spun at 200g for 5 minutes and then incubated for 18 hours (37°C, 5% CO_2) before being gently pipetted using wide bore pipette tips and transferred to 100mm bacteriological grade petri dishes. Approximately 600 spheroids were transferred per plate and cultured in 10mL MSC complete media (described above) in suspension culture on an orbital rotary at 65rpm for up to 4 days.

3.2.3 Treatment of MSCs with Prolyl Hydroxylase Inhibitors

Dimethyloxalyl Glycine(DMOG, R&D Systems), 3,4-Dihydroxybenzoate(AKA Protocatechuic acid ethyl ester, Sigma), and IOX2 (Tocris) were dissolved in DMSO at a concentration of 100mM, 137mM, and 21mM respectively. Stock solutions were added directly to culture media at appropriate concentrations. For vehicle controls, equal volumes of sterile DMSO were added to the media.

3.2.4 Conditioned Media Collection

At day four of culture, spheroids and media were transferred to a 15mL conical tube where spheroids were centrifuged (100g, 5min). Conditioned media was collected and stored at -20°C until further analysis. VEGF secretion was determined using a DuoSet ELISA kit for human VEGF (R&D systems). Spheroids were rinsed with PBS, pelleted, and then frozen and stored at -80°C overnight until cell number was determined using a

CyQUANT Cell Proliferation Kit (Life Technologies) according to the manufacturer's protocol.

3.2.5 Human Cytokine Antibody Array

A membrane based human cytokine antibody array (Abcam, ab133998) containing 80 cytokines involved in immune response and angiogenesis was performed according to manufacturer's protocol. Briefly, membranes were blocked with the blocking buffer (30min, RT), and then 1mL of conditioned media (pooled from four samples) was added to the membrane for overnight incubation at 4°C under gentle shaking. Membrane was washed thoroughly with wash buffer I and wash II before biotin-conjugated anti-cytokines were added and incubated (2h, RT). Membranes were washed as described previously and incubated with HRP-conjugated streptavidin (diluted 1:1000, 2h, RT). After washing, the membranes were blot-dried and incubated with the detection buffer (2 min, RT), and then were imaged using ODYSSEY Infrared Imaging System (LiCor, Lincoln, NE). Intensity of individual dots was quantified by densitometric analysis using ImageJ software (National Institutes of Health). The membrane incubated with sample collected from monolayer vehicle group was assigned as reference membrane. The normalized signal density of each dots was then calculated using the formula: X(Ny)=X(y)*P1/P(y), where P1=mean density of Positive control spots on reference array; P(y)=mean signal density of Positive control spots on Array "y"; X(y)= signal density for spot "x" on array for sample "y", and X(Ny)=normalized signal intensity for spot "X" on array "y". A complete list of cytokines included on the array are found in the appendix (A1).

3.2.6 Statistical Analysis

Statistical analysis was performed using Prism (GraphPad, Inc). Data is represented as mean +/- standard error (n=4, unless otherwise stated). One-way or two-way Analysis of

Variance coupled with Tukey's post-hoc test was used to determine statistical significance. P-values less than 0.05 were considered significant.

3.3 Results

3.3.1 Effects of various small molecule PHDi on VEGF Secretion of MSCs

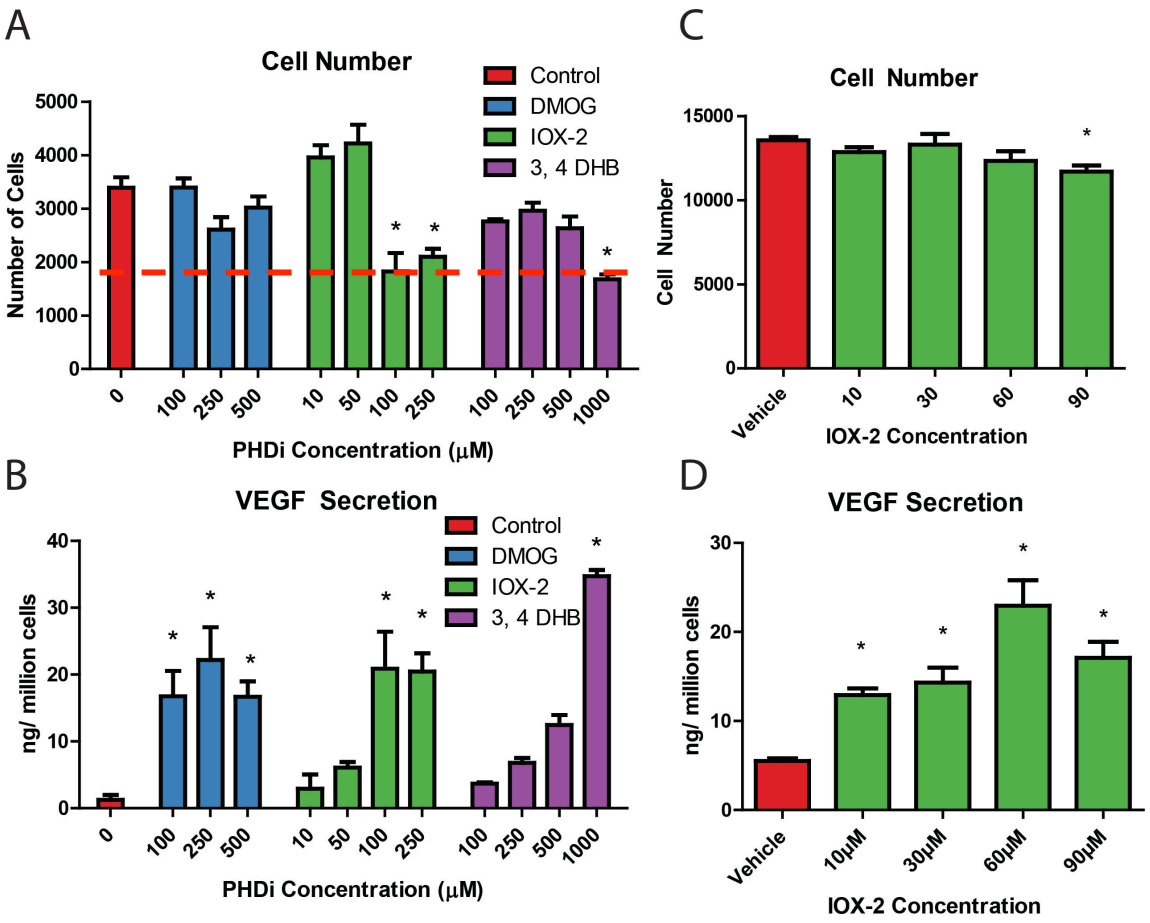

Figure 1:VEGF response of MSCs to three Prolyl Hydroxylase inhibitors: DMOG, IOX2, and 3,4-DHB.

MSCs cultured in monolayer for three days were treated with various concentrations of DMOG, IOX2 or 3,4 DHB. Cell count (A) and VEGF secretion normalized to cell number (B) after treatment with PHDi demonstrated varying responses for each respective PHDi. Additional IOX2 dose response was performed to determine optimal concentration based on cell number (C) and VEGF secretion (D) in response to IOX2. *indicates significantly different to Control (P<0.05).

A range of concentrations for each PHDi was screened in order to determine appropriate dosages and compare the relative potencies of the different inhibitors. VEGF was chosen as the primary means of assessment due to its well established activation by the HIF-1α pathway and role in angiogenesis. Cell number was quantified as an assessment of cell

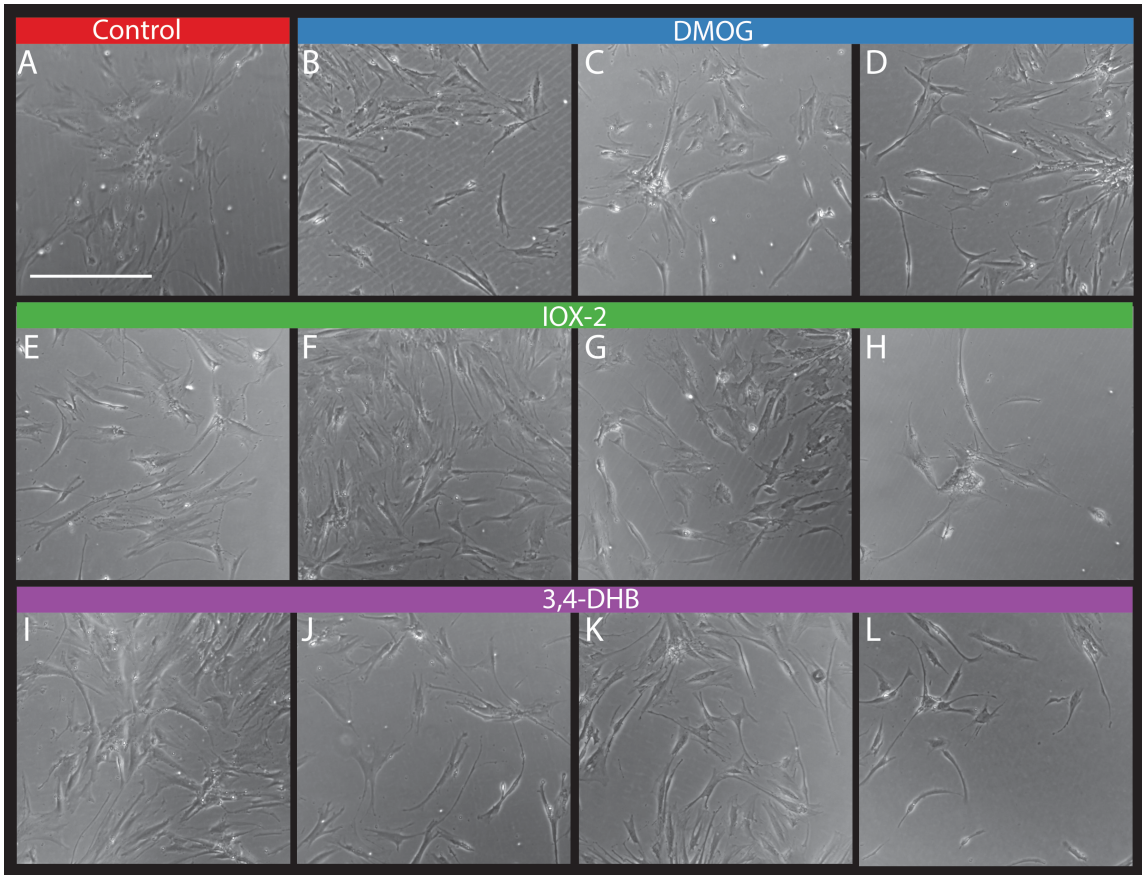

Figure 2: The effect of PHDi on MSC morphology. MSCs treated with DMOG at 100μM(B), 250μM(C), and 500μM (D) looked similar to MSCs that were not treated with PHDi (A). The morphology of IOX2 treated MSCs appeared normal for 10μM(E), 25μM(F) and 50μM(G), but 100μM IOX2 (H) appeared sparse. 3,4-DHB cells (I-L, 100, 250, 500, 1000μM respectively) had normal morphology but were more sparse than the control.

survival and growth. There was no significant decrease in cell number due to PHDi treatment with DMOG. However, MSCs treated with IOX2 at 100μM and 250μM had almost 40% less cells after three days of treatment (p-value <0.001). The highest dosage of 3,4-DHB at 1000μM resulted in 50% fewer cells at day 3 than non-treated MSCs(p-value <0.001). The VEGF secretion (Figure 1B) by MSCs treated with DMOG was increased 13- to 18-fold compared to untreated MSCs, but did not vary significantly over the range of DMOG concentrations examined. The VEGF response to IOX2 was increased approximately 17-fold at higher concentrations of 100μM and 250μM, which were also the two groups with significantly decreased cell numbers. An additional range of IOX2 concentrations were examined in order to determine an effective concentration

that did not have a negative effect on cell growth. IOX2 concentrations between 10µM and 90µM were assesed. This study revealed an optimal concentration of 60µM for IOX2 due to a peak in the VEGF response at 23ng VEGF/million cells (Figure 1D) and no significant decrease in cell number (Figure 1C). In contrast, the highest concentration of 3,4-DHB stimulated a 29-fold increase in VEGF secretion, however, this was accompanied by a 3-fold reduction in cell number (Figure 2L).

3.3.2 Transient Response to PHDi

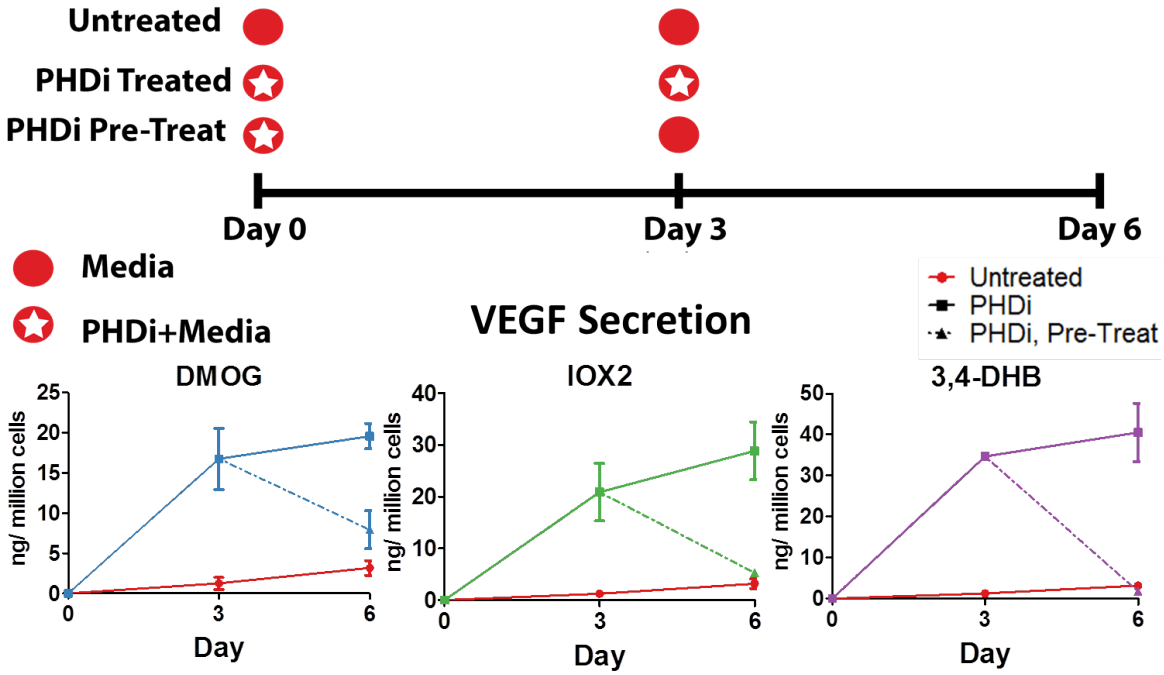

Figure 3: Transient Response to PHDi. Persistence of VEGF response was assessed by removal of PHDi from the culture media at Day 3, and testing for VEGF in Day 6 conditioned media. Concentrations of 100µM, 50µM, and 1000µM are shown for DMOG, IOX2, and 3,4-DHB respectively.

