The Effects of Oxygen and Glucose Culture on Mouse Bone Marrow Mesenchymal Stem Cells

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ABSTRACT

A key limitation for bulk tissue engineered scaffold development is the inability to deliver oxygen to cells found within scaffolds – which can result in hypoxia and tissue necrosis. The objective of this study was to determine the necessary requirements for anoxic MSC survival up to 13 days. Initially, unmodified and Erythropoietin (Epo) gene-modified mouse bone marrow mesenchymal stem cells (Epo-MSCs) were cultured for 7 days in normoxic (21%) and anoxic (<1%) oxygen conditions. In the second set of experiments, unmodified MSCs were cultured in anoxia for up to 13 days in high (4.5g/L) and regular (1g/L) glucose media. Cell viabilities were determined for up to 13 days. We observed from our oxygen experiments, that while both groups of MSCs showed similar viabilities (78-88%) when compared to normoxic controls (85-91%), Epo-MSCs showed a significantly higher cell count ($10.1x10^4 \pm 4.5$) compared to unmodified MSCs ($7x10^4 \pm 3.8$) when cultured in anoxia. In anoxia, addition of high glucose media to unmodified MSCs led to a significant increase in cell viability percentage when compared to regular glucose media at 13 days. Our results indicate that both groups of MSCs can survive for up to 13 days in the absence of oxygen, with higher cell counts seen for Epo-MSCs. Protein production was unaffected by the absence of oxygen compared to normoxic controls. Additional glucose allowed for a 37% increase in MSC survival in anoxia. These results may lead to future research in scaffold designs that do not require vasculature to maintain cell survival for extended periods of time.

RÉSUMÉ

Incapacité à fournir de l'oxygène aux cellules constituant les matrices tissulaires constitue aujourd'hui l'une des principales limitations au développement de l'ingénierie tissulaire. Cette difficulté peut mener à une hypoxie et à une nécrose des tissus. L'objectif de notre étude était de déterminer quelles conditions sont favorables à la survie des cellules souches mésenchymateuses (CSM) anoxiques durant 13 jours de culture. Une première expérience a consisté à mettre des CSMs issues de la moëlle osseuse de souris contrôle ou modifiées pour le gène codant pour l'Erythropoietin (Epo) en culture durant 7 jours en conditions normoxiques (21% O_2) ou anoxiques (<1% O_2). Dans un second groupe d'expériences, des CSMs non-modifiées ont été cultivées en conditions anoxiques pendant 13 jours dans un milieu fortement ou faiblement concentré en glucose (4.5g/L et 1g/L, respectivement). La viabilité cellulaire a ensuite été déterminée. Nous avons observé que les deux groupes de CSMs cultivées en conditions anoxiques présentent un taux de survie similaire (78-88%) à celui des cellules cultivées en conditions normoxiques (85-91%). Cependant, nous avons constaté que les cultures de CSMs modifiées pour Epo possédaient un nombre de cellules plus important ($10.1x10^4±4.5$) que les CSMs non-modifiées ($7x10^4±3.8$) en conditions anoxiques. De plus, une augmentation significative de la viabilité des CSMs non-modifiées en anoxie a été observée lorsque celles-ci étaient cultivé dans un milieu riche en glucose. Ainsi, nos résultats montrent que les deux groupes de CSMs peuvent survivre jusqu'à 13 jours en absence d'oxygène, et que les cultures de CSMs modifiées pour Epo présentent un nombre plus important de cellules. Une forte concentration de glucose dans le milieu de culture permet de plus une augmentation de 37% de la survie des CSMs en anoxie. La production de protéines demeure inchangée en absence ou en présence d'oxygène. Ces résultats pourraient permettre une avancée significative dans la conception de nouvelles matrices tissulaires, pour lesquelles la vascularisation ne serait pas nécessaire à la survie à long terme des cellules.

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LIST OF ABBREVIATIONS

ATP	**Adenosine triphosphate**
Ang	**Angiopoietin**
BDNF	**Brain derived neurotrophic factor**
bFGF	**Basic fibroblast growth factor**
CCL	**Chemokine ligand**
CO2	**Carbon dioxide**
CoA	**Coenzyme A**
DNA	**Deoxyribonucleic acid**
Epo	**Erythropoietin**
EpoR	**Erythropoietin receptor**
FADH2	**Flavin adenine dinucleotide (reduced form)**
FBS	**Fetal bovine serum**
HIF-1	**Hyoxia-inducible factor-1**
HGF	**Hepatocyte growth factor**
HLA-DR	**Human leukocyte antigen - DR isotype**
HO	**Heme oxygenase**
HSCs	**Hematopoietic stem cells**
IFN	**Interferon**
IGF	**Insulin growth factor**
ISCT	**International Society for Cellular Therapy**
LDHA	**Lactate dehydrogenase A**
MCP	**Monocyte chemoattractant protein**
mEPO	**Mouse Erythropoietin**
MHC	**Major histocompatibility complex**
MSCs	**Mesenchymal stem cells**
N2	**Nitrogen**
NAD+	**Nicotinamide adenine dinucleotide (oxidized form)**
NADH	**Nicotinamide adenine dinucleotide (reduced form)**
NGF	**Nerve growth factor**

NIH	**American National Institute of Health**
P/S	**Penicillin/Streptomycin**
PBS	**Phosphate buffered saline**
PDGF	**Platelet derived growth factor (PDGF)**
PDH	**Pyruvate dehydrogenase**
Rpm	**Revolutions per minute**
SCF	**Stem cell factor**
TCA	**Tricarboxylic acid**
TGF-β	**Transforming growth factor beta**
TNF	**Tumor necrosis factor**
VEGF	**Vascular endothelial growth factor**
WT	**Wild-type**
α-MEM	**Alpha Minimal Essential Medium**

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CHAPTER 1: INTRODUCTION

1.1 TISSUE AND ORGAN TRANSPLANTS

In 1954, Joseph Murray and David Hume performed the first successful organ transplant of a kidney in Boston, Massachusetts [1]. Since then, organ transplantation techniques have evolved and spread throughout the world, with a reported 32% increase in transplant procedures performed within Canada in 2016 since 2007 [2]. Presently, organ transplants are commonly used to treat end-stage organ failures to help extend the lives of patients. Strict regulations with regards to consent and how the donor died (must be pronounced brain dead by medical doctors prior to retrieval of organs) must be followed before an organ is deemed eligible for use in transplants [3]. While this technique has revolutionized medicine and helped extend the lives of many individuals, there still exists a few recurring universal problems such as: organ shortage, lack of donors, high procedure costs, and possibility of organ rejection post transplant [4], [5].

End-stage organ failure has become one of the most expensive and damaging problems for modern medicine [6]. In 1989, a total of 17,917 patients were on the United States transplant wait list, this number increased to an alarming 79,062 by 2015 and has since been on the rise [6]. Even with the large amounts of individuals who become donors on a daily basis, there is still a national shortage of organs; with a reported value of approximately 21 patients who will die per day while on wait list for a transplant [6]. In Canada between 2006 – 2015, a reported total of 2,570 organ transplants were performed and a total of 4,333 patients were on the Canadian transplant wait list in 2017 [7]. On average, 1,600 patients are added to the Canadian transplant wait list on a yearly basis [8]. Additionally, the development of larger donor registries in some countries is thwarted

by various legal, social and cultural factors implemented by the countries themselves. The use of organs from live donors instead of only from the deceased for transplantation procedures is an alternative solution (although it does still pose as an ethical quandary to some), however, many countries ban the purchase and sale of organs from live donors [9], [10].

While there are many problems related to the field of organ transplantation, the main challenge after each transplant procedure is the possibility of organ rejection by the patient's body. In order to prevent rejection, patients are subjected to life-long immunosuppressive medication to ensure prolonged survival and acceptance of the new organ by the patient's immune system. Although this may allow for a successful transplant, patients can develop life-threatening complications such as increased risks for infections and cardiovascular complications [11], [12], [13], [14], [15]. Another reason why organ transplant patients tend to be more susceptible to complications that arise from the immunosuppressive drugs is due to the fact that their bodies are already frail prior to the surgery [16]. The complicated procedures involved take a large toll on their immune system, which, combined with the drugs can further weaken their system [16]. Due to well-known risks involved with the use of immunosuppressive medication, scientists have begun to look for alternative solutions that will provide similar effectiveness of current medications, but with reduced side effects [11]. One such alternative that has started to emerge as a promising field in the scientific community of regenerative medicine is tissue engineering. This field delves into the abilities adults may possess in order to restore form

and function of the damaged tissues and organs on their own without the formation of scar tissues [16], [17].