To assess the persistence of the effects of PHDi on VEGF secretion, PHDi was either included or omitted from the culture media at day 3 of feeding and changes in VEGF level were assessed at day 6. (Day 6+ or Day 6-, respectively) for MSCs previously treated with 100µM DMOG, 50µM IOX2, and 1000µM 3,4-DHB. It was evident that the omission of PHDi from culture media caused VEGF levels to be attenuated indicating a

transient increase in VEGF secretion by MSCs in response to PHDi in all three PHDi tested.

3.3.3 Effect of DMOG and IOX2 on VEGF secretion of MSC spheroids

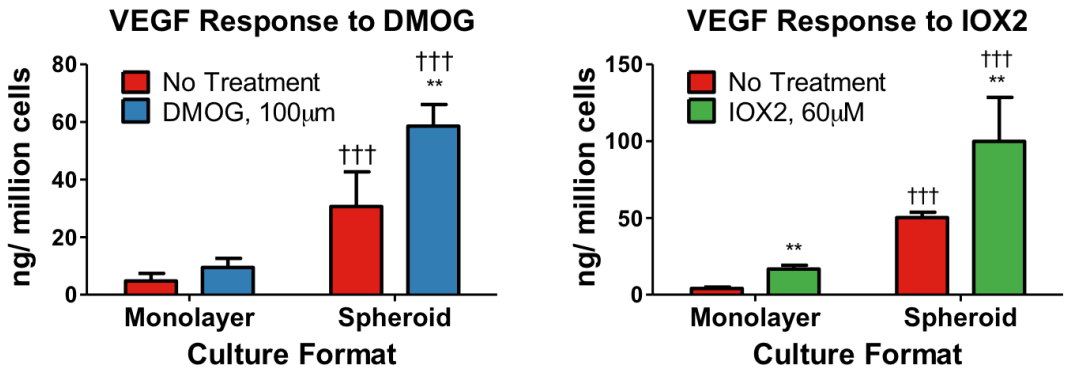

Figure 4: Effects of PHDi on the VEGF secretion of MSC Spheroids. VEGF secretion of MSCs in response to DMOG(A) or IOX2(B) was assessed when cultured either as monolayer or spheroids. ** indicates statistically different to no treatment (p-value <0.01). ††† indicates statistically different to monolayer.

DMOG and IOX2 were chosen for further investigation into their effects on MSCs when cultured as three dimensional spheroids. As observed previously, culturing of MSCs as spheroids instead of adherent monolayers induced a six-fold increase in VEGF secretion (per cell) in the absence of PHDi or hypoxic conditions(Figure 4). The addition of PHDi to the culture media of MSC spheroids led to an approximate 2-fold increase for both 100μM DMOG and 60μM IOX2. These results demonstrate that treatment of MSCs with PHDi augments secretion of VEGF when cultured as spheroids rather than monolayer.

3.3.4 Semi-Quantitative screen of cytokines affected by IOX2

A membrane based antibody array analyzing conditioned media from MSCs treated with 60μM IOX2 in monolayer or spheroid culture (Figure 5A) revealed at least a 10% increase in cytokine concentration in 61 and 68 of the 80 cytokines assayed for monolayer and spheroid treated cells, respectively. Additionally, levels of two cytokines, IL-8 and MCP-1 were decreased upon IOX2 treatment in both monolayer and spheroid

cultures. To examine the most responsive cytokines, a threshold of a 2 fold change from no treatment was established (Figure 5B). Nine cytokines had 2-fold or higher increase in secretion upon IOX2 treatment and included IL-10, TARC, VEGF, Eotaxin, IGFBP-2, IGF-BP3, NT-3, Leptin, and TGF-β1. Also of note are cytokines that responded differently upon IOX2 treatment across the different culture platforms. RANTES was increased upon IOX2 treatment in spheroid culture but was unaffected upon treatment with IOX2 in monolayer culture relative to untreated MSCs. The relative levels of GRO in conditioned media of IOX2 treated spheroids decreased whereas the levels slightly increased upon treatment in monolayer culture.

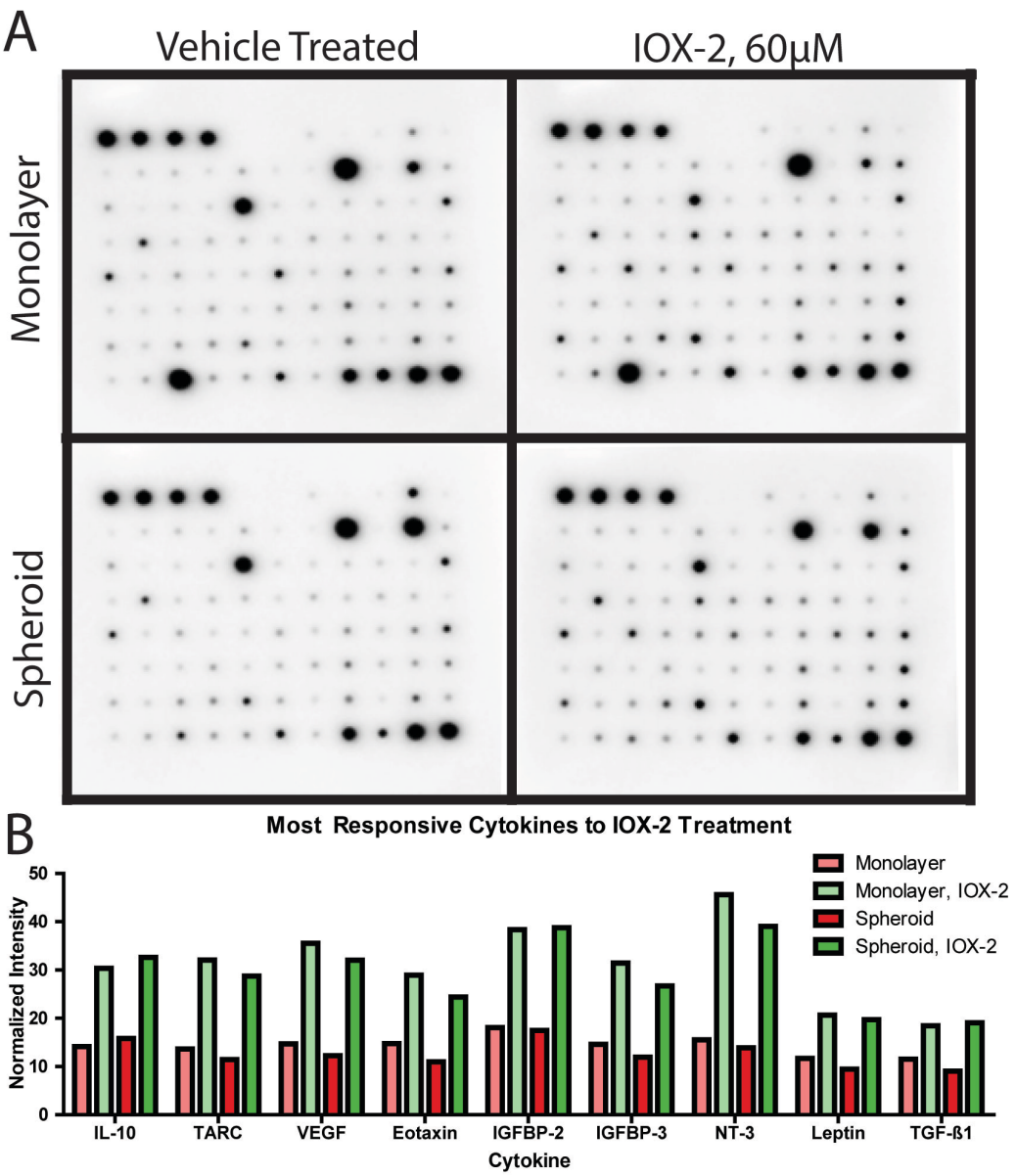

Figure 5: A semi-quantitative membrane based antibody array screened for 80 human cytokines present in MSC conditioned media (A). Densitometry analysis normalized to positive controls revealed that secretion of at least 75% of the cytokines was increased with IOX2 treatment. Cytokines that were upregulated by at least two fold are shown (B).

3.4 Discussion

DMOG, IOX2, and 3,4-DHB were all able to increase the VEGF secretion of MSCs in monolayer culture. However, upon removal of PHDi treatment, VEGF secretion was attenuated suggesting the presence of PHDi is necessary to maintain the effect on VEGF secretion. DMOG and IOX2 treatment in spheroid culture further increased VEGF secretion by 6-fold. A semi-quantitative antibody array detected increased levels of the majority of the 80 cytokines screened in the conditioned media of IOX2 treated MSCs relative to untreated MSCs for both monolayer and spheroid culture many of which were related to angiogenesis.

The differences observed in cell growth, morphology, and VEGF secretion of MSCs in response to DMOG, IOX2, and 3,4 DHB is interesting to note, and likely due to differences in specificity of each PHDi. Previous reports in other cell lines have demonstrated low responsiveness of cells to 3,4-DHB[53]. The results here found an increase in VEGF secretion to 3,4-DHB was only apparent at the highest concentration of 1000μM. However, this concentration also caused reduced cell number and a change in cell morphology, rendering this treatment undesirable. Thus it was concluded that 3,4-DHB should not be investigated further.

Cytotoxicity has not been reported at DMOG concentrations of up to 1 mM and activation of HIF-1α can be achieved at as low as 100μM[9], [54]. The concentrations of DMOG examined here fall well within that range and concentrations between 100 and 500μM DMOG were effective at producing an increase in VEGF secretion, while not effecting cell growth and survival. DMOG is a non-selective PHDi and may also work through inhibition of the enzyme factor inhibiting HIF (FIH). Although DMOG is non-specific it is effective at inducing an increase in VEGF secretion across a large effective range before having an effect on cell number, which could be beneficial in pharmaceutical applications. However, IOX2 is more potent than DMOG with an IC50 250 fold lower than DMOG, and more specific for PHD over other 2-oxoglutarate

dependent enzymes such as histone demethylases[10]. IOX2 is one of the most potent inhibitors of PHD commercially available and thus the effects of IOX2 on paracrine factor secretion of MSCs was assessed further through a semi-quantitative screen that compared relative levels of cytokines in MSC conditioned media. This study demonstrated that the small molecule drug candidate IOX2 enhances the secretion of a multitude of cytokines commonly involved in wound healing.

The cytokine array results support the hypothesis that IOX2 will lead to increased paracrine secretion of factors implicated in angiogenesis. In addition to VEGF, the secretion of 8 other cytokines was increased by two-fold or more which include: IL-10, TARC, eotaxin, IGFBP-2, IFGBP-3, NT-3, Leptin, and TGF-β3. Of specific interest, IL-10 was increased almost 3 fold with PHDi treatment. IL-10 is an anti-inflammatory cytokine and has been shown to be critical in wound healing for its role in preventing neutrophil infiltration into the wound[55]. Additonally, IL-8 was decreased upon IOX2 treatment by approximately 30% which may also lead to decreased neutrophil recruitment since IL-8 is a known chemoattractant to neutrophils[22]. This is important because the persistence of high amounts of neutrophils is indicative of a chronic non-healing wound environment, thus PHDi treatment may improve chronic wound healing through not only increasing angiogenic paracrine factor secretion but also decreasing neutrophil infiltration.

An increased secretion of TARC by IOX2 treated MSCs was observed in the antibody array. TARC is a chemokine that serves for the recruitment and migration of T-helper type II cells which are known to secrete anti-inflammatory cytokines IL-4, IL-10, and IL-13 [56]. Also, TARC has been shown to induce MSC migration *in vitro*[40]. Eotaxin, a chemoattractant known to cause the infiltration of eosinophils, was also present in the IOX2 treated MSCs. An established link between eotaxin and angiogenesis has been established in previous studies through an increase of endothelial cell migration *in vitro* as well as CAM assays and matrigel plug assays[57].

IGF binding proteins are known to be involved in angiogenesis. IGFBP-2 has been studied heavily in the cancer field, linking it with tumor angiogenesis and enhancement of VEGF gene promoter activity in neuroblastoma cells[58]. Additionally IGFBP-2 increases with HIF-1α expression so it is not surprising that it is upregulated in IOX2 treated MSCs since IOX2 acts by stabilizing HIF-1α[59]. IFGBP-3, was also upregulated in IOX2 treated MSCs and has been shown to induce angiogenesis both *in vitro* in tube formation assays and *in vivo* wound healing studies[60].

The VEGF secretion of MSCs after PHDi was removed from the culture media revealed a transient response to PHDi, with VEGF levels returning close to basal levels at 72 hours. There is limited literature studying the lasting effects of PHDi on paracrine factor secretion. Studies have shown, however, functional differences in cells that have been pre-conditioned by hypoxia or PHDi approximately 18 hours after pre-conditioning[4], [61]. The results here suggest that an increase in paracrine factor secretion is transient after PHDi pre-conditioning.

Overall, the results here demonstrate that both DMOG and IOX2 are promising candidates for enhancing the pro-angiogenic factor secretion of MSCs. However, since the increase in VEGF secretion is attenuated after PHDi removal simple pre-conditioning of spheroids in PHDi may not be the most effective method for preparing MSC spheroids for wound healing therapies. The ability to prolong the enhancement of paracrine factor secretion of MSC spheroids would likely improve the therapeutic efficacy of MSC spheroids to lead to improve wound healing of chronic non-healing wounds.

CHAPTER 4 Localized Delivery of PHDi to MSC spheroids via Microparticles for Enhanced Angiogenic Factor Secretion of MSCs

The ability of PHDi's to enhance the angiogenic properties of MSCs appears to be a promising approach for increasing the potential therapeutic efficacy of MSC spheroids for wound healing. However, the short-lived effects of pre-treating MSCs with PHDi added to culture media is likely not ideal since wound healing is a relatively long process that takes approximately 2 weeks to complete. Warnecke *et al* studied the effects of three PHDi: L-Mim, 3,4-DHB, and S956711. When delivered systemically it was found that L-Mim and S956711 caused an increase in HIF-1α expression in the kidneys but nowhere else examined. When injected repeatedly into a rat sponge model there was a strong increase in invasion of vascularized tissue into the sponge[5]. Similarly, Ding et al found that PHDi treatment improved bone healing capacity of ASCs in a critically sized calvarial defect. In this study, ASCs were implanted into the defect using a hydrogel composite. Cells were pretreated with DMOG for 24 hours and DMOG was also added to the hydrogel. The DMOG hydrogel group outperformed the group with cells engineered to overexpress HIF-1α. This was most likely due to the soluble DMOG in the hydrogel that had effects on the host response and may have recruited host MSCs or endothelial cells to the hydrogel[9]. *These studies demonstrate that the use of materials to localize the pro-angiogenic effects of PHDi is an important factor for successful clinical use.*

One method to both localize delivery of PHDi and reduce off-target risk is the delivery of PHDi via microparticles incorporated within MSC spheroids. Previously, we have shown that microparticles can be incorporated into stem cell aggregates to deliver 12-fold less growth factor compared to soluble delivery and still maintain similar effects[13]. Additionally, this may allow for sustained release and exposure of a PHDi to MSCs to prolong the pro-angiogenic effects. The objective of this study was to compare the encapsulation efficiencies of DMOG or IOX2 into PLGA microparticles and

determine the effect of the PHDi microparticles on the pro-angiogenic factor secretion of MSCs when incorporated into MSC spheroids.

4.1 Methods

4.1.1 PLGA Microparticle Formation

PLGA microparticles were made using an oil-in-water emulsion technique at a 1:10 ratio of oil to water. The oil phase consisted of a 2% (w/v) PLGA solution in dichloromethane. Different types of co-solvents were used to dissolve the drug into the oil phase. This ranged from 10-40% DMSO and also 12% DMF in order to find conditions that allowed for homogeneous microparticle formation and efficient encapsulation. The oil phase consisting of either PLGA+cosolvent in DCM or PLGA+Drug+cosolvent in DCM were added dropwise to a 2% Poly Vinyl Alcohol(Acros Organics, 88% Hydrolyzed, M.W:22,000) solution while being homogenized at 3000rpm for 2 minutes. The emulsion was then left on a magnetic stir plate for 3 hours to allow for solvent evaporation. The emulsion was centrifuged at 1500 g for 5 minutes and washed three times with water. Microparticles were then lyophilized for 48 hours and stored at -20°C until further use.

4.1.2 Analysis of DMOG or IOX2 concentrations

DMOG (Figure 6B) was detected using High Performance Liquid Chromatography. This was necessary since it was not able to be detected using standard spectroscopy. A standard curve was made using known concentrations of DMOG (Figure 6A). IOX2(Figure 6D) was detected by reading the absorbance at 330nm and comparing the O.D readings of the unknown sample to readings on a standard curve. The optimal absorbance reading was found by doing a spectral scan of IOX2 (Figure 6C) and determined to be at 330nm (Figure 6C, yellow line). A known amount of microparticles were dissolved in the solvent acetonitrile and read against a standard curve. As

demonstrated in the spectral scan, the background PLGA did not interfere with the absorbance reading of dissolved IOX2 MPs (Figure 6C, Green Triangles).

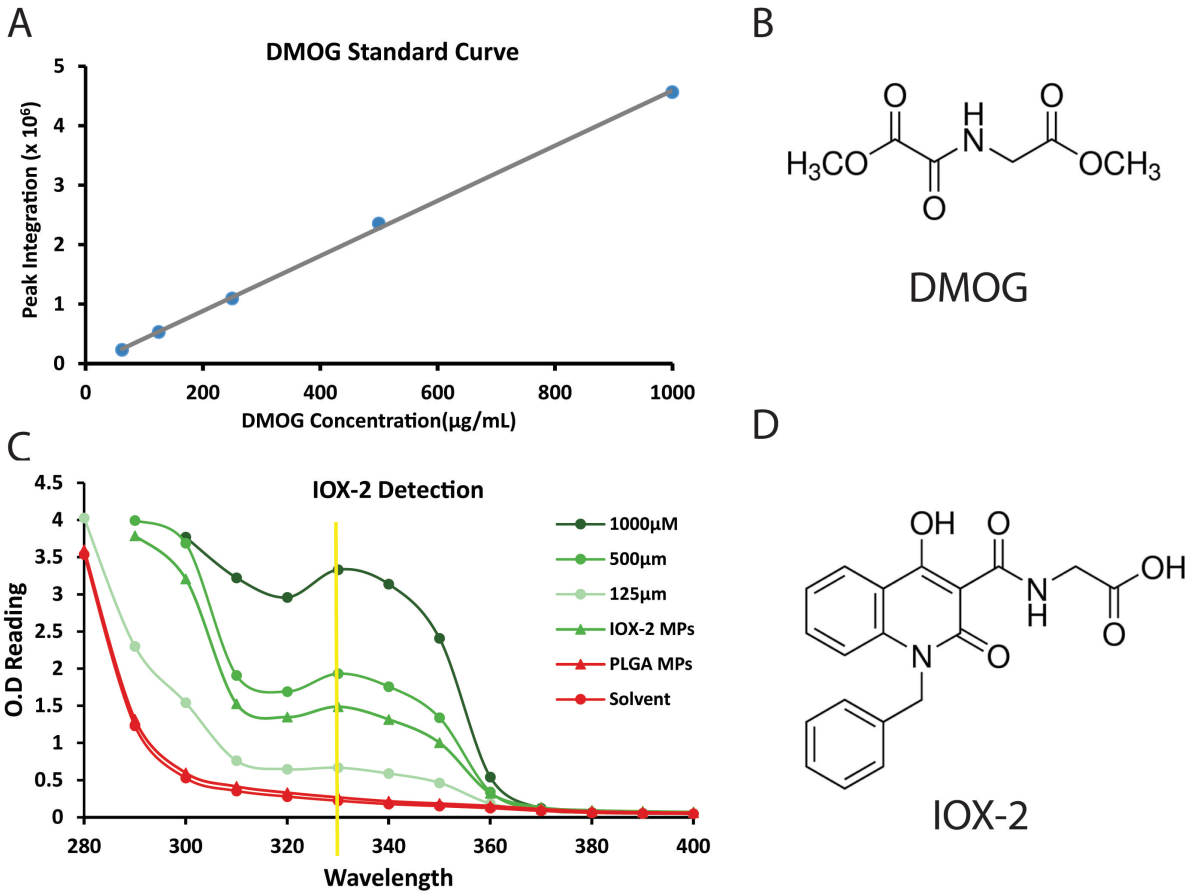

Figure 6: Small Molecule Detection: DMOG(B) was detected using a standard made from HPLC readings(A). IOX2 (D) was detected using absorbance readings at 330nm (yellow line), which clearly falls within the spectral range of the molecule(C).

4.1.3 Release from PLGA Microparticles

The release kinetics of IOX2 from PLGA microparticles was determined by resuspending 1 mg of microparticles in 1 mL of 0.1% BSA Solution (Bovine Serum Albumin, Millipore) and incubating at 37°C on a rotisserie. At desired timepoints (3h, 4d, 7d) the microparticles were centrifuged at 1500g for 5 minutes and 250µL of supernatant was removed and stored at -20°C for later analysis. The volume removed was replaced with an equivalent amount of 250µL of 0.1% BSA.

4.1.4 Incorporation of Microparticles into MSC Spheroids

Microparticles were incorporated into MSC spheroids using a modified protocol of the forced aggregation technique described previously in chapter 3. After cell centrifugation, 5.4 x 10^6 microparticles were added to each micro-well. The plate was subsequently centrifuged for a third time before overnight incubation (37°C, 5% CO_2) to allow for spheroid formation. The spheroids were removed and washed with media twice to remove unincorporated microparticles before culture. Efficiency of microparticle incorporation was determined by lysing a known number of spheroids in RIPA buffer and counting the resulting microparticles on a hemacytometer.

4.1.5 HUVEC Migration Assay

Human umbilical vein endothelial cells(HUVECs) were cultured according to Lonza protocols using EGM-2MV media (Lonza, EGM-2MV Bullet Kit). Briefly, HUVECS were plated at a density of 2,500 cells/cm^2 in 5mL of HUVEC media per 25cm^2 and grown to approximately 70% confluence. Cells were passaged by rinsing the cells with PBS and brief exposure to room temperature 0.05% trypsin (approximately 1-3 minutes). Cells were collected and centrifuged at 200g for 5 minutes before being used or plated for further expansion. If being used for a HUVEC migration assay, cells were labeled with CellTracker Green CMFDA(5-chloromethylfluorescein diacetate; Life Technologies) for 30 minutes prior to trypsinization. The migration of HUVECs was determined using FluorBlok cell culture inserts (8μm pore size, BD Biosciences). Cell culture inserts were coated with 10μg/mL fibronectin prior to use for 30minutes and placed into transwell companion plates(BD Biosciences). Labeled HUVECs were transferred into 24-well inserts at a density of 30,000 cells per insert. Approximately 750μL of basal media (EBM, Lonza) was placed in the bottom chamber with MSCs that had previously been plated or day 4 spheroids. After 4 hours the cells were read at 485/525nm (Synergy H4 Hybrid plate reader, BioTek). Prior to reading the wells using a plate reader, transwell

inserts were transferred to empty companion plates to allow for fluorescence readings that were not disrupted by MSC spheroids or cells disrupting the light path. Relative fluorescent intensities readings were used to compare pro-migratory effect of substance in the basal chamber.

4.1.6 HUVEC Scratch Assay

Human Umbilical Vein Endothelial Cells (Lonza) were grown to confluence in 24-well tissue culture plates using EGM-2MV growth media. HUVECs were serum starved overnight in basal EGM media before a scratch was made using a pipette tip. The media was changed to remove cell debris and replaced with conditioned media. Images were take immediately after the scratch and 12 hours post-scratch. The width of the scratch was measured using ImageJ.

4.1.7 Statistical Analysis

Statistical analysis was performed using Prism(GraphPad, Inc). Data is represented as mean +/- standard error (n=4, unless otherwise stated). One-way or two-way Analysis of Variance coupled with Tukey's post-hoc test was used to determine statistical significance. P-values less than 0.05 were considered significant.

4.2 Results

4.2.1 DMOG encapsulation into PLGA microparticles

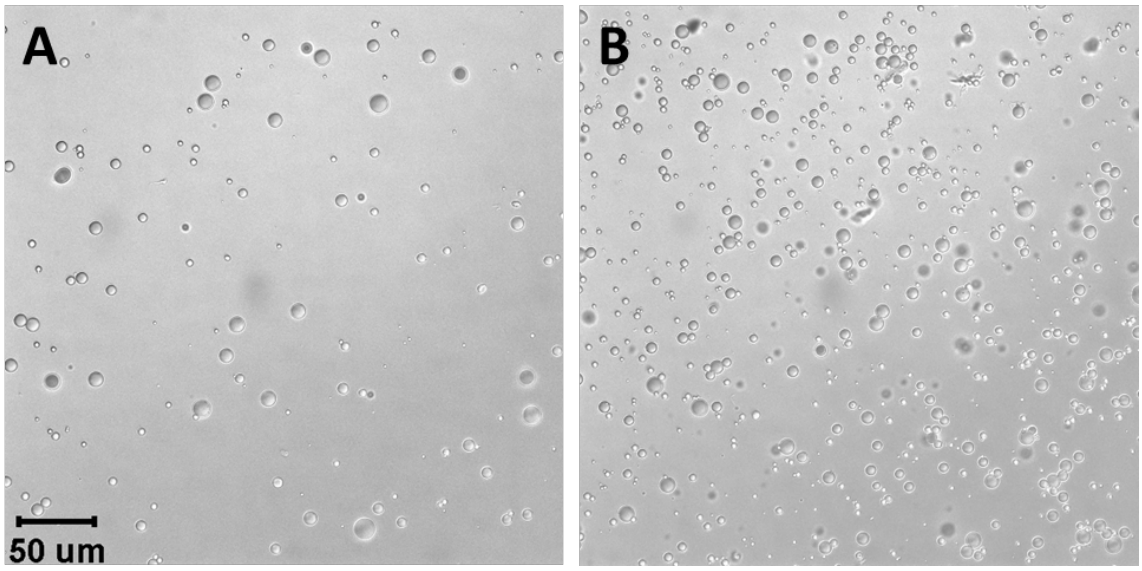

Figure 7: DMOG Microparticle formation. PLGA microparticles (A) and DMOG encapsulated microparticles (B) have similar morphology and size.