1.1.1 REGENERATIVE MEDICINE

Regenerative medicine is the combination of molecular, cell biology and tissue engineering; whose three fields come together to create a unique area of translational science [18]. Translational science is the integration of basic and clinical research with the main goal of public health improvement [19]. The main target of this topic of research is to stimulate and promote the body's natural ability to heal itself. Hopefully through this type of medicine, a patient may once again have a fully functional and competent organ that would have originally required a transplant [18]. In order for this to be achieved, various methods exist through the use of stem cells to stimulate healing, such as: 1) use of exogenous stem cells from donors after the culture expansion of their stem cells before administration into the patients, 2) stimulating the recruitment of endogenous stem cells within the patient to the site of tissue injury, and 3) transplantation of functional organs completely grown from stem cells of patients within laboratories [18]. The field of regenerative medicine presents itself as a desirable solution to conquer diseases that can not be treated with drugs or other therapies [20]. As modern medicine evolves and progresses with all the new discoveries being made, it now moves in a direction that tries to limit the need for transplant procedures and to seek alternatives that will provide a higher quality of life for patients [21], [16], [22].

1.1.2 TISSUE ENGINEERING

Tissue engineering was first discovered in 1933 by Vincenzo Bisceglie when he observed that no immune response was triggered when mouse tumour cells encapsulated in a polymer were inserted into the abdominal cavity of a pig, allowing for the survival of the tumour cells [6]. Since then, other tissue engineering studies have been performed to look for alternative solutions using biological substitutes. An example of an early study after the initial discovery by Bisceglie was in 1975 by Chick et al. who tried to control glucose within patients with diabetes mellitus through the encapsulation of their pancreatic-islet cells [23]. These initial discoveries have led to the search for suitable materials that can be safely used within the human body to help with the restoration and improvement of damaged tissues and organs. For over 30 years, the field of tissue engineering has become an alternative solution for organ and tissue reconstruction and transplantation - with its' most unique feature being the ability to regenerate a patient's individual organ or tissue without provoking any immune responses [24]. Tissue engineering uses a combination of cells, synthetic or biological scaffolds and molecular signals such as growth or differentiation factors in order to create special constructs that help stimulate and promote the regeneration of tissues [17], [24], [25], [23], [26]; with the ultimate goal of functional organ reconstruction [6].

Essentially, the concept of tissue engineering is to utilize cells that can contribute to the regeneration process through the introduction of growth factors or genes to help create a functional tissue within the adult human body. While cells alone contribute enormously to this technique, at times, scaffolds are required for larger and more complex

structures [27]. In order to recreate large organs or tissues by growing cells and embedding them within scaffolds or 3D printing using bio-inks, scaffolds play an important role in providing structure and support to cells that become embedded within them. Scaffolds help with the process of proliferation, differentiation and generation of cells at the site of injury and prevent foreign cells from integrating and disrupting the regeneration process [24].

Various types of scaffolds exist that are used in tissue engineering, ranging from synthetic, organic and metal materials, with their use dependent on their intended applications. Synthetic materials are easy to manipulate in terms of chemical and physical properties which allows for better control over their mechanical and degradation properties. This type of material will allow for cell embedment and will slowly degrade over time to allow for the incorporation of cells to the area of interest. Organic materials are normally used as a biomimetic environment for stem cells and lastly, metal scaffolds provide for applications that require a sturdy bone like support structure, such as reproduction of odontoblast-like cells [28]. Design of the constructs in tissue engineering is extremely important to ensure that whatever materials are chosen will allow for maximum incorporation and integration into the specific area of interest [28].

To date, over 20 different types of tissues have been produced in laboratories, i.e. skin, bone, cartilage, blood vessels, heart tissues, and fatty tissues, etc. [20], [23], [29]. While a large amount of money has been invested into this field of research, despite the appealing potentials of tissue engineering and how it could change modern medicine -

very few clinical applications of tissue engineering within patients currently exist (a total of 79 studies according to clinicaltrials.gov) [27]. However, major advances have and are still occurring within the field of tissue engineering. Some of these include the generation of pluripotent cells directly from patients by reprogramming of their somatic cells through the use of various transcription factors [30], use of drug tests and disease modelling through microtissue platforms to help with the reproduction of large scale drug delivery pharmacokinetics [31], and the creation of lab grown artificial organs in the hopes of alleviating the national organ shortage [32]. With the continuous advancements and emergence of new applications, there still exists great potential that these emerging techniques can be used for clinical applications in the future [32]. Many barriers exist before a construct is created, such as origin of cells, cell signalling, manipulation of cells, tissue expression and the successful integration of the tissue construct into the patients' body. To overcome these barriers, more information with regards to this field needs to be discovered, along with further optimization of procedures used during the creation of constructs. If the construct is successfully created and transplanted into patients, concerns also follow with regards to immune responses, and proper integration of the new tissue with the nerves and vascular system [27]. A complex and persistent issue with tissue engineering is the inadequate supply of oxygen and nutrients to cells that are found embedded deep within the 3D tissue constructs after a transplant procedure. When distances of 100-200um (or over) between cells and blood vessels exist [33], oxygen deficiency can occur. Without sufficient amounts of oxygen and nutrients, important cellular functions such as cellular migration, neo-vascularization and regenerative abilities become hampered. The environment which surrounds the cells becomes hypoxic, and

ultimately tissue necrosis may occur [4], [34], [35]. Cell death in these cases largely occur because cells rely on aerobic respiration and are unable to respire anaerobically. Aerobic respiration produces adenosine triphosphate (ATP) molecules important in many cellular chemical reactions to allow for cell survival. When oxygen is not present, free radicals are produced which cause damage to the DNA of cells and may introduce several mutations which can result in cell apoptosis and cell death. Due to this, scientists have made many attempts to supply oxygen to areas that lack vascularization such as through the development of oxygen-rich fluids or implanting engineered tissues near tissues abundant with vasculature [4], [36]. Although these solutions work temporarily, a constant supply of oxygen for an extended period of time until new vasculature incorporates into tissue scaffolds still proves to be a difficult task [4]. Thus, the ability to support large numbers of cells without the presence of vasculature would be beneficial as neo-vascularization rates are normally too slow to maintain the viability of the seeded cells found within these constructs [37]. Benefits of cells that do not require vasculature will reduce the chances of tissue necrosis from occurring within complex organ constructs.

1.2 MESENCHYMAL STEM CELLS (MSCs)

In the past, it was believed that all tissue specific adult stem cells could only differentiate into the same lineages that they originate from [38], [39], [40]. The presence of non-hematopoietic stem cells in the bone marrow was first described by Friedenstein and his colleagues in 1960s to 1970s who showed that the osteogenic differentiation abilities of cells obtained from bone marrow was due to a small group of cells found within the bone marrow itself which possessed the unique ability to adhere to plastic, which facilitated the *in vitro* expansion of these particular cells. Also, from a single isolated cell,

they could produce an entire colony of cells *in vitro*. Friedenstein et al., later coined these cells as colony forming unit fibroblasts (CFU-F) [38], [41], [18], [42], [43].

Within the adult bone marrow, two types of stem cell populations exist: Hematopoietic stem cells (HSCs) and non-hematopoietic stem cells, also known as Mesenchymal stem cells (MSCs) - with MSCs the least known of the two, although still the second most studied stem cell within the human body after HSCs [44], [28], [45]. Both *in vivo* and *in vitro* studies have been conducted on MSCs since their discovery which has led to the unveiling of their multipotency [38], [46]. It was first found that differentiation into various cell types such as osteogenic, chondrogenic and adipogenic tissue lineages could be achieved by bone marrow MSCs if cultured in specific conditions that promoted each differentiation process [47]. Later, additional studies have led to the expansion of the number of possible cell types that MSCs can differentiate into such as neuronal, cardiogenic and myogenic cells [48]–[51], [52]–[55].