The morphology of DMOG encapsulated microparticles appeared normal and homogenous in size (Figure 7). However, the amount of DMOG encapsulated into PLGA microparticles was very low. One batch of microparticles (approximately 20mg) was dissolved in acetonitrile, and was undetectable within the standard curve determined via HPLC(Figure 6A). Extrapolation of the small signal would suggest encapsulation of approximately 345pmol/mg. This low encapsulation efficiency is believed to be due to the amphiphillic nature of DMOG(Figure 6B) which allows DMOG to diffuse into the water phase at a faster rate than the solvent, thus leading to very low encapsulation.

4.2.2 First generation IOX2 Microparticles

The first generation of PLGA microparticles were made in a small batch with a total emulsion volume of 20mL and 40% DMSO as a co-solvent. The IOX-2 was dissolved in DMSO at a concentration of 83mM. The resulting IOX2 microparticles contained rod-like debris (Figure 8B). The particles were larger than expected and spanned a larger range than is typical for PLGA MPs produced under similar conditions. The size of the PLGA MPs were 5.87µM +/-3.9µm and the IOX2 microparticles were 6.68+/- 4.055µm (Figure 8C). The IOX2 microparticles contained 683 nmoles/ mg and released approximately 400nmoles over the course of one week (Figure 8D).

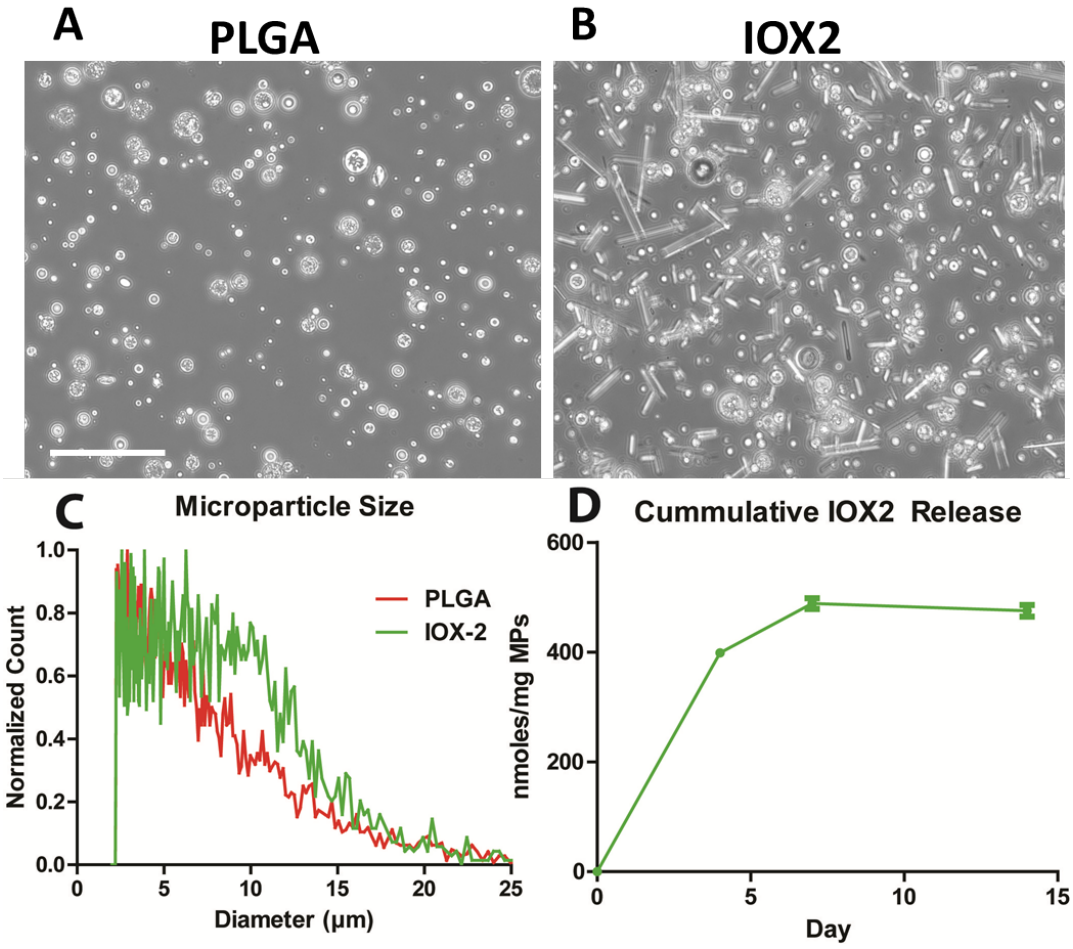

Figure 8: First Generation IOX2 Microparticle Characterization. First generation MPs formed were heterogeneous(A) and IOX2 MPs contained rod-like debris (B). The size distribution (C) indicated a large variance in the size. Approximately 400 nmoles were released over the course of one week(D).

4.2.3 Incorporation of first generation IOX2 microparticles into MSC Spheroids

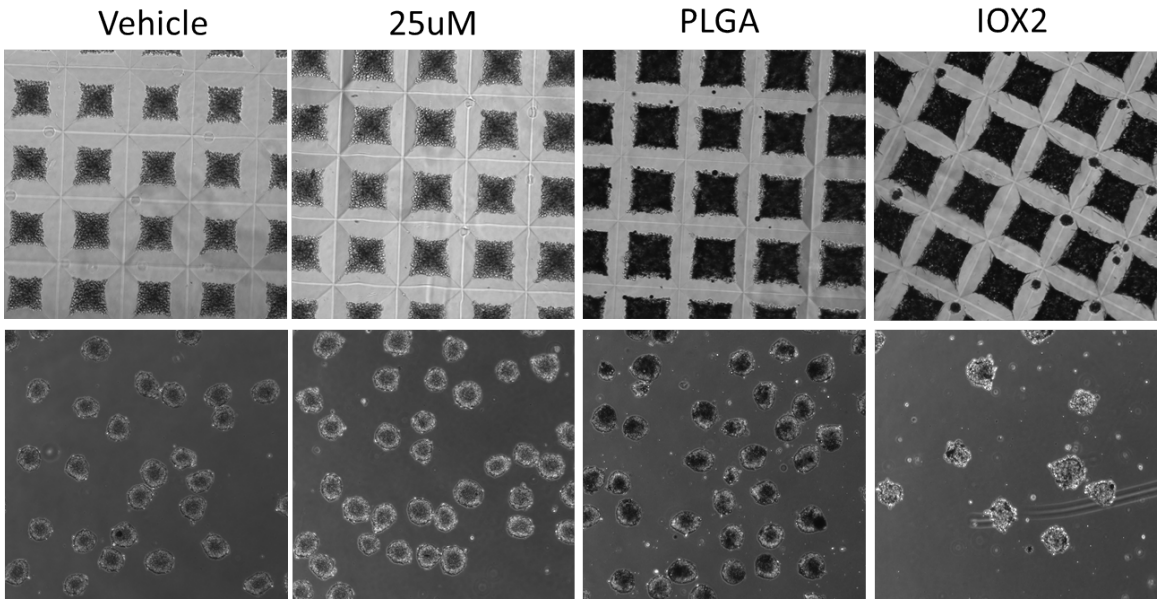

Figure 9: First generation IOX2 microparticle incorporation. Microparticles were incorporated using forced aggregation and shown in wells after centrifugation(top row) and out of wells on day 1 (bottom row).

Microparticles incorporated into spheroids successfully via forced aggregation and spheroids appear to be of similar size. PLGA MPs can clearly be seen in the MSC spheroids as demonstrated by the dark spots within the spheroids. There appeared to be less IOX2 MP incorporation compared to PLGA MP incorporation as evident by the smaller areas of dark spots in the MSC spheroids on day 1 (Figure , bottom row). This motivated a quantification of microparticle incorporation on day 1 in future studies. Additionally, the rod-like debris was evident on day 0 (Figure , top row) but was not evident at day 1. This led to the hypothesis that the rod-like debris was actually crystallized IOX2. This was ultimately confirmed by dissolving pure IOX2 in DMSO at high concentrations and observing the same debris, which did not persist at lower concentrations. The presence of IOX2 crystals was deemed unacceptable since it does not allow for a large amount of control on dosing of IOX2 as would be desired.

4.2.4 Effect of first generation MP incorporation on VEGF secretion

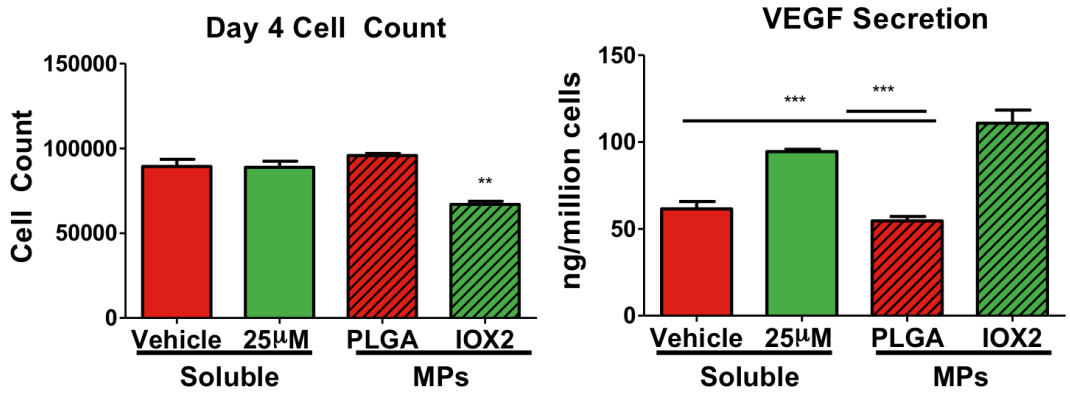

Figure 10: First Generation MP effects on MSC Spheroids. Cell number (A) and VEGF secretion (B) were assessed. ** indicates significantly different than vehicle control (p-value<0.01), * indicates significantly different than vehicle control (p-value<0.001),**

MSC spheroids were cultured for 3 days before the conditioned media and cell pellets were collected for analysis. The VEGF secretion was assessed via ELISA. The final cell number at day 4 of culture was decreased in IOX2 MP treated groups (Figure 10B, p-value<0.001). Both the soluble IOX2 and IOX2 MP treated groups secreted approximately 2-fold more VEGF per cell than the vehicle or PLGA treated groups (Figure 10B). The increase in VEGF secretion was encouraging since a significant effect in MP treated groups was observed, although, the presence of IOX2 crystals at day 1 makes it difficult to conclude that this was microparticle mediated and not confounded by the presence of IOX2 crystals. Thus, additional microparticle formulations were investigated that may lead to more homogeneous microparticle batches that do not have excessive amounts of IOX2 crystals in the system.

4.2.5 Second generation microparticle synthesis

4.2.5.1 Effect of co-solvent percentage

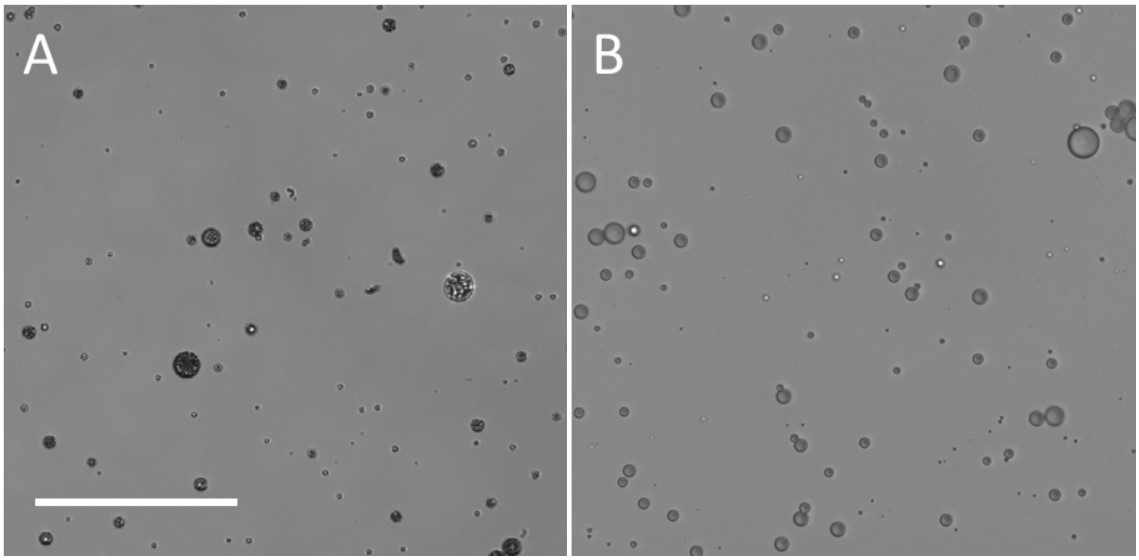

Figure 11: Effect of co-solvent percentage on microparticle formulation. PLGA microparticles made from 40% DMSO (A) and 12% DMSO as co-solvents differ in morphology and surface appearance.

The large variance in size of the first generation microparticles and the irregular surface appearance was hypothesized to be due to the high DMSO percentage used as the co-solvent in the emulsion process which is typically limited to 10-15%. To assess if lower co-solvent percentage would lead to more homogeneously sized MPs, a 12% DMSO co-solvent PLGA microparticle was synthesized. These microparticles appeared to be more homogeneously sized than the 40% DMSO co-solvent microparticles and the surfaces appeared to be smoother (Figure 11A and 11B). Thus 12% co-solvent was deemed a more acceptable co-solvent percentage.