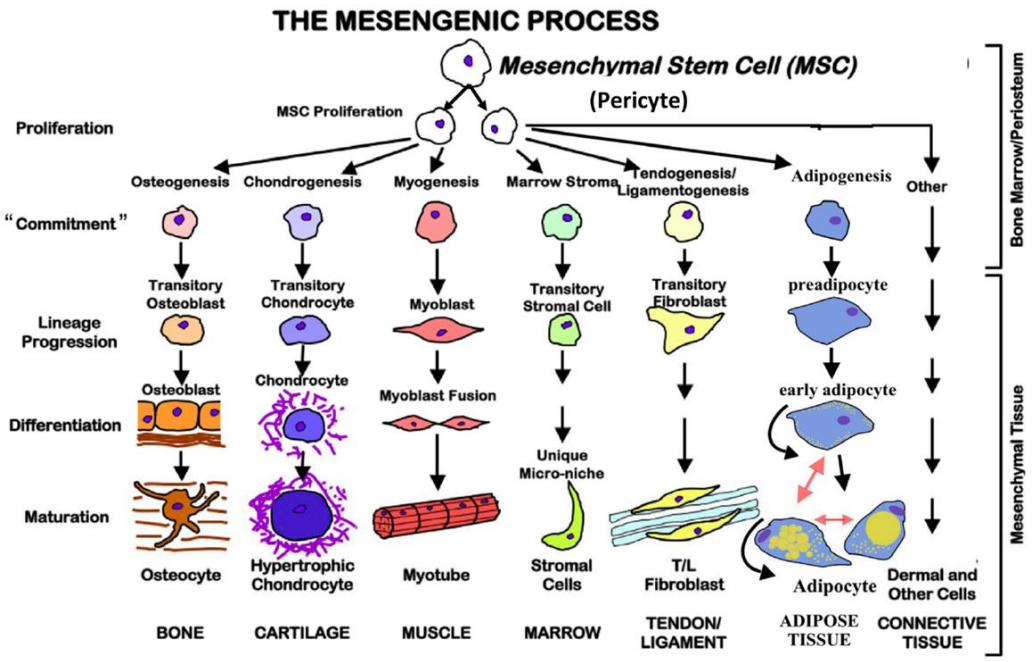

Figure 1. Multipotency of MSCs. [56]

1.2.1 SOURCES OF MSCs

MSCs can be harvested from various tissue sources such as: bone marrow, adipose tissue, umbilical cord, dental pulp, muscle and almost all types of connective tissues found within the adult human body with minimal difficulties, and are known to be easily expanded and handled within laboratory environments [57], [24], [58], [59], [60].

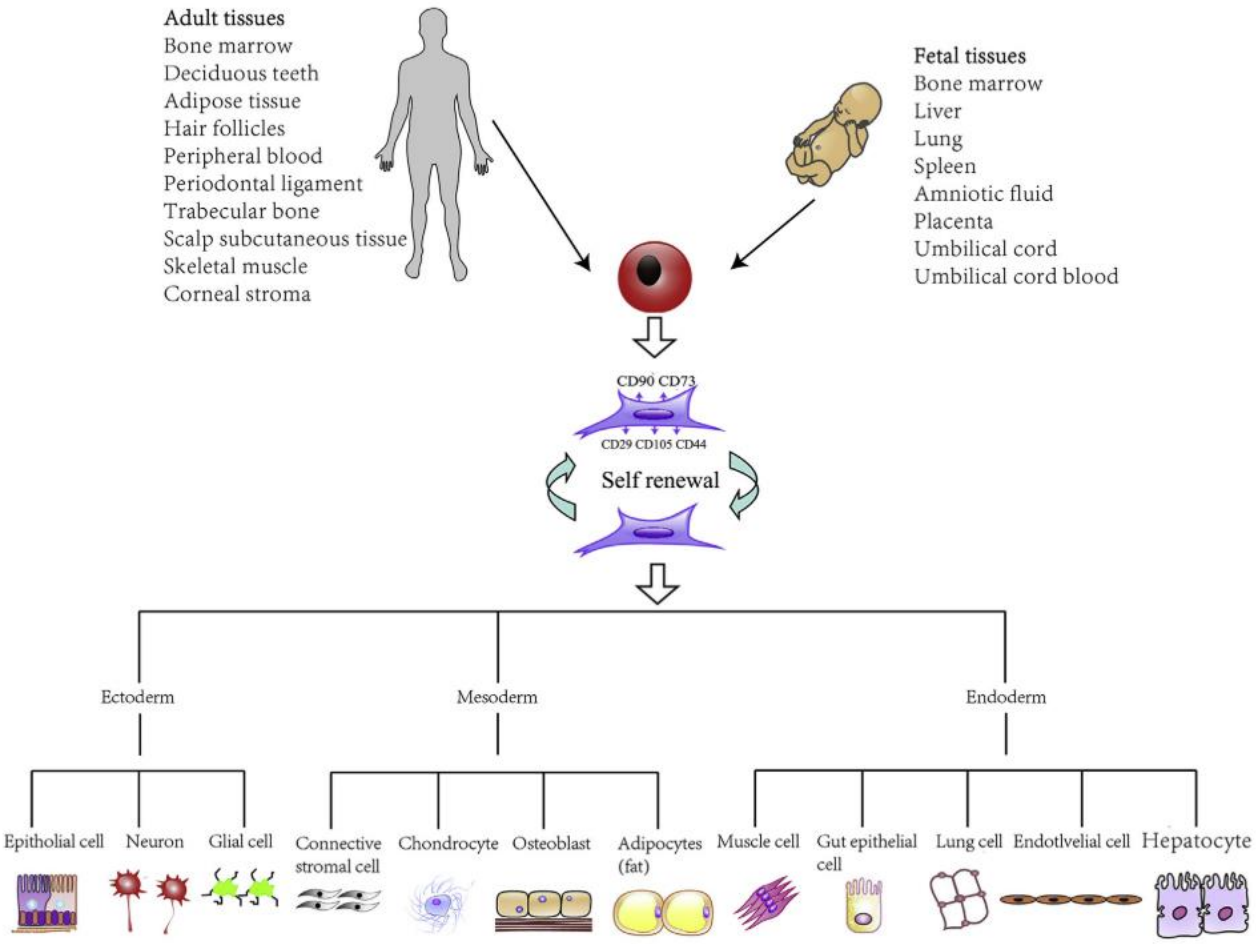

Figure 2. Sources of MSCs within the human body. [61]

1.2.2 DEFINING MSCs

To be defined as MSCs, the International Society for Cellular Therapy (ISCT) in 2006 developed a list of requirements that cells should possess in order to be considered legitimate MSCs [62]. ISCT states that the cells must adhere to the following three standards [63] : 1) be plastic adherent, 2) must express the following cell surface antigens: CD105, 73 and 90 in over 95% of the entire population and not express CD45, 34, 14 and 11b, 79a, 19 and HLA-DR [64], [65], and 3) must show capabilities to differentiate into the following three different lineages: osteoblasts, adipocytes and chondrocytes when

cultured in appropriate conditions [18] [66] [67]. In particular, it was their unique abilities to adhere to plastic that allowed for their successful isolation from bone marrow which ultimately led to the considerable amount of *in vitro* work performed on MSCs in culture to this date [18], [68], [43] .

1.2.3 CHARACTERISTICS OF MSCs

Recent studies have shown that with MSCs, what is even more important than the differentiation abilities of these cells are their paracrine effects, i.e., what these MSCs produce and secrete [53], [69]–[71]. This MSC “secretome” includes a wide variety of cytokines, chemokines and growth factors, such as: Angiopoietin (Ang), basic fibroblast growth factor (bFGF), brain derived neurotrophic factor (BDNF), chemokine ligand (CCL), heme oxygenase (HO), hepatocyte growth factor (HGF), insulin growth factor (IGF), monocyte chemoattractant protein (MCP), nerve growth factor (NGF), platelet derived growth factor (PDGF), stem cell factor (SCF), and vascular endothelial growth factor (VEGF) etc.

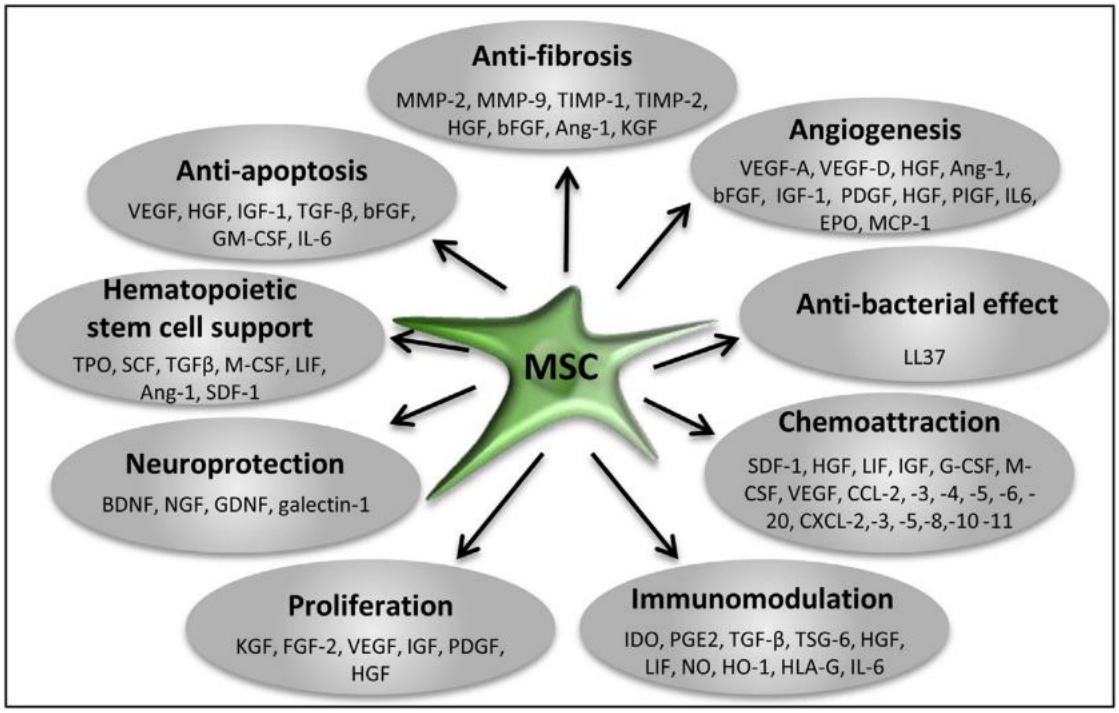

Figure 3. Paracrine factors released by MSCs. [72]

These factors released by MSCs are dependent on the microenvironment and can lead to various effects such as anti-apoptosis, anti-fibrosis, angiogenesis, cell proliferation, tissue protection, immunomodulation, HSC support, and others.

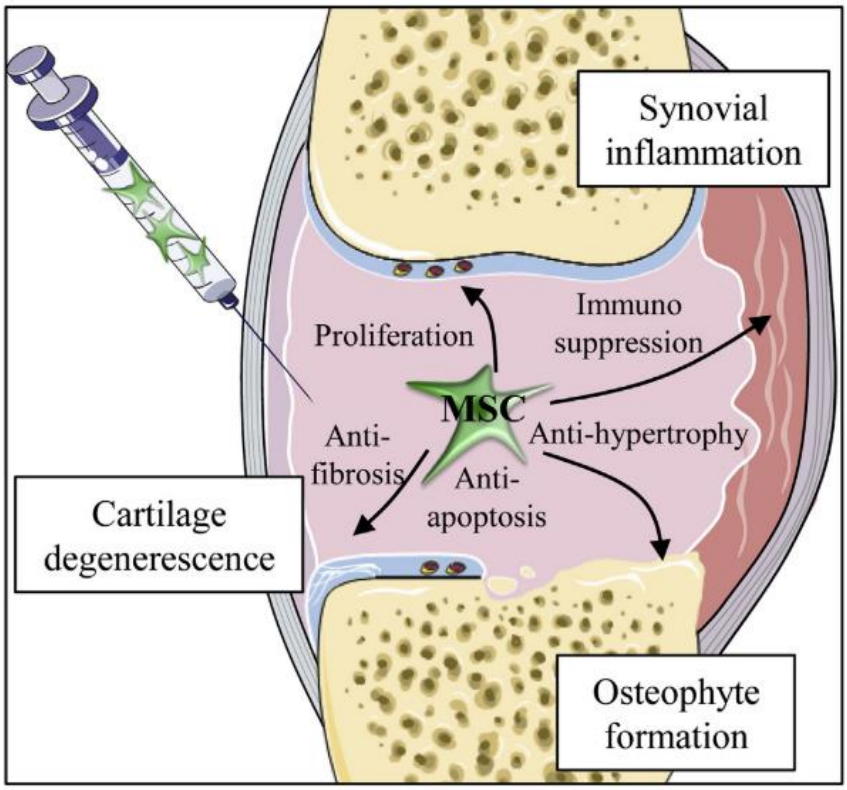

Figure 4. MSC-based therapies through the release of paracrine factors. [72]

Therefore, other than for their ability to differentiate into a large variety of cell types, MSCs have numerous medical applications due to their paracrine effects [53], [69]–[71]. Clinical uses of MSCs include cardiovascular, orthopedic, spinal cord, neural, and autoimmune diseases, as well as wound healing [71].

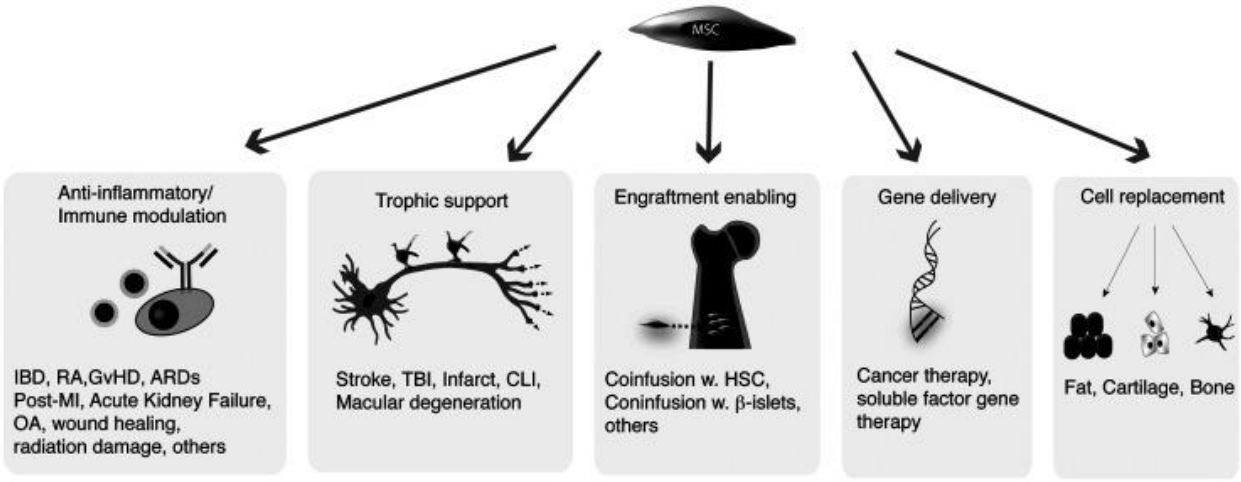

Figure 5. Multiple modes of action of MSCs used in tissue engineering and cell therapy. Figure from Mary Ann Liebert, Inc., New Rochelle, NY [73].

The paracrine signalling effect is a result of nearby cell to cell communication through the release of proteins that help regulate cellular function within the nearby vicinity to help with injuries [24], [74]. As it is believed that up to 80% of the therapeutic effects of MSCs is due to this unique effect, scientists propose that the paracrine signalling abilities of MSCs may play an essential role in the beneficial therapeutic effectiveness of MSCs in the field of regenerative medicine [74], [75]. Research is also currently being conducted to gain a better understanding of what allows for the production and release of these paracrine factors and how this process is controlled, although observations have shown that stress on MSCs such as hypoxia stimulate the secretion of these proteins [75], [76]. Due to this unique range of secreted proteins, also known as the secretome, scientists have also begun to conduct secretome profiling studies on MSCs to get a better understanding of the various proteins that are important in the regulation of different cellular functions such as proliferation, inflammatory responses, and cell survival [75]. Interestingly, scientists have discovered that the secretome profiles

of MSCs may vary based on their origins, for example MSCs obtained from Wharton's jelly were found to secrete the largest amounts of immunomodulatory factors, and varying levels of neuroregulatory factors were found in the secretomes of MSCs obtained from adipose tissue, bone marrow and umbilical cords [75], [77], [78].

Currently, paracrine signalling has become the main focus for the therapeutic basis of many MSC-based tissue repair standards to help with the development of future tissue engineering applications that will contain tailored microenvironments mimicking the secretome of MSCs unique to the desired application [75].

Other than the tissue repair and wound healing abilities of MSCs, MSCs also possess strong immunomodulatory properties that can help to both upregulate and downregulate cellular immune responses during injuries [79], [80]. While MSCs can suppress B cell proliferation and activate natural killer (NK) cells, lymphocytes and dendritic cells (DCs) through their paracrine signalling, this may largely depend on the interactions between MSCs and the sites of injury [81], [82]. It was postulated that for MSCs to have their reparative functions, the presence of inflammatory cells and secreted factors is what drives the wound healing abilities of MSCs [81]. This idea was based on two specific observations by scientists: 1) MSCs tend to migrate to sites of injury which may be due to the build up of chemokines released by inflammatory cells, which in turn attract MSCs to these sites due to the presence of these chemokine receptors found on MSCs, and 2) MSCs require the presence of inflammatory factors IFN-γ and TNF-α to activate their immunosuppressive potentials [81], [83], [84]. Interestingly, while MSCs are

largely known for their immunosuppressive capabilities, they have also been shown to enhance immune responses within the sites of injury [79], [82]. MSCs have been found to also promote B cell differentiation and expansion as well as to direct macrophages to sites of injuries [81], [82]. The lack of MSCs tend to encourage DCs to increase T cell activation whereas high concentrations of MSCs discourage this process [82], [85]. Finally, low levels of IFN-γ allow MSCs to act as antigen-presenting cells while high levels of IFN-γ decrease the presence of class II major histocompatibility complex (MHC-II) on MSCs and limit the release of anti-inflammatory factors by MSCs [82], [86], [87].