4.2.5.2 Choice of co-solvent

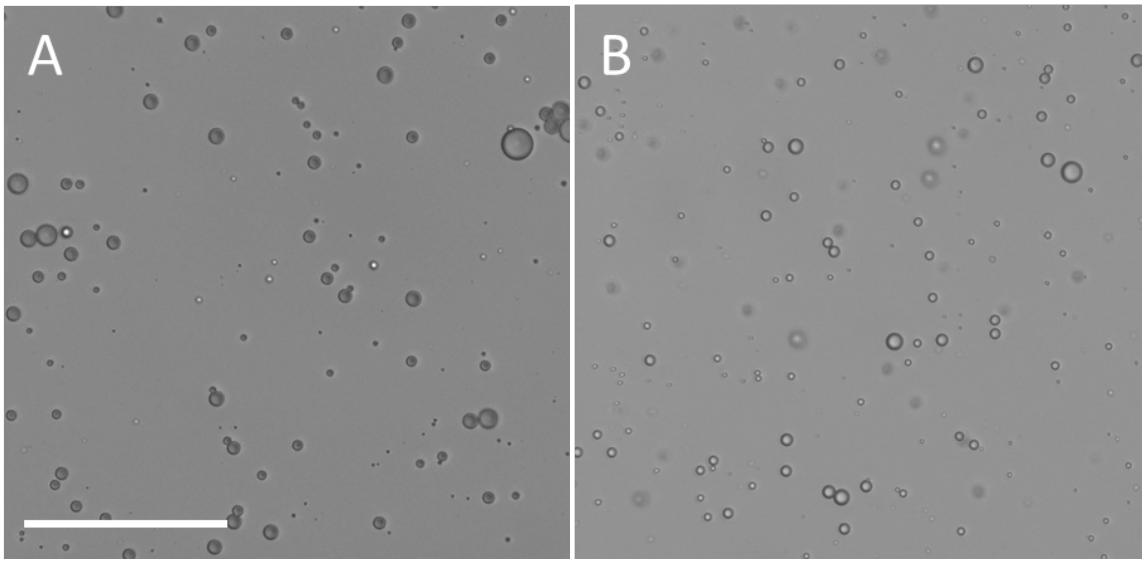

Figure 12: Effect of co-solvent on microparticle formation. PLGA microparticles formed with 12% DMSO(A) or 12% DMF(B) appear similar in morphology.

It was evident that IOX2 was not being completely dissolved at the concentration of 83mM in DMSO even though the solubility in DMSO is reported to be up to 100mM[62]. An alternative manufacturer states the solubility of IOX2 to be 14mM in DMSO and 40mM in DMF[63]. The reported increased solubility motivated the use of DMF as a co-solvent instead of DMSO. PLGA MPs made using 12% DMF formed successfully(Figure 12B) and maintained a homogenous morphology similar to the 12% DMSO PLGA MPs(Figure 12A). IOX2 was dissolved in DMF at the suggested concentration of 40mM and used for microparticle formation at a 12% co-solvent ratio. This final formulation led to both homogeneously sized PLGA MPs and successfully removed the presence of the IOX2 crystals(Figure 13B).

4.2.6 Second generation microparticle characterization

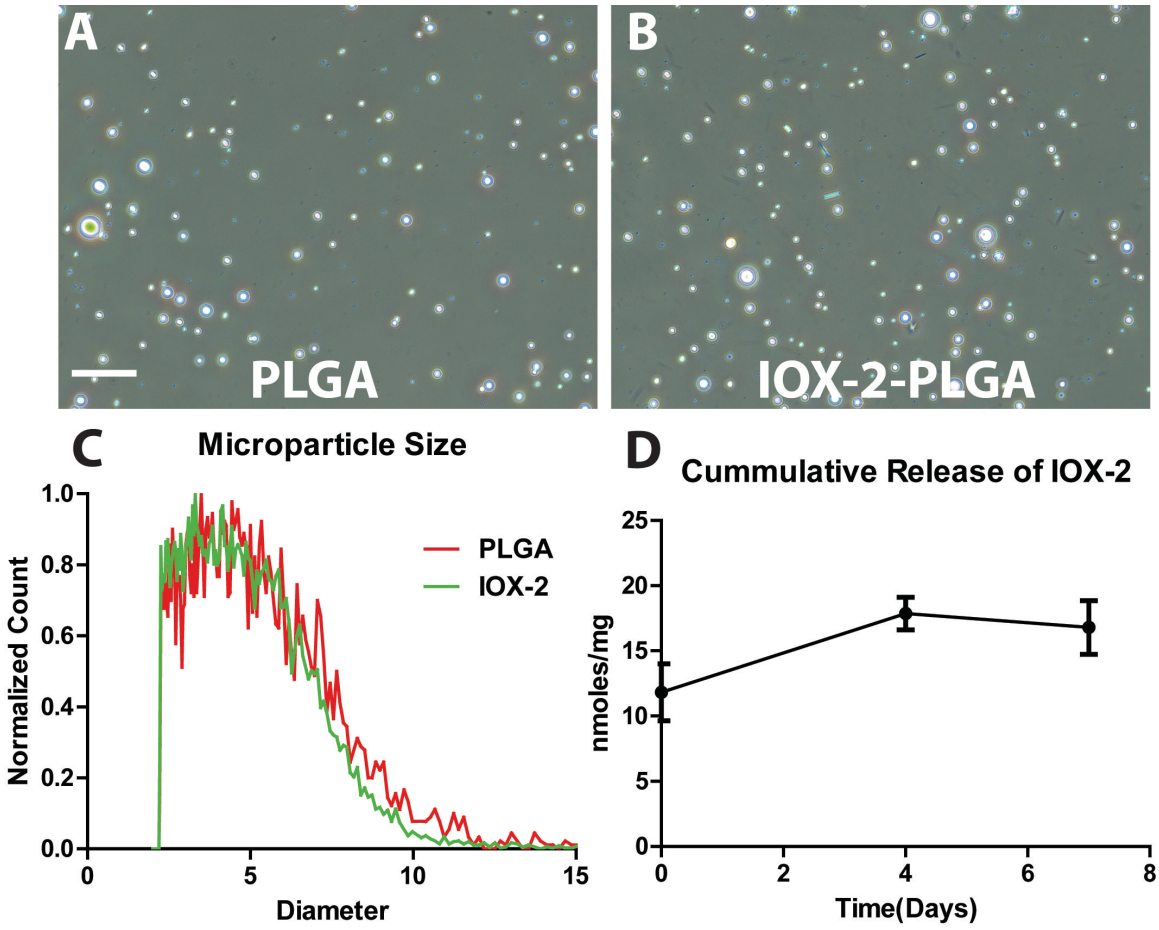

Figure 13: Second generation IOX2 microparticle characterization. PLGA MPs (A) and IOX2 MPs (B) appeared homogeneous and IOX2 crystals were not observed. The size (C) of the particles was less variable than first generation MPs(C). Release of IOX2 from the particles was assessed over the course of one week(D).

IOX2 was successfully incorporated into PLGA microparticles at an amount of 76.4 nmoles/mg MPs and without the appearance of IOX2 crystals (Figure 13A and B). The amount of IOX2 encapsulated was almost 10 fold less than the amount of first generation microparticles. This finding was to be expected, however, due to the lower IOX2 concentration being added during MP synthesis (83mM compared to 40mM from the first generation) and a decrease in co-solvent compound decreasing from 40% to 12%. The average size of the PLGA control MPs and IOX2 MPs were 4.7µm +/- 2.2µm and 4.4+/- 1.8µm, respectively (Figure 13C). These were not only smaller but also had decreased variance than the first generation microparticles. A release assay over 10 days revealed

that after the initial burst release, negligible amount of IOX2 was detected indicating no sustained release from the microparticles. (Figure 13D). While the MPs contained less IOX2, the smaller size theoretically should improve MP incorporation which was thought may compensate for the decreased amount of IOX2 encapsulated per MP.

4.2.7 Second generation microparticle incorporation

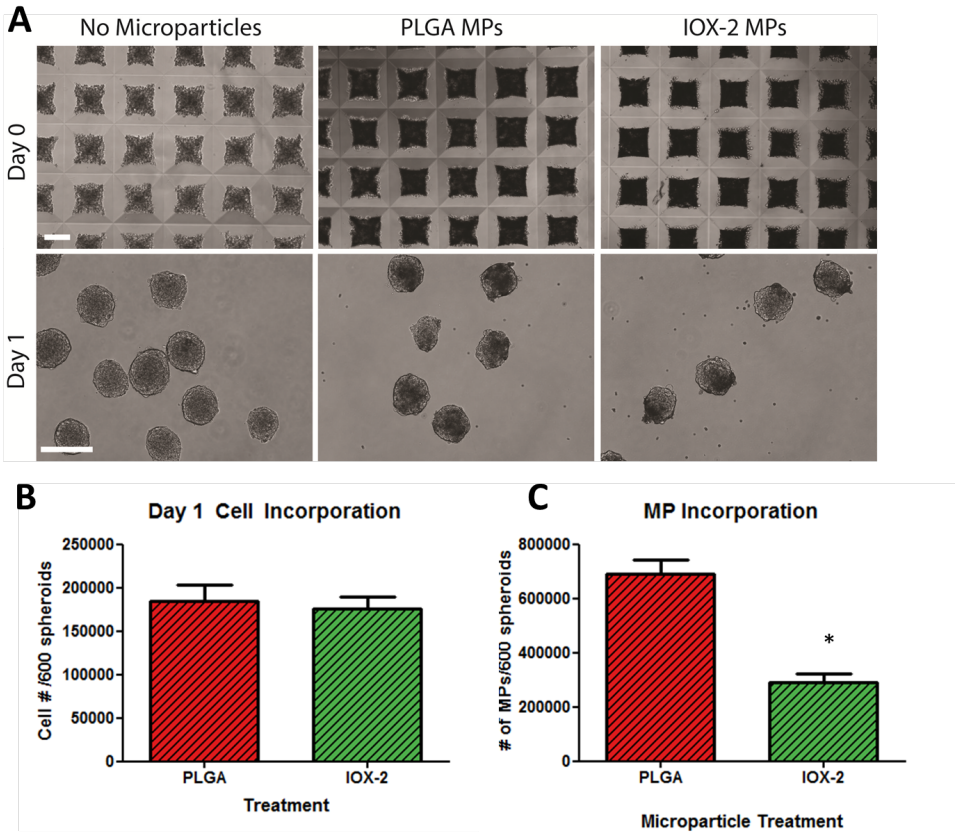

Figure 14: Second generation microparticle incorporation into MSC spheroids. Microparticles were incorporated into MSC spheroids (A) and cultured for 3 days. Cell number(B) and VEGF secretion were analyzed(C). ** indicates p-value<0.01

Second generation microparticles incorporated successfully into the aggregates, leading to $6.9x10^5$ and 2.9×10^5 MPs incorporated per plate of spheroids, respectively (Figure C). Incorporation of microparticles appears similar to the incorporation observed in first generation microparticles (Figure A and Figure , respectively). There was no difference in initial cell number incorporated between the microparticle groups (Figure B).

4.2.8 Effect of IOX2 MPs on the VEGF Secretion of MSC Spheroids

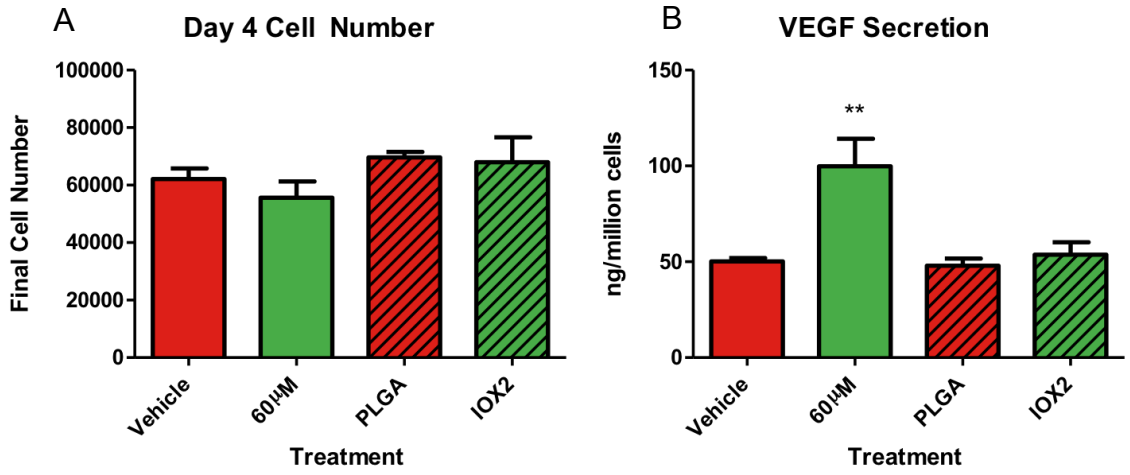

Figure 15: VEGF secretion of microparticle treated MSC spheroids. Cell number was similar amongst all groups (A) and VEGF secretion was only effected in the soluble IOX2 treated group. **indicates P-value<0.01.

The final cell number was similar amongst all the groups, however, approximately 2-fold lower than the amount at day 1 (Figure A). VEGF secretion(Figure B) was increased in the 60μM soluble treatment group similar to that observed in the first experiment. However, unlike the first generation microparticles, incorporation of PLGA MPs or IOX2 MPs had no effect on the final cell number nor the VEGF secretion of the cells. These results indicate that insufficient PHDi was being delivered within the system to elicit an increase in VEGF secretion when using the second generation microparticles.

4.2.9 HUVEC migration response to IOX2 treated Spheroids

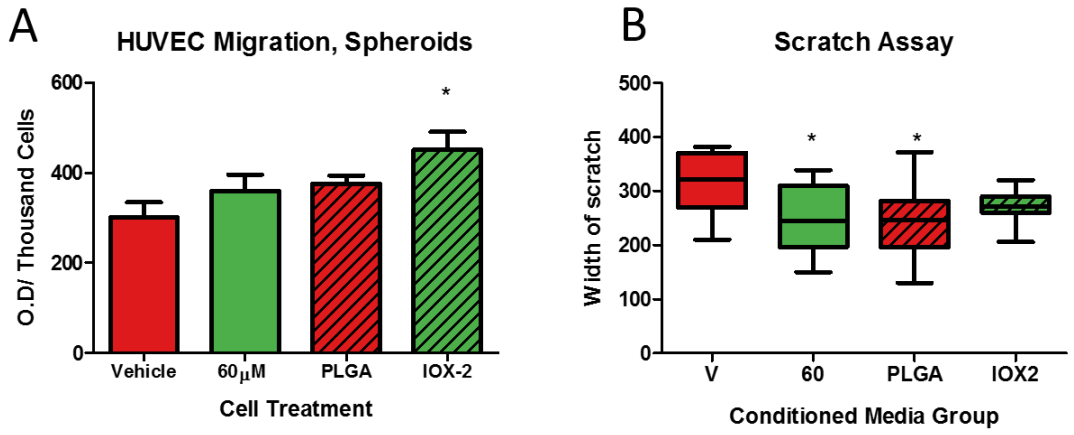

Figure 16: *in vitro* Functional Assessment of Pro-Angiogenic effects of treated MSC spheroids. A HUVEC migration assay(A) and scratch assay demonstrate effects of IOX2 on HUVEC migration(B)

A HUVEC migration assay and scratch assay was performed since it may be a more robust overall indicator of angiogenic potency compared to the assessment of VEGF secretion using ELISA. Spheroids treated with IOX2 MP had a significant improvement on HUVEC migration(Figure 16A), but soluble treatment did not. A scratch assay revealed improved HUVEC migration within a scratch when exposed to conditioned media of both 60μM IOX2 treated spheroids and PLGA MP treated spheroids(Figure 16B).

4.3 Discussion and Conclusions

Encapsulation of both DMOG and IOX2 into PLGA microparticles proved to be difficult due to the amphiphillic nature of DMOG and the low solubility of IOX2. First generation IOX2 microparticles successfully increased VEGF secretion of MSC spheroids to levels comparable to soluble treatment. However, the irregular morphology and presence of IOX2 crystals necessitated adjustment of parameters for microparticle formulation. Multiple iterations of microparticles were necessary for successful IOX2 encapsulation, while maintaining normal microparticle morphology. The final parameters yielded microparticles with 76nmoles IOX2/ mg MPs, which was approximately 9-fold

less encapsulation than the first generation microparticles. Second generation microparticles did not have an effect on VEGF secretion of MSC spheroids, however, IOX2 MP treated spheroids did have an effect on HUVEC migration in a transwell HUVEC migration assay suggesting the IOX2 MPs may have had a response on other angiogenic factors besides VEGF.

While DMOG may be a satisfactory small molecule PHDi to induce a hypoxic response, its amphiphillic nature makes drug delivery a challenge. The hydrophobicity of IOX2 however, is more amenable to encapsulation. Importantly, IOX2 is a more potent and specific PHD inhibitor than DMOG[10], thus it is concluded that IOX2 is the more promising PHD inhibitor for drug delivery applications. IOX2 could be encapsulated in PLGA microparticles using both DMSO and DMF as the co-solvent for encapsulation of the drug. However, due to the lower solubility of IOX2 in DMSO, a large amount of IOX2 crystals formed during the emulsion. In the first generation MP spheroid study IOX2 crystals were incorporated into the aggregate as evidenced by the phase images of the wells in which the crystal structures could be seen. It is likely that once the cells were returned to the incubator, the IOX2 was able to dissolve due to the higher temperature since this was observed empirically with the soluble stock solution in DMSO. Due to this confounding factor, it is impossible to know whether the effects on the VEGF secretion of the MSC spheroids were due to actual release from the microparticles or the IOX2 crystal gradually dissolving. Exposure of the MSCs to high local concentrations of IOX2 would likely have toxic effects as evidenced from monolayer studies in chapter 3. The observation that there were less cells in the IOX2 MP treated spheroids at day 4 of culture may support the hypothesis that high local levels of IOX2 are present within the MSC spheroids. Since the dissolution of IOX2 crystals is unable to be controlled, it was evident that improvements in the microparticle synthesis were necessary. Using DMF as a co-solvent at a lowed co-solvent percentage (12% rather than 40%) led to microparticles of an acceptable size with a more narrow size distribution compared to the first generation

MPs and eliminated crystal formation. The release of IOX2 from the particles over the course of one week, mostly occurred in the first 4 days and resulted in the release of approximately 20% of total PHDi. However, degradation of PLGA microparticles is thought to occur at a faster rate in biological systems than *in vitro* assays due to the presence of cells and enzymes that may expedite degradation[64].

The incorporation of second generation PLGA MPs and IOX2 MPs did not negatively affect MSC formation nor cell incorporation into spheroids. The incorporation efficiency of PLGA MPs was 2-fold higher than IOX-2 MPs. The reasons for this are unclear, but may be due to a difference in degradation of the microparticles. Further studies are necessary to determine the cause of the lower incorporation efficiency. One potential method for improving microparticle incorporation would be to treat the microparticles with an adhesive protein such as gelatin which may promote improved incorporation into the MSC spheroids. This has previously been done within mouse embryonic stem cell aggregates, although it was unlear how much of an improvement in incorporation was achieved[65].

In this study, it was observed that delivery of IOX2 via second generation microparticles within MSC spheroids had no effect on the VEGF secretion of MSCs at day 4 of culture. This was not surprising since the amount of IOX2 delivered was 250 times less than an effective soluble dose of 25μM IOX2. This was calculated using the known microparticle incorporation per plate, number of MPs/ mass conversion (approximately 12.3 million MPs/mg) and the encapsulation efficiency determined previously. The estimated amount delivered in the first generation microparticles was 50 times greater than the second generation (assuming similar incorporation efficiency and using 3.5 million MPs/mg conversion within the calculation). This effective first generation MP dose was 5 times less than an effective soluble dose suggesting that localized delivery may be more potent than soluble delivery. Additionally, it is encouraging to observe the presence of PLGA MPs alone did not cause a negative effect

on cell number or VEGF secretion of MSC spheroids suggesting that if drug loading challenges can be solved, a microparticle method for delivery may still be a viable option for enhancing the pro-angiogenic factor secretion of MSC spheroids.

IOX2 MP treated spheroids were able to successfully promote the migration of HUVECs, a common functional *in vitro* assay demonstrating effects on their ability to enhance angiogenesis. The effect on HUVEC migration but not VEGF may indicate that IOX2 MPs are causing an increase in secretion of an alternative pro-angiogenic factor that was not assessed in this study. The effect on HUVEC migration was not observed in soluble IOX2 treated groups. The reason for this is unclear but may be an effect due to the presence of the particles.

Overall, the major barrier to successful delivery of PHDi to MSC spheroids is synthesis of a microparticle that can efficiently encapsulate either DMOG or IOX2 and be successfully incorporated into spheroid. This particle must be able to degrade to release most of its contents within 7 to 14 days and be non-toxic to the MSCs. Further optimization of IOX2 encapsulation using the current oil-in water emulsion technique may be worthwhile, however, the investigation of more advanced microparticle synthesis such as layer by layer technology [66]may be necessary to obtain desired effects.

Chapter 5: Future Work and Considerations

Treatment of chronic non-healing wounds with MSCs is currently being investigated in pre-clinical trials with promising results. However, engraftment of MSCs is very low, and it is likely that the beneficial wound healing effects are through paracrine factor secretion. Thus, methods to enhance paracrine factor secretion during the time frame that MSCs persist (< 1 week) would be beneficial. The results here demonstrate that PHDi enhance MSC pro-angiogenic factor secretion and verifies that IOX2 and DMOG are both effective PHDi treatments for MSCs. Additionally, these results are in agreement with previous literature that culture of MSCs as spheroids further enhances paracrine factor secretion, however technical challenges remain in adequately delivering PHDi to spheroid cultures in order to achieve the combinatorial effects of PHDi treatments with 3D culture.

Future work should investigate pro-angiogenic factor secretion at earlier timepoints after PHDi removal. In this study, it appears that the effect of PHDi on VEGF secretion is diminished once PHDi is removed from the system. While other studies have found that pre-conditioning of MSCs in a soluble treatment of PHDi can have lasting effects on engraftment and cell migration up to 18 hours days after treatment [4], no thorough studies on lasting effects on paracrine factor secretion have been performed.

The angiogenic properties of MSCs is just one mechanism of action intended for the use of MSCs in wound healing. MSCs immunomodulatory properties are thought to be critical for improving chronic non healing wounds. Thus the effects of IOX2 and DMOG on the immunomodulatory effects of MSCs should be investigated. Hypoxia has been shown to either promote or maintain the immunomodulatory properties of MSCs[67][68]. The results of the 80 cytokine array support these findings demonstrating an increase in relevant immunomodulatory cytokines. This finding is encouraging for

potential future investigations of the effects of DMOG or IOX2 on immunomodulatory properties of MSCs.

While delivery of PHDi via microparticles within the spheroids appears promising, the ability to deliver an effective dose in the time frame desired was not achieved. Future work, will investigate methods for improved delivery of IOX2 via microparticles to allow for delivery of an effective dose. One potential approach to this would be the synthesis of Layer by Layer(LbL) nanoparticles. The LbL is a very adaptable technique and is well suited for small molecules that have low aqueous solubilities[66]. This technique has previously been used to deliver doxorubicin via gold nanoparticles by conjugating doxorubicin to a polymer using a proteoytically degradeable linker[69]. This technique could be promising for IOX2 as they have similar functional groups. DMOG would not be promising for this technique as its structure would not facilitate conjugation.

Alternative PLGA-PEG based materials should also be investigated to improve the efficiency of encapsulation of IOX2. Co-polymers of PLGA-PEG can be formed into both nano/microparticles or polymeric micelles with a hydrophobic core for the aqueous IOX2 solution[70]. The formation of nanoparticles using a water-oil-water emulsion of PLGA-PEG has previously been shown to encapsulate both hydrophilic and hydrophobic drugs[71] which may make this technique a viable option for encapsulation of DMOG within PLGA-PEG nanoparticles. Mallarde et al compared PLGA-PEG microspheres with PLGA microspheres and found a faster drug release of the compound Teverelix[72] which is also beneficial for this system as release on a shorter timeframe of 7 days is preferred for wound healing. The synthesis of PLGA-PEG micelles should also be investigated for encapsulation of IOX2, however, typically loading efficiency with micelles is less than with particles formation.

To further build upon this research and previous research in the field, an advanced method for microparticle synthesis that allows for dual drug delivery and release may

prove to be the most effective treatment for wound healing. Dual drug delivery would allow for enhancement of the appropriate properties of MSCs at different phases of wound healing. For example, in the first three days of wound healing a molecule that enhances the immunomodulatory properties of MSCs such as IFN-γ could be released from microparticles to enhance their immunomodulatory response[73] and preventing a chronic inflammatory environment from forming. During the next phase of wound healing, when angiogenesis and recruitment of fibroblasts and endothelial cells is necessary, IOX2 could be released to enhance MSC paracrine secretion of VEGF. This could be achieved using the Layer by Layer technique discussed previously[66].

In conclusion, soluble treatment with both DMOG and IOX2 are successful for the enhancement of angiogenic paracrine factor secretion of MSCs in both monolayer and spheroid culture formats. However, delivery of these molecules to MSC spheroids using microparticles has proven to be challenging. DMOG was determined to be unfavorable for the encapsulation technique used here, and would likely be unfavorable for more advanced techniques that would require more functional groups for successful conjugation to polymers. The encapsulation of IOX2 into MPs was more successful than DMOG, but was not sufficient to have the desired effects on VEGF secretion of MSC spheroids.

.