1.2.4 CURRENT APPLICATIONS USING MSCs

Canada is currently one of the top three countries within the world known for their research on stem cells in both the academic and commercial sectors [88]. This enormous research program was formed through the help of The Stem Cell Network who helped gather scientists, bioengineers and clinicians from across Canada to come together as one [88]. Within the United States alone, it was reported that regenerative stem cell therapy could benefit over 128 million individuals throughout their lifetimes [89], [90]. Especially with increased life expectancies, individuals become more susceptible to diseases that have life-long effects such as diabetes, Alzheimer's, Parkinson's, and stroke to name a few [89].

MSCs are considered to be the cells with the most potential in the field of regenerative medicine [18]. This can be attributed to the minimal presence of MHC-II on their cell surface [59], [91], [92], [93], [94]. Due to this, scientists are interested in their characterization, and method refinements that will allow for better harvest and expansions

of these cell types so that they can be used to help with tissue injuries and repairs [18]. As previously mentioned, another aspect that makes MSCs particularly appealing for their use in this field of science is that they can be easily expanded in culture. Therefore, large amounts of cells can be obtained from a single individual without concerns that more than one individual may be needed to acquire the desired amount of cells. Moreover, MSCs can easily be gene-enhanced within laboratories once they are isolated from their natural environments, thus, MSCs have also become popular gene delivery vehicles for several gene delivery applications [47], [95], [96].

Large amounts of animal studies and some clinical studies have used MSCs to try to treat diseases such as: acute myocardial infarction, ischemic stroke, Crohn's disease, and acute kidney failure, etc.. Further applications can be found in Table 1 [45], [97]. It was through such studies that scientists were able to discover that MSCs regulate immunomodulatory functions through a paracrine effect instead of the previously accepted theory of an autocrine effect [45], [98], [99]. To date, there are 901 clinical trials worldwide designed to incorporate the use of MSCs to treat an array of diseases (obtained November 28, 2018 on clinicaltrials.gov); with the first reported study performed in 1999, which involved the transplantation of allogenic MSCs into children that had osteogenic imperfecta [45], [100].

Table 1. Various Clinical Applications of MSCs. Data within table depicts some of the clinical applications of MSCs listed in www.clinicaltrials.gov, accessed on December 1, 2018.

Status	Conditions	Location
Completed	Solid Tumor Metastatic Cancer	Montreal, Quebec, Canada
Recruiting	Multiple Sclerosis	Winnipeg, Manitoba, Canada Ottawa, Ontario, Canada
Recruiting	Bronchopulmonary Dysplasia	Chongqing, China
Recruiting	Refractory Pulmonary Diseases	Chongqing, China
Completed	Urticaria Autoimmune diseases Immune system diseases Skin diseases	Manisa, Turkey
Completed	Septic Shock	Ottawa, Ontario, Canada
Completed	Hematopoietic stem cell transplantation	Ankara, Turkey
Recruiting	Blood and marrow transplantation Adult respiratory distress syndrome	Houston, Texas, USA
Completed	Articular cartilage disorder of knee Osteoarthritis, knee	Amman, Jordan
Unknown	Respiratory Distress Syndrome, Adult	Seoul, Korea
Recruiting	Tracheal Stenosis Laryngeal Stenosis	Minsk, Belarus
Unknown	Severe Aplastic Anemia	Guangzhou, China
Active, not recruiting	Rotator cuff tear	Znojmo, Czech Republic
Completed	Graft versus host disease	Rawalpindi, Pakistan
Unknown	Ulcerative colitis Unbilical Cord	Qingdao, China
Completed	Tibial Fracture	Tehran, Iran
Unknown	Diabetes Mellitus 1	Qingdao, China
Completed	Erectile dysfunction	N/A
Unknown	Duchenne muscular dystrophy	Istanbul, Turkey
Completed	Mandibular fractures	N/A
Unknown	Spinal cord injury	Santiago, Chile

Recruiting	Hormone Deficiency	Hanoi, Vietnam
Completed	Chronic renal failure Polycystic kidney disease	Tehran, Iran
Not yet recruiting	Systemic lupus erythematosus	Shijiazhuang, China
Unknown	Dilated cardiomyopathy	Qingdao, China
Recruiting	Type 2 Diabetes	Shanghai, China
Unknown	Ischemic cardiomyopathy	Shijiazhuang, China
Recruiting	Ketoacidosis, diabetic	Nanjing, China
Recruiting	Chrohn's disease	Tehran, Iran
Active, not recruiting	Osteoarthritis of knee	Toronto, Ontario, Canada
Active, not recruiting	Chron’s disease	London, Ontario, Canada
Recruiting	Parkinson's disease	Shijiazhuang, China
Completed	Klinefelter syndrome Azoospermia	Cairo, Egypt
Completed	Graft versus host disease	Calgary, Alberta, Canada Halifax, Nova Scotia, Canada London, Ontario, Canada
Completed	Myocardial Infarction	Montreal, Quebec, Canada
Terminated	Acute kidney injury	Hamilton, Ontario, Canada Toronto, Ontario, Canada Montreal, Quebec, Canada
No longer available	Graft versus host disease	Calgary, Alberta, Canada Montreal, Quebec, Canada
Recruiting	Cystic fibrosis	Shaoxing, China
Completed	Acute respiratory distress syndrome	Various location in USA
Recruiting	Inflammatory bowel diseases	Amman, Jordan
Completed	Motor neuron disease	Sao Paulo, Brazil
Completed	Crohn’s disease	London, Ontario, Canada Toronto, Ontario, Canada
Recruiting	Chronic heart failure	Victoria, British Colombia, Canada
Completed	Lung transplant reject Bronchiolitis obliterans	Jacksonville, Florida, USA

While MSCs have enormous potential in the field of regenerative medicine, some scientists have indicated that MSCs may become genetically unstable [89], [101], [102], [103]. While this problem may exist, there are solutions that allow scientists to overcome it through the screening of MSCs prior to use in patients, such as through chromosomal karyotype analysis.

Several studies on MSCs have shown low survival of MSCs when incorporated into material scaffolds used for bone, cardio or kidney replacement applications [104], [105], [106], [107], [108]. Although MSC use still requires improvements, within the past 10 years, MSCs have been used for instance to help with heart repair and regeneration through either direct (engraftments) or indirect (paracrine effect) methods [109], [110], [111], [112]. Furthermore, there have been other successful stem cell therapies using human MSCs such as for the regeneration of cornea, skin, and the reinstallation of blood flow by promoting angiogenesis for the neovascularization and restoration of blood vessels through their paracrine effects [113], [114], [115]. This demonstrates that there is still a lot of hidden potential that has yet to be discovered for MSCs. As the scientific world progresses and advances their technologies, more light will be shed on MSCs to allow for a better understanding of this unique type of cell.

1.3 ERYTHROPOIETIN

Initially discovered in 1906 by Carnot and Deflandre, and successfully cloned in the mid 1980s, Erythropoietin (Epo) is a hematopoietic growth factor produced in the kidney and involved in the regulation of erythropoiesis [116], [117]. In homeostatic conditions, Epo is normally detected in low levels within the bloodstream until stress such

as hypoxia or anemia stimulate its production and release [118], [119], [120], [121]. Circulation of Epo within the bloodstream promotes erythropoiesis by stimulating the production of red blood cells through the binding and subsequent activation of the high affinity receptor (EpoR) expressed on the surfaces of immature erythroid cells which activates the Jak/STAT signalling cascade [118], [122], [123], [124], [125]. Studies have shown that Epo has tissue protective and reparative effects against cytotoxicity from oxidants, such as in acute kidney injuries, through a heterodimeric receptor [126]. Among various cytoprotective applications, Epo has also been shown to possess neuroprotective capabilities to help with focal brain ischemia injuries, concussion related injuries, as well as damages caused by autoimmune encephalomyelitis *in vivo* [127], [128], [129], [130].