APPENDIX

A.1. Appendix

A.1.1. Semi-Quantitative Antibody Array Layout

	A	B	C	D	E
1	PC	PC	PC	PC	NC
2	I-309	IL-1α	IL-1β	IL-2	IL-3
3	IL-12 (p40/p70)	IL-13	IL-15	IFN-γ	MCP-1
4	MIP-1δ	RANTES	SCF	SDF-1	TARC
5	Oncostatin M	Thrombopoietin	VEGF	PDGF-BB	Leptin
6	FGF-4	FGF-6	FGF-7	FGF-9	Fit-3 Ligand
7	IGFBP-3	IGFBP-4	IL-16	IP-10	LIF
8	NT-4	Osteopontin	Osteopotegerin	PARC	PIGF

	F	G	H	I	J	K
1	Neg	ENA-78	GCSF	GM-CSF	RGO	RGO-α
2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
3	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1b
4	TGF-β1	TNF-α	TNF-β	EGF	IGF-1	Angiogenin
5	BDNF	BLC	CK β 8-1	Eotaxin	Eotaxin-2	Eotaxin-3
6	Fractalkine	GCP-2	GDNF	HGF	IGFBP-1	IGFBP-2
7	LIGHT	MCP-4	MIF	MIP-3α	NAP-2	NT-3
8	TGF-β2	TGF-β3	TIMP-1	TIMP-2	PC	PC

A.1.2. Semi-Quantitative Antibody Array Results

Cytokine	Monolayer, Vehicle	Monolayer, IOX2	Spheroid, V	Spheroid, IOX-2
ENA-78	10.9	12.4	8.9	14.8
GCSF	10.4	14.0	8.7	13.5
GM-CSF	10.1	14.5	8.4	12.7
GRO	17.9	22.9	44.1	24.0
GRO-α	9.9	15.4	9.0	13.4
I-309	12.7	15.1	8.8	14.5

IL-1α	12.7	16.3	9.4	15.3
IL-1β	13.2	15.9	10.6	14.2
IL-2	11.7	12.9	9.6	12.8
IL-3	13.1	16.6	12.5	17.4
IL-4	10.0	11.6	8.1	12.5
IL-5	11.3	12.8	8.5	13.4
IL-6	163.8	192.5	160.9	132.3
IL-7	13.3	16.3	12.4	13.4
IL-8	57.9	47.0	139.6	99.9
IL-10	14.1	30.4	15.8	32.6
IL-12 (p40/p70)	15.4	19.1	13.6	20.8
IL-13	11.0	12.9	8.2	13.4
IL-15	11.8	16.3	8.9	14.9
IFN-γ	12.4	14.3	10.5	13.1
MCP-1	101.3	57.9	106.4	71.6
MCP-2	12.6	13.5	10.2	13.7
MCP-3	11.7	13.0	9.1	12.6
MCSF	14.6	16.7	10.6	14.1
MDC	12.8	16.8	10.1	13.8
MIG	11.9	13.0	10.0	12.2
MIP-1b	30.5	34.1	32.3	34.1
MIP-1δ	11.6	12.8	9.4	13.7
RANTES	28.9	29.4	25.4	36.5
SCF	12.7	15.4	9.5	15.4
SDF-1	13.4	15.4	10.7	14.6
TARC	13.6	32.1	11.4	28.7
TGF-β1	11.5	18.4	9.0	19.1
TNF-α	14.4	20.7	11.0	20.1
TNF-β	13.4	20.9	10.2	18.8
EGF	14.9	17.4	11.6	16.0
IGF-1	13.8	14.7	11.4	13.9
Angiogenin	11.4	13.5	9.5	12.4
Oncostatin M	28.6	32.2	24.2	33.5
Thrombopoietin	11.9	13.2	9.3	13.2
VEGF	14.7	35.5	12.2	32.0
PDGF-BB	11.1	18.6	9.0	17.1
Leptin	11.7	20.7	9.5	19.7
BDNF	33.0	31.2	12.5	23.0
BLC	13.0	15.3	9.6	14.7
CK β 8-1	18.9	19.5	14.7	18.4
Eotaxin	14.8	28.9	11.0	24.4

Eotaxin-2	19.3	28.1	16.9	25.6
Eotaxin-3	27.0	29.0	28.2	32.4
FGF-4	11.3	12.8	9.7	12.7
FGF-6	11.6	16.0	9.3	15.8
FGF-7	11.5	14.4	9.4	12.7
FGF-9	14.2	19.3	12.7	16.8
Fit-3 Ligand	11.2	13.0	9.4	12.5
Fractalkine	13.6	14.4	11.0	13.4
GCP-2	14.7	14.9	11.7	14.3
GDNF	24.6	25.5	18.8	25.6
HGF	15.2	16.7	11.7	14.6
IGFBP-1	17.4	21.0	14.3	19.3
IGFBP-2	18.1	38.4	17.5	38.8
IGFBP-3	14.6	31.4	11.9	26.7
IGFBP-4	12.1	15.6	9.9	14.0
IL-16	16.1	19.9	14.8	16.1
IP-10	16.2	30.6	16.1	26.5
LIF	24.1	45.3	27.6	44.6
LIGHT	14.0	15.6	12.3	14.2
MCP-4	11.9	12.9	9.1	11.9
MIF	21.4	26.1	18.7	28.1
MIP-3α	13.5	14.2	9.6	12.4
NAP-2	28.0	30.8	23.7	27.7
NT-3	15.5	45.6	13.9	39.0
NT-4	10.7	13.7	10.0	12.2
Osteopontin	15.5	27.2	12.9	17.2
Osteopotegerin	150.7	160.6	27.6	23.0
PARC	17.0	22.4	13.8	16.4
PIGF	14.2	17.6	14.6	15.0
TGF-β2	34.6	55.5	33.4	49.3
TGF-β3	13.9	13.5	11.0	13.6
TIMP-1	77.6	75.1	76.3	72.4

REFERENCES

[1] C. E. Fife, M. J. Carter, D. Walker, and B. Thomson, "Wound Care Outcomes and Associated Cost Among Patients Treated in US Outpatient Wound Centers: Data From the US Wound Registry | WOUNDS," *WOUNDS*, vol. 24, no. 1, pp. 10–17, 2012.

[2] Y. Wu, L. Chen, P. G. Scott, and E. E. Tredget, "Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis.," *Stem Cells*, vol. 25, no. 10, pp. 2648–59, Oct. 2007.

[3] L. Chen, E. E. Tredget, P. Y. G. Wu, and Y. Wu, "Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing.," *PLoS One*, vol. 3, no. 4, p. e1886, Jan. 2008.

[4] I. Rosová, M. Dao, B. Capoccia, D. Link, and J. A. Nolta, "Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells.," *Stem Cells*, vol. 26, no. 8, pp. 2173–82, Aug. 2008.

[5] C. Warnecke, W. Griethe, A. Weidemann, J. S. Jürgensen, C. Willam, S. Bachmann, Y. Ivashchenko, I. Wagner, U. Frei, M. Wiesener, and K.-U. Eckardt, "Activation of the hypoxia-inducible factor-pathway and stimulation of angiogenesis by application of prolyl hydroxylase inhibitors.," *FASEB J.*, vol. 17, no. 9, pp. 1186–8, Jun. 2003.

[6] S. H. Bhang, S. Lee, J.-Y. Shin, T.-J. Lee, and B.-S. Kim, "Transplantation of cord blood mesenchymal stem cells as spheroids enhances vascularization.," *Tissue Eng. Part A*, vol. 18, no. 19–20, pp. 2138–47, Oct. 2012.

[7] T. J. Bartosh, J. H. Ylöstalo, A. Mohammadipoor, N. Bazhanov, K. Coble, K. Claypool, R. H. Lee, H. Choi, and D. J. Prockop, "Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 107, no. 31, pp. 13724–9, Aug. 2010.

[8] X.-B. Liu, J.-A. Wang, M. E. Ogle, and L. Wei, "Prolyl hydroxylase inhibitor dimethyloxalylglycine enhances mesenchymal stem cell survival.," *J. Cell. Biochem.*, vol. 106, no. 5, pp. 903–11, Apr. 2009.

[9] H. Ding, Y.-S. Gao, Y. Wang, C. Hu, Y. Sun, and C. Zhang, "Dimethyloxaloylglycine increases the bone healing capacity of adipose-derived stem cells by promoting osteogenic differentiation and angiogenic potential.," *Stem Cells Dev.*, vol. 23, no. 9, pp. 990–1000, May 2014.

[10] R. Chowdhury, J. I. Candela-Lena, M. C. Chan, D. J. Greenald, K. K. Yeoh, Y.-M. Tian, M. A. McDonough, A. Tumber, N. R. Rose, A. Conejo-Garcia, M. Demetriades, S. Mathavan, A. Kawamura, M. K. Lee, F. van Eeden, C. W. Pugh, P. J. Ratcliffe, and C. J. Schofield, "Selective small molecule probes for the hypoxia inducible factor (HIF) prolyl hydroxylases.," *ACS Chem. Biol.*, vol. 8, no. 7, pp. 1488–96, Jul. 2013.

[11] S. H. Bhang, S.-W. Cho, W.-G. La, T.-J. Lee, H. S. Yang, A.-Y. Sun, S.-H. Baek, J.-W. Rhie, and B.-S. Kim, "Angiogenesis in ischemic tissue produced by spheroid grafting of human adipose-derived stromal cells.," *Biomaterials*, vol. 32, no. 11, pp. 2734–47, Apr. 2011.

[12] N.-C. Cheng, S.-Y. Chen, J.-R. Li, and T.-H. Young, "Short-term spheroid formation enhances the regenerative capacity of adipose-derived stem cells by

promoting stemness, angiogenesis, and chemotaxis.," *Stem Cells Transl. Med.*, vol. 2, no. 8, pp. 584–94, Aug. 2013.

[13] A. M. Bratt-Leal, A. H. Nguyen, K. A. Hammersmith, A. Singh, and T. C. McDevitt, "A microparticle approach to morphogen delivery within pluripotent stem cell aggregates.," *Biomaterials*, vol. 34, no. 30, pp. 7227–35, Oct. 2013.

[14] H. M. Kwon, S.-M. Hur, K.-Y. Park, C.-K. Kim, Y.-M. Kim, H.-S. Kim, H.-C. Shin, M.-H. Won, K.-S. Ha, Y.-G. Kwon, D. H. Lee, and Y.-M. Kim, "Multiple paracrine factors secreted by mesenchymal stem cells contribute to angiogenesis.," *Vascul. Pharmacol.*, vol. 63, no. 1, pp. 19–28, Oct. 2014.

[15] C. K. Sen, G. M. Gordillo, S. Roy, R. Kirsner, L. Lambert, T. K. Hunt, F. Gottrup, G. C. Gurtner, and M. T. Longaker, "Human skin wounds: a major and snowballing threat to public health and the economy.," *Wound Repair Regen.*, vol. 17, no. 6, pp. 763–71, Jan. .

[16] J. Grey, K. G. Harding, and S. Enoch, "Pressure Ulcers," *BMJ*, vol. 332, pp. 472–475, 2006.

[17] R. Nunan, K. G. Harding, and P. Martin, "Clinical challenges of chronic wounds: searching for an optimal animal model to recapitulate their complexity.," *Dis. Model. Mech.*, vol. 7, no. 11, pp. 1205–13, Nov. 2014.

[18] T. N. Demidova-Rice, M. R. Hamblin, and I. M. Herman, "Acute and impaired wound healing: pathophysiology and current methods for drug delivery, part 1: normal and chronic wounds: biology, causes, and approaches to care.," *Adv. Skin Wound Care*, vol. 25, no. 7, pp. 304–14, Jul. 2012.

[19] M. LOOTS, “Fibroblasts derived from chronic diabetic ulcers differ in their response to stimulation with EGF, IGF-I, bFGF and PDGF-AB compared to controls,” *Eur. J. Cell Biol.*, vol. 81, no. 3, pp. 153–160, Mar. 2002.

[20] O. Z. Lerman, R. D. Galiano, M. Armour, J. P. Levine, and G. C. Gurtner, “Cellular Dysfunction in the Diabetic Fibroblast,” *Am. J. Pathol.*, vol. 162, no. 1, pp. 303–312, Jan. 2003.

[21] J. M. Reinke and H. Sorg, “Wound repair and regeneration.,” *Eur. Surg. Res.*, vol. 49, no. 1, pp. 35–43, Jan. 2012.

[22] S. A. Eming, T. Krieg, and J. M. Davidson, “Inflammation in wound repair: molecular and cellular mechanisms.,” *J. Invest. Dermatol.*, vol. 127, no. 3, pp. 514–25, Mar. 2007.

[23] L. A. DiPietro, M. Burdick, Q. E. Low, S. L. Kunkel, and R. M. Strieter, “MIP-1alpha as a critical macrophage chemoattractant in murine wound repair.,” *J. Clin. Invest.*, vol. 101, no. 8, pp. 1693–8, Apr. 1998.

[24] L. A. DiPietro and P. J. Polverini, “Role of the macrophage in the positive and negative regulation of wound neovascularization.,” *Behring Inst. Mitt.*, no. 92, pp. 238–47, Aug. 1993.

[25] R. Clark, “Regulation of Fibroplasia in Cutaneous Wound Repair,” *Am. J. Med. Sci.*, vol. 306, no. 1, 1993.

[26] H. Thangarajah, D. Yao, E. I. Chang, Y. Shi, L. Jazayeri, I. N. Vial, R. D. Galiano, X.-L. Du, R. Grogan, M. G. Galvez, M. Januszyk, M. Brownlee, and G. C. Gurtner, “The molecular basis for impaired hypoxia-induced VEGF expression in

diabetic tissues.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 32, pp. 13505–10, Aug. 2009.

[27] D. Stiefel, C. Schiestl, and M. Meuli, "Integra Artificial Skin for burn scar revision in adolescents and children.," *Burns*, vol. 36, no. 1, pp. 114–20, Feb. 2010.

[28] W. A. Marston, J. Hanft, P. Norwood, and R. Pollak, "The Efficacy and Safety of Dermagraft in Improving the Healing of Chronic Diabetic Foot Ulcers: Results of a prospective randomized trial," *Diabetes Care*, vol. 26, no. 6, pp. 1701–1705, Jun. 2003.

[29] G. C. M. Steffens, C. Yao, P. Prével, M. Markowicz, P. Schenck, E. M. Noah, and N. Pallua, "Modulation of angiogenic potential of collagen matrices by covalent incorporation of heparin and loading with vascular endothelial growth factor.," *Tissue Eng.*, vol. 10, no. 9–10, pp. 1502–9, Jan. 2004.

[30] M. A. Cohen and W. H. Eaglstein, "Recombinant human platelet-derived growth factor gel speeds healing of acute full-thickness punch biopsy wounds.," *J. Am. Acad. Dermatol.*, vol. 45, no. 6, pp. 857–62, Dec. 2001.

[31] T. J. Wieman, J. M. Smiell, and Y. Su, "Efficacy and Safely of a Topical Gel Formulation of Recombinant Human Platelet-Derived Growth Factor-BB (Becaplermin) in Patients With Chronic Neuropathic Diabetic Ulcers: A phase III randomized placebo-controlled double-blind study," *Diabetes Care*, vol. 21, no. 5, pp. 822–827, May 1998.

[32] N. Papanas and E. Maltezos, "Benefit-Risk Assessment of Becaplermin in the Treatment of Diabetic Foot Ulcers," *Drug Saf.*, vol. 33, no. 6, pp. 455–461, Jun. 2010.

[33] S. Maxson, E. A. Lopez, D. Yoo, A. Danilkovitch-Miagkova, and M. A. LeRoux, "Concise Review: Role of Mesenchymal Stem Cells in Wound Repair," *Stem Cells Transl. Med.*, vol. 1, no. 2, pp. 142–149, Feb. 2012.

[34] S. Kirana, B. Stratmann, C. Prante, W. Prohaska, H. Koerperich, D. Lammers, M. H. Gastens, T. Quast, M. Negrean, O. A. Stirban, S. G. Nandrean, C. Götting, P. Minartz, K. Kleesiek, and D. Tschoepe, "Autologous stem cell therapy in the treatment of limb ischaemia induced chronic tissue ulcers of diabetic foot patients.," *Int. J. Clin. Pract.*, vol. 66, no. 4, pp. 384–93, Apr. 2012.

[35] W. Tse, J. Pendleton, W. Beyer, M. Egalka, and E. Guinan, "Suppresion of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation.," *Transplantaion*, vol. 75, no. 3, pp. 389–397, 2003.

[36] A. Krasnodembskaya, Y. Song, X. Fang, N. Gupta, V. Serikov, J.-W. Lee, and M. A. Matthay, "Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37.," *Stem Cells*, vol. 28, no. 12, pp. 2229–38, Dec. 2010.

[37] M. Duijvestein, A. C. W. Vos, H. Roelofs, M. E. Wildenberg, B. B. Wendrich, H. W. Verspaget, E. M. C. Kooy-Winkelaar, F. Koning, J. J. Zwaginga, H. H. Fidder, A. P. Verhaar, W. E. Fibbe, G. R. van den Brink, and D. W. Hommes, "Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study.," *Gut*, vol. 59, no. 12, pp. 1662–9, Dec. 2010.

[38] "STREAMS: A Phase 2 Clinical Trial Of Autologous Mesenchymal Stem Cells In Active Multiple Sclerosis (S23.005)," *Neurology*, vol. 82, no. 10 Supplement, Apr. 2014.

[39] S. Aggarwal and M. F. Pittenger, “Human mesenchymal stem cells modulate allogeneic immune cell responses.,” *Blood*, vol. 105, no. 4, pp. 1815–22, Feb. 2005.

[40] M. Sasaki, R. Abe, Y. Fujita, S. Ando, D. Inokuma, and H. Shimizu, “Mesenchymal Stem Cells Are Recruited into Wounded Skin and Contribute to Wound Repair by Transdifferentiation into Multiple Skin Cell Type,” *J. Immunol.*, vol. 180, no. 4, pp. 2581–2587, Feb. 2008.

[41] T. Kinnaird, E. Stabile, M. S. Burnett, C. W. Lee, S. Barr, S. Fuchs, and S. E. Epstein, “Marrow-Derived Stromal Cells Express Genes Encoding a Broad Spectrum of Arteriogenic Cytokines and Promote In Vitro and In Vivo Arteriogenesis Through Paracrine Mechanisms,” *Circ. Res.*, vol. 94, no. 5, pp. 678–685, Mar. 2004.

[42] R. Schweizer, P. Kamat, D. Schweizer, C. Dennler, S. Zhang, J. T. Schnider, S. Salemi, P. Giovanoli, D. Eberli, V. Enzmann, D. Erni, and J. A. Plock, “Bone marrow-derived mesenchymal stromal cells improve vascular regeneration and reduce leukocyte-endothelium activation in critical ischemic murine skin in a dose-dependent manner.,” *Cytotherapy*, Jun. 2014.

[43] A. Aguirre, J. A. Planell, and E. Engel, “Dynamics of bone marrow-derived endothelial progenitor cell/mesenchymal stem cell interaction in co-culture and its implications in angiogenesis.,” *Biochem. Biophys. Res. Commun.*, vol. 400, no. 2, pp. 284–91, Sep. 2010.

[44] C.-C. Tsai, Y.-J. Chen, T.-L. Yew, L.-L. Chen, J.-Y. Wang, C.-H. Chiu, and S.-C. Hung, “Hypoxia inhibits senescence and maintains mesenchymal stem cell

properties through down-regulation of E2A-p21 by HIF-TWIST.," *Blood*, vol. 117, no. 2, pp. 459–69, Jan. 2011.

[45] D.-C. Yang, M.-H. Yang, C.-C. Tsai, T.-F. Huang, Y.-H. Chen, and S.-C. Hung, "Hypoxia inhibits osteogenesis in human mesenchymal stem cells through direct regulation of RUNX2 by TWIST.," *PLoS One*, vol. 6, no. 9, p. e23965, Jan. 2011.

[46] M. Wagegg, T. Gaber, F. L. Lohanatha, M. Hahne, C. Strehl, M. Fangradt, C. L. Tran, K. Schönbeck, P. Hoff, A. Ode, C. Perka, G. N. Duda, and F. Buttgereit, "Hypoxia promotes osteogenesis but suppresses adipogenesis of human mesenchymal stromal cells in a hypoxia-inducible factor-1 dependent manner.," *PLoS One*, vol. 7, no. 9, p. e46483, Jan. 2012.

[47] S.-C. Hung, R. R. Pochampally, S.-C. Hsu, C. Sanchez, S.-C. Chen, J. Spees, and D. J. Prockop, "Short-term exposure of multipotent stromal cells to low oxygen increases their expression of CX3CR1 and CXCR4 and their engraftment in vivo.," *PLoS One*, vol. 2, no. 5, p. e416, Jan. 2007.

[48] E. Olson, L. Demopoulos, T. F. Haws, E. Hu, Z. Fang, K. M. Mahar, P. Qin, J. Lepore, T. A. Bauer, and W. R. Hiatt, "Short-term treatment with a novel HIF-prolyl hydroxylase inhibitor (GSK1278863) failed to improve measures of performance in subjects with claudication-limited peripheral artery disease.," *Vasc. Med.*, vol. 19, no. 6, pp. 473–82, Dec. 2014.

[49] J. Liu, H. Hao, H. Huang, C. Tong, D. Ti, L. Dong, D. Chen, Y. Zhao, H. Liu, W. Han, and X. Fu, "Hypoxia regulates the therapeutic potential of mesenchymal stem cells through enhanced autophagy.," *Int. J. Low. Extrem. Wounds*, vol. 14, no. 1, pp. 63–72, Mar. 2015.

[50] M. Y. Emmert, P. Wolint, S. Winklhofer, P. Stolzmann, N. Cesarovic, T. Fleischmann, T. D. L. Nguyen, T. Frauenfelder, R. Böni, J. Scherman, D. Bettex, J. Grünenfelder, R. Schwartlander, V. Vogel, M. Gyöngyösi, H. Alkadhi, V. Falk, and S. P. Hoerstrup, "Transcatheter based electromechanical mapping guided intramyocardial transplantation and in vivo tracking of human stem cell based three dimensional microtissues in the porcine heart.," *Biomaterials*, vol. 34, no. 10, pp. 2428–41, Mar. 2013.

[51] S. H. Bhang, S. Lee, J.-Y. Shin, T.-J. Lee, and B.-S. Kim, "Transplantation of cord blood mesenchymal stem cells as spheroids enhances vascularization.," *Tissue Eng. Part A*, vol. 18, no. 19–20, pp. 2138–47, Oct. 2012.

[52] I. Sekiya, B. L. Larson, J. R. Smith, R. Pochampally, J.-G. Cui, and D. J. Prockop, "Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality.," *Stem Cells*, vol. 20, no. 6, pp. 530–41, Jan. 2002.

[53] X. Shen, C. Wan, G. Ramaswamy, M. Mavalli, Y. Wang, C. L. Duvall, F. D. Lian, R. E. Guldberg, A. Eberhart, T. L. Clemens, and S. R. Gilbert, "Prolyl hydroxylase inhibitors increase neoangiogenesis and callus formation following femur fracture in mice," *J. Orthop. Res.*, vol. 27, no. 10, pp. 1298–1305, 2009.

[54] E. P. Cummins, E. Berra, K. M. Comerford, A. Ginouves, K. T. Fitzgerald, F. Seeballuck, C. Godson, J. E. Nielsen, P. Moynagh, J. Pouyssegur, and C. T. Taylor, "Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 103, no. 48, pp. 18154–9, Nov. 2006.

[55] Y. Sato, T. Ohshima, and T. Kondo, “Regulatory role of endogenous interleukin-10 in cutaneous inflammatory response of murine wound healing.,” *Biochem. Biophys. Res. Commun.*, vol. 265, no. 1, pp. 194–9, Nov. 1999.

[56] T. Imai, “Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine,” *Int. Immunol.*, vol. 11, no. 1, pp. 81–88, Jan. 1999.

[57] R. Salcedo, H. A. Young, M. L. Ponce, J. M. Ward, H. K. Kleinman, W. J. Murphy, and J. J. Oppenheim, “Eotaxin (CCL11) Induces In Vivo Angiogenic Responses by Human CCR3+ Endothelial Cells,” *J. Immunol.*, vol. 166, no. 12, pp. 7571–7578, Jun. 2001.

[58] W. J. Azar, S. H. X. Azar, S. Higgins, J.-F. Hu, A. R. Hoffman, D. F. Newgreen, G. A. Werther, and V. C. Russo, “IGFBP-2 Enhances VEGF Gene Promoter Activity and Consequent Promotion of Angiogenesis by Neuroblastoma Cells,” *Endocrinology*, Jul. 2011.

[59] D. Feldser, F. Agani, N. V. Iyer, B. Pak, G. Ferreira, and G. L. Semenza, “Reciprocal Positive Regulation of Hypoxia-inducible Factor 1{{alpha}} and Insulin-like Growth Factor 2,” *Cancer Res.*, vol. 59, no. 16, pp. 3915–3918, Aug. 1999.

[60] R. Granata, L. Trovato, E. Lupia, G. Sala, F. Settanni, G. Camussi, R. Ghidoni, and E. Ghigo, “Insulin-like growth factor binding protein-3 induces angiogenesis through IGF-I- and SphK1-dependent mechanisms.,” *J. Thromb. Haemost.*, vol. 5, no. 4, pp. 835–45, Apr. 2007.

[61] C.-C. Tsai, T.-L. Yew, D.-C. Yang, W.-H. Huang, and S.-C. Hung, "Benefits of hypoxic culture on bone marrow multipotent stromal cells.," *Am. J. Blood Res.*, vol. 2, no. 3, pp. 148–59, Jan. 2012.

[62] "IOX 2 Supplier | CAS 931398-72-0 | Tocris Bioscience | IOX2." [Online]. Available: http://www.tocris.com/dispprod.php?ItemId=331885#.VV3rUvlVhBc. [Accessed: 21-May-2015].

[63] "IOX2 (CAS 931398-72-0) || Cayman Chemical | Supplier." [Online]. Available: https://www.caymanchem.com/app/template/Product.vm/catalog/11573. [Accessed: 21-May-2015].

[64] H. K. Makadia and S. J. Siegel, "Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier.," *Polymers (Basel).*, vol. 3, no. 3, pp. 1377–1397, Sep. 2011.

[65] R. L. Carpenedo, A. M. Bratt-Leal, R. a Marklein, S. a Seaman, N. J. Bowen, J. F. McDonald, and T. C. McDevitt, "Homogeneous and organized differentiation within embryoid bodies induced by microsphere-mediated delivery of small molecules.," *Biomaterials*, vol. 30, no. 13, pp. 2507–15, May 2009.

[66] R. Smith, M. Riollano, A. Leung, and P. Hammond, "Layer-by-Layer Platform Technology for Small-Molecule Delivery," *Angew. Chemie*, vol. 121, no. 47, pp. 9136–9139, Nov. 2009.

[67] C. M. Jiang, J. Liu, J. Y. Zhao, L. Xiao, S. An, Y. C. Gou, H. X. Quan, Q. Cheng, Y. L. Zhang, W. He, Y. T. Wang, W. J. Yu, Y. F. Huang, Y. T. Yi, Y. Chen, and J. Wang, "Effects of hypoxia on the immunomodulatory properties of human

gingiva-derived mesenchymal stem cells.," *J. Dent. Res.*, vol. 94, no. 1, pp. 69–77, Jan. 2015.

[68] M. Roemeling-van Rhijn, F. K. F. Mensah, S. S. Korevaar, M. J. Leijs, G. J. V. M. van Osch, J. N. M. Ijzermans, M. G. H. Betjes, C. C. Baan, W. Weimar, and M. J. Hoogduijn, "Effects of Hypoxia on the Immunomodulatory Properties of Adipose Tissue-Derived Mesenchymal Stem cells.," *Front. Immunol.*, vol. 4, p. 203, Jan. 2013.

[69] G. F. Schneider, V. Subr, K. Ulbrich, and G. Decher, "Multifunctional cytotoxic stealth nanoparticles. A model approach with potential for cancer therapy.," *Nano Lett.*, vol. 9, no. 2, pp. 636–42, Feb. 2009.

[70] N. Nishiyama and K. Kataoka, "Current state, achievements, and future prospects of polymeric micelles as nanocarriers for drug and gene delivery.," *Pharmacol. Ther.*, vol. 112, no. 3, pp. 630–48, Dec. 2006.

[71] H. Wang, Y. Zhao, Y. Wu, Y. Hu, K. Nan, G. Nie, and H. Chen, "Enhanced anti-tumor efficacy by co-delivery of doxorubicin and paclitaxel with amphiphilic methoxy PEG-PLGA copolymer nanoparticles.," *Biomaterials*, vol. 32, no. 32, pp. 8281–90, Nov. 2011.

[72] D. Mallardé, F. Boutignon, F. Moine, E. Barré, S. David, H. Touchet, P. Ferruti, and R. Deghenghi, "PLGA–PEG microspheres of teverelix: influence of polymer type on microsphere characteristics and on teverelix in vitro release," *Int. J. Pharm.*, vol. 261, no. 1–2, pp. 69–80, Aug. 2003.

[73] J. A. Zimmermann and T. C. McDevitt, “Pre-conditioning mesenchymal stromal cell spheroids for immunomodulatory paracrine factor secretion.,” *Cytotherapy*, vol. 16, no. 3, pp. 331–45, Mar. 2014.