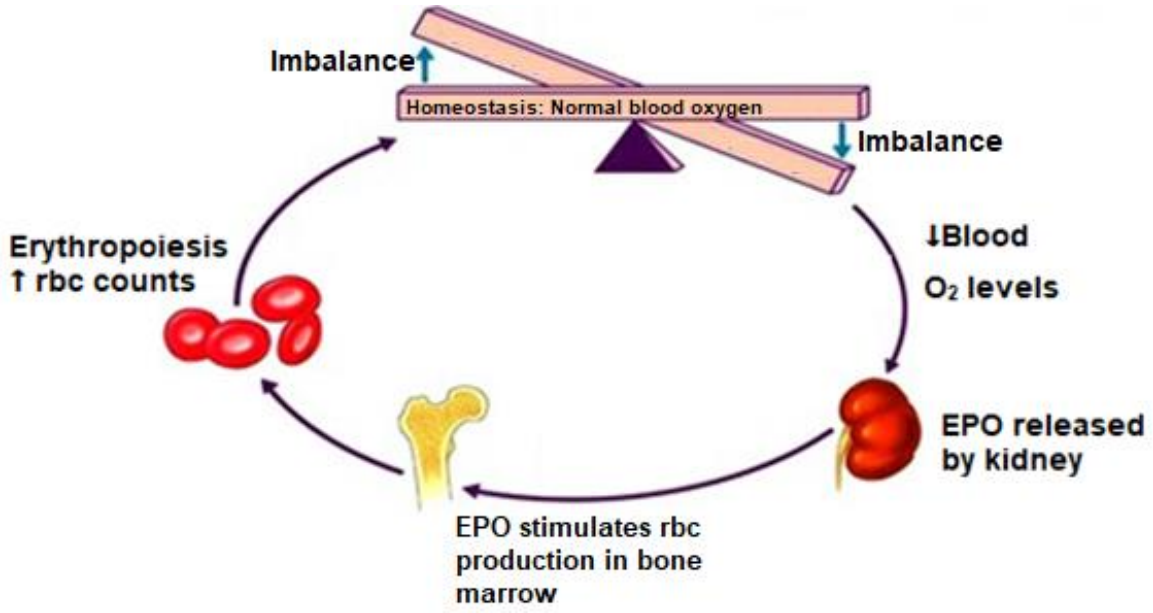

Figure 6. Mechanism by which Epo levels within the human body are maintained. Redrawn from the following reference: [131]

1.4 FACTORS FOR CELL SURVIVAL

1.4.1 OXYGEN

Oxygen is essential for the survival of cells as it is required in various metabolic pathways responsible for maintaining cellular function [132], [133], [134]. It is transported throughout our bodies by the circulation of red blood cells that travel through our vascular network to provide all organs with a constant supply of oxygen and nutrients, while removing any waste produced [133]. As each of our organs have their own separate function, they also require different amounts of oxygen to be supplied to them [133]. Due to this, experiments conducted within supposed normoxic conditions *in vitro* may produce misleading results as the cells within these types of studies are in fact exposed to hyperoxic conditions (21% Oxygen, with the addition of 5% CO_2), as opposed to physioxic levels found within our bodies that range from 1 to 11% Oxygen [133].

Table 2. Physioxic levels of oxygen exposure for organs within the human body. Table redrawn from the following source: [133]

	mmHg	Percentage (%)
Air	160	21.1
Inspired air (in the tracheus)	150	19.7
Air in the alveoli	110	14.5
Arterial blood	100	13.2
Venous blood	40	5.3
Cell	9.9 - 19	1.3 - 2.5
Mitochondria	<9.9	<1.3
Brain	33.8 ± 2.6	4.4 – 0.3
Lung	42.8	5.6
Skin (sub-papillary plexus)	35.2 ± 8	4.6 – 1.1
Skin (dermal papillae)	24 ± 6.4	3.2 – 0.8
Skin (superficial region)	8 ± 3.2	1.1 – 0.4
Intestinal tissue	57.6 ± 2.3	7.6 – 0.3
Liver	40.6 ± 5.4	5.4 – 0.7
Kidney	72 ± 20	9.5 – 2.6
Muscle	29.2 ± 1.8	3.8 – 0.2
Bone marrow	48.9 ± 4.5	6.4 – 0.6

Oxygen levels also affect the proliferation and differentiation processes of stem cells, with previous studies demonstrating their preference to remain undifferentiated in hypoxic conditions [135], [136], [137], [138], [139]. Clinical studies have also shown that wound healing processes become delayed in environments with reduced oxygen levels [132], [140]. This further strengthens the importance that oxygen has on cellular functions as it increases specific processes during wound healing such as cell proliferation and synthesis of collagen which help to strengthen and repair damaged tissues [132], [134]. Past studies have extensively looked into the effect of hypoxia on different cells types

such as fibroblasts and MSCs, but few have looked into the effects of anoxia on cells. It was only in the recent years that research on anoxic affects on MSCs were performed [141], [104].

1.4.2 GLUCOSE

Glucose plays an important role in providing energy for cell growth and survival. It is metabolized through the glycolysis or oxidative phosphorylation pathways into pyruvate which subsequently enters the tricarboxylic acid (TCA) cycle; also known as Krebs cycle, to generate large amounts of ATP through the process of oxidative phosphorylation during aerobic respiration [142]. Glucose also plays a role in the generation of new progenitor cells as it provides all the necessary precursor chemical constituents such as amino acids, nucleotides, and lipids; important for the creation of macromolecules used throughout the cell division process [142], [143], [144].

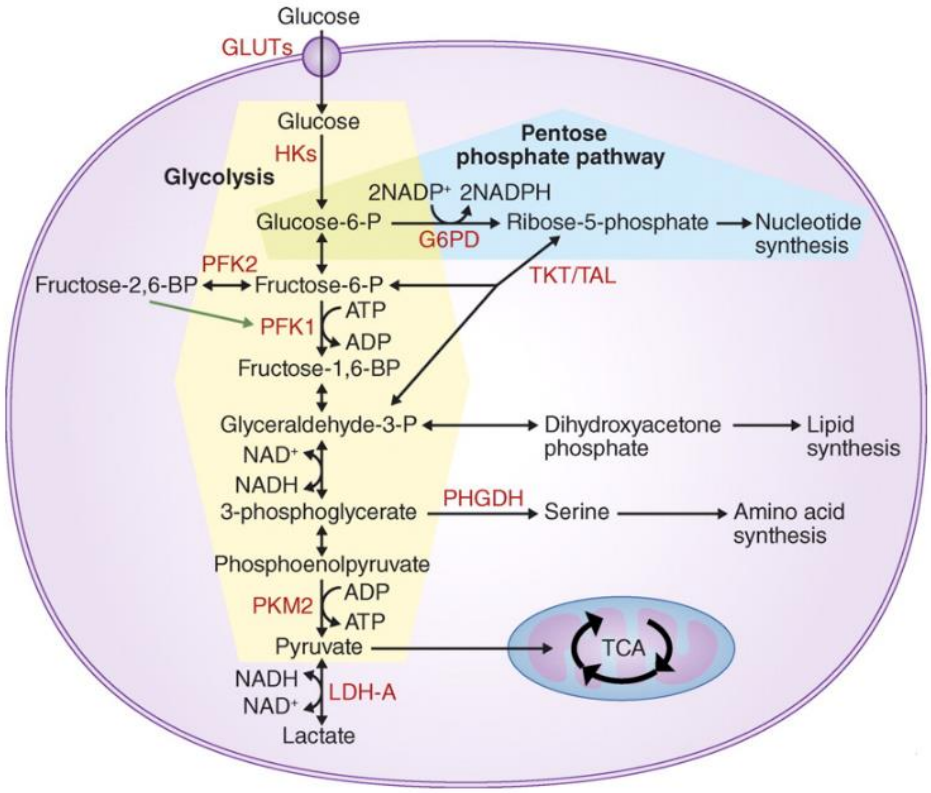

Figure 7. Metabolic pathways that involve glucose metabolism. [145]

1.5 CELLULAR ENERGY

There exist two types of cellular respiration pathways that cells can take to obtain cellular energy for their survival: 1) aerobic and 2) anaerobic [146]. While both pathways follow similar initial steps in the conversion of glucose into pyruvate through glycolysis which generates 2 ATP molecules, they differ greatly in their next steps [147].

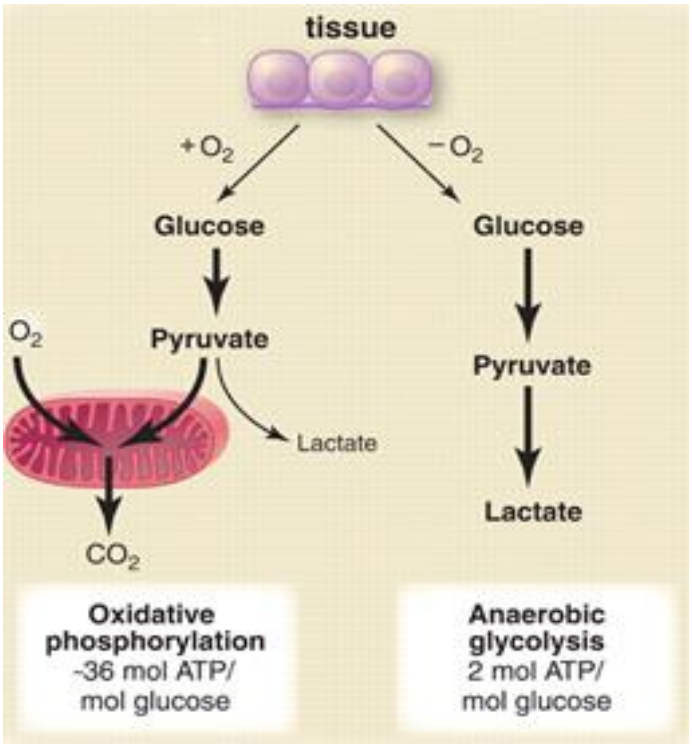

Figure 8. Cellular respiration. [148]

1.5.1 AEROBIC RESPIRATION

Within conditions where oxygen is abundant, only approximately 10% of ATP is produced within the cytoplasm through glycolysis, while the overall 90% generation of ATP originates from within the mitochondria through oxidative phosphorylation by the TCA cycle [147]. During aerobic respiration, pyruvate enters the mitochondria where it is broken down into acetyl-CoA by pyruvate dehydrogenase (PDH) in order to enter the TCA cycle. This produces the reducing equivalents NADH and $FADH_2$ which drive the electron transport chain to generate additional ATP through oxidative phosphorylation [147], [149].

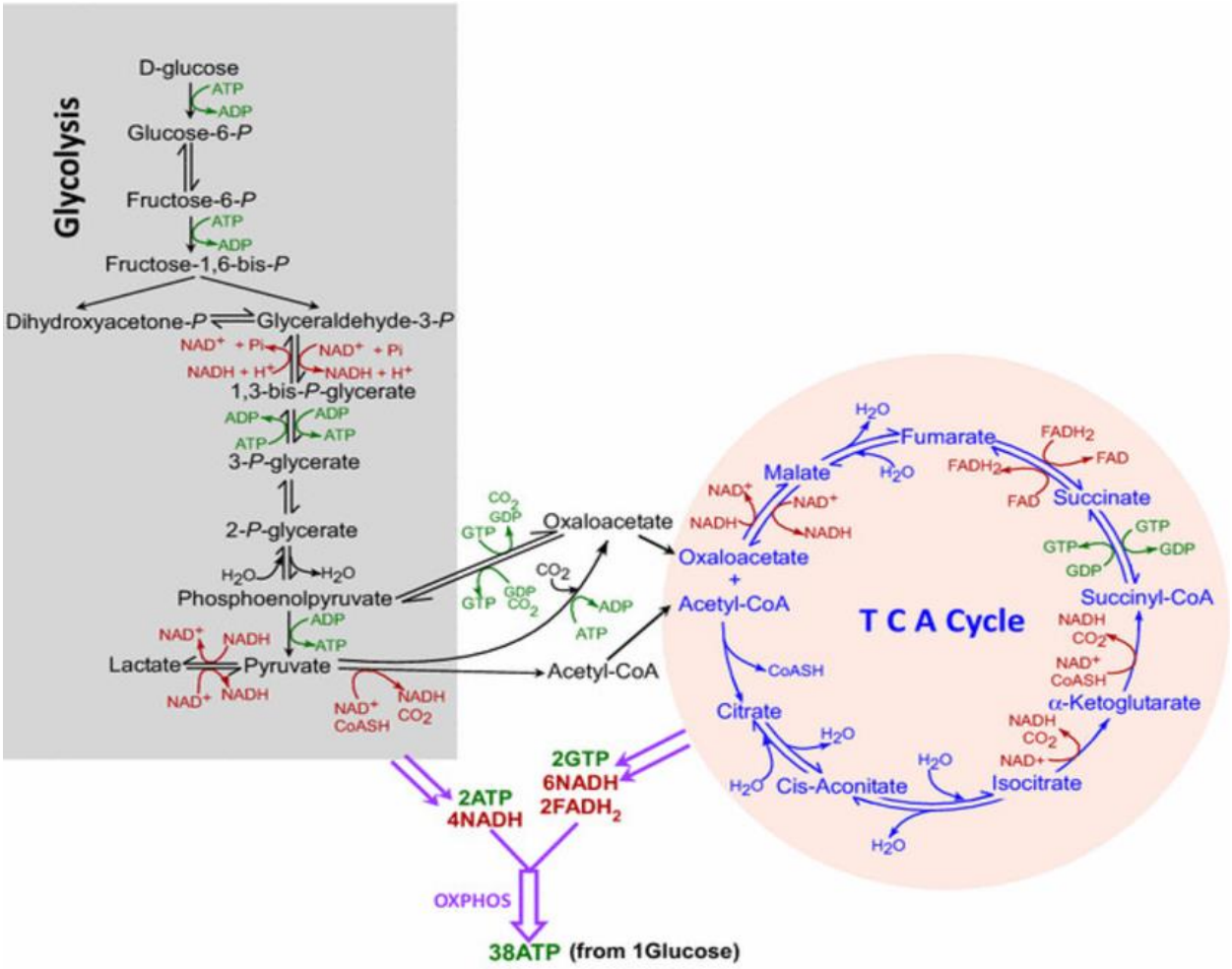

Figure 9. Metabolic steps involved in aerobic respiration. [150]

1.5.2 ANAEROBIC RESPIRATION

Hypoxia-inducible factor-1 (HIF-1) is a crucial transcription factor in cellular metabolism that responds specifically to changes in oxygen levels and is important for the survival of cells in hypoxic (2-9% oxygen) conditions [135] . HIF-1 is a heterodimer composed of two subunits: HIF-1a and HIF-1b. HIF-1a is normally unstable in normoxic conditions and degrades in the presence of oxygen through hydroxylation. In hypoxic conditions, hydroxylation is inhibited, which allows for HIF-1a stability [135], [151]. HIF-1b is constantly produced regardless of oxygen levels. Under hypoxic conditions, when

HIF-1a and HIF-1b associate, they activate promoters of glycolytic genes to help cells adapt to hypoxic environments through anaerobic glycolysis [147], [135].

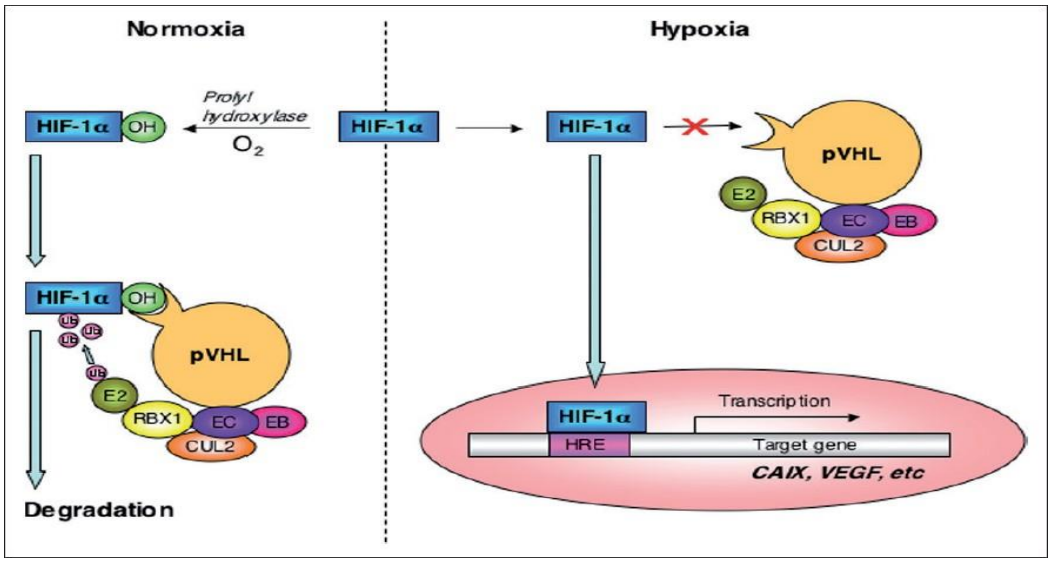

Figure 10. Hypoxia inducible factor-1 signalling pathway. [152]

Unlike aerobic respiration, anaerobic respiration occurs when oxygen levels are low. In these situations, pyruvate and NADH are instead converted into lactate and NAD^+ by lactate dehydrogenase A (LDHA) through a fermentation process. This enzyme is activated by HIF-1 to ensure that pyruvate does not accumulate within the cell because with lack of oxygen, the TCA cycle becomes suspended and can no longer utilize pyruvate [147], [153], [154]. Once lactate is produced, it is removed from the cell and NAD^+ re-enters into the glycolysis pathway to contribute to the oxidative conversion step of glyceraldehyde 3-phosphate [147]. This leads to the production of pyruvate and allows for continuous cycles of glycolysis to occur as an alternative solution for cells to obtain cellular energy when the TCA cycle is halted [147].

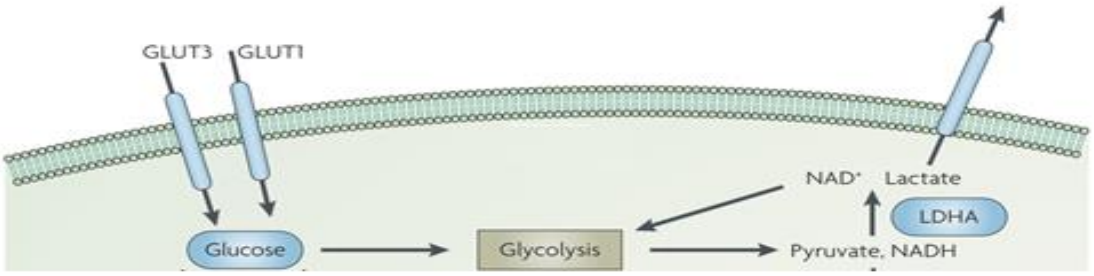

Figure 11. Continuous glycolysis cycle during anaerobic respiration. Redrawn from the following reference: [147]

1.6 RATIONALE FOR RESEARCH PROJECT

While there have been many advances in the field of tissue engineering and regenerative medicine to help find an alternative solution to organ transplants and tissue rejection, maintenance of cells and their survival in tissue engineered constructs post transplantation remains a difficult step for scientists to overcome. Therefore, the search for ways to maintain cell survival while in construct, along with looking into the adaptability of cells themselves to different environments continue in hopes that scientists can uncover a solution to these obstacles. While a lot of research has been done on MSCs in hypoxic conditions, little research has been done to determine the effects of anoxia on MSCs – the environmental condition that most closely mimics the conditions cells embedded within scaffolds are exposed to post transplantation.

Through additional research into anoxic conditions that mimic the environment cells are exposed to when embedded within tissue engineered constructs, and the requirements necessary for cell survival based on these conditions, this could allow for the possibility of generating tissue engineered constructs that do not rely on the delivery of nutrients and oxygen by the presence of vasculature to survive for extended periods of

time. As the neovascularization process takes weeks in order for vascular ingrowth to occur within implants [155], this study defines “extended periods of time” as 13 days. These additional 2 weeks will allow for sufficient time for the neovascularization process to begin within the implant so that prior to this, MSCs will be self sustaining until they can begin to rely on the presence of the new network of vasculature that will begin to spread within the implant. If possible, the ability for MSCs to self-sustain long enough within the implant until a new network of vasculature is incorporated may lead to a ground-breaking solution to the problems that currently exist within the field of tissue engineering. By answering this question, this will help advance the field so that the use of MSCs in tissue engineered constructs will become more promising for clinical use.

1.7 HYPOTHESIS

I hypothesize that:

1) Mesenchymal stem cells may be able to adapt to survive *in vitro* without the presence of oxygen (anoxia) - which will present itself as a novel study as no previous research has been done to look at the effects of anoxia on mesenchymal stem cells *in vitro*. If so,
2) By changing the amount of glucose available to cells within anoxic cultures, it may further alter the length of their survival. Therefore,
3) This could indicate the possibility of a metabolic switch from aerobic to anaerobic cellular respiration, which will be demonstrated through an increase in L-Lactate production and accumulation within the conditioned media collected from MSCs in anoxic conditions.

1.8 OBJECTIVES

The objectives of this study are to:

1) determine whether MSC viability and function are affected after a week of anoxic exposure
2) extend and prolong the survival of MSCs in anoxic cultures for close to two weeks through the introduction of high glucose media
3) demonstrate the capacity of MSCs to retain their stem cell properties after being cultured in anoxia, and
4) analyze L-Lactate production and glucose consumption by MSCs in oxygen (Normoxia vs. Anoxia) conditions, as well as glucose (Regular 1g/L vs. High 4.5g/L) conditions

CHAPTER 2: MATERIALS AND METHODS

2.1 ISOLATION OF MOUSE MESENCHYMAL STEM CELLS

MSCs were isolated from the bone marrow of C57BL/6 mice. Isolation of the cells from the bone marrow required the sacrifice of two C57BL/6 mice by cervical dislocation. Mice were rinsed with 70% ethanol and an incision made around the perimeter of the hind limbs where they attach to the trunk. The skin was pulled towards the foot and cut at the anklebone to eliminate contamination from the animal's fur. Dissection of hind limbs from the trunk of the body was done through an incision with care not to damage the femur underneath the hood. Each limb was bisected at the knee joint with the removal of muscles and connective tissues from both tibia and femurs by gently cutting away tissue surrounding the entire bone and removing any residual tissue by wiping with sterile tissue.

Bone marrow was obtained by cutting the ends of both tibia and femur at each epiphysis with the use of sharp sterile scissors. A 25-gauge needle attached to a 30-ml syringe containing 20ml complete media (α-MEM containing 10% FBS, 1% P/S and 1% L-Glutamine) was inserted into one end of the spongy bone and the marrow was flushed out of the other end of the bone with the media, and collected in a 50ml conical tube.

Separation of cell clusters into single cells by homogenate was achieved using 20ml syringes with 18 gauge needles for approximately 5 times. Cell suspensions were cultured in T75 flasks (P0) at 37C, with 5% CO_2. Fresh α-MEM complete media was changed after 5 days in order to discard the non-adherent hematopoietic cells. Media changes were done every 3 days until the occurrence of large colonies of the adherent MSCs. The MSCs were then trypsinized and reseeded in new flasks (P1). Cells were

continuously expanded and passaged about once a week until at least P8 or higher, Flow cytometry analysis was performed to confirm that the cells were positive for cell surface antigens CD44, CD73, CD105, H-2Kb (Kb), and were negative for CD31, CD34, CD45, and I-Ab.

2.2 GENERATION OF ERYTHROPOIETIN GENE-MODIFIED MOUSE MSCs

2.2.1 GENERATION OF RETROVECTOR (CONSTRUCT) AND RETROVIRUS-PRODUCING CELLS

The mouse erythropoietin (mEpo) cDNA was retrieved from our previously reported retroviral plasmid pEpo-IRES-EGFP [156] by *Bam*HI digestion, and then inserted into our *Bg/*II-digested retroviral plasmid pEmptyVector [157]. This generated the retroviral plasmid construct pEpo which contained the cDNA for mEpo [157]. Co-transfection of the retroviral construct pEpo with VSV-G expression vector into 293GP2 retroviral packaging cells lines generated retroviral particle production by the 293GP2 packaging cells. Retroviral particles were then collected and filtered using a 0.45um filter and concentrated through the use of ultra high centrifugation [158].

2.2.2 ERYTHROPOIETIN GENE MODIFICATION OF MOUSE MSCs

A 15-20g female C57BL/6 mouse was sacrificed and bone marrow-derived MSCs were isolated as described above. Retroviral transduction was performed on the MSCs for a total of 5 days as previously reported [158] where each round of transduction consisted of the exposure of MSCs to 0.45um filtered retroviral supernatant collected from 293GP2 cells transfected with the mEpo retroviral construct and VSV-G vector in 6ug/ml lipofectamine reagent (Invitrogen) [158].

To quantify the levels of Epo secretion by the newly Epo – gene modified MSCs, conditioned media (CM) was collected and used for mEpo ELISA (R&D Systems, Minneapolis, MN). Likewise, to ensure that only the Epo-gene modified MSCs produced mEpo, ELISA was performed on non gene-modified MSCs (WT-MSCs), and no Epo secretion was detected [158].

2.3 ANOXIC ENVIRONMENT

2.3.1 OXYGEN DEPENDENCY EXPERIMENTS

WT-MSCs (Passage (P) 5-20) and Epo-gene modified MSCs (Epo-MSCs) (P4-5) were plated at 850 cells/cm^2 in 35mm tissue culture plates in triplicates with 2ml of complete α-MEM media (Invitrogen) supplemented with 10% FBS (Wisent), 1% Penicillin/Streptomycin (Wisent) and 1% L-Glutamine (Invitrogen) and incubated overnight in a 37C incubator with 5% CO_2 to allow for cell attachment. Media was aspirated, plates were placed into small glass jars and freshly flushed (using a needle connected to gas tanks that provided a consistent flow of gas inserted into the appropriate container with 95% N_2 and 5% CO_2) α-MEM complete media was added to the plates, and rubber stoppers used to seal the glass jars. All samples were then flushed with the same mixture of 95% N_2 and 5% CO_2 for a total of 15 minutes each, then placed in a 37C incubator with 5% CO_2 to culture for a total of 7 days.

At the 7 day timepoint, the supernatant of each plate were collected in 15ml conical tubes and centrifuged at 1500rpm for 11 minutes to remove dead cells found within supernatants. Supernatants were collected and frozen at -20C for later determination of glucose and lactate levels for anaerobic respiration measurements. All cells were

trypsinized using 0.4ml of 0.05% trypsin (Wisent) for 45 seconds to a minute and then 0.1ml of complete α-MEM media containing 60% FBS was used to neutralize the trypsin. 60% FBS media was used in order to reduce the volume of media required to neutralize the trypsin in order to increase the total number of cells visible during the cell counting process. Trypan blue exclusion using trypan blue stain (Sigma Life Sciences) was used to determine the number of live and dead cells for each sample.

2.3.2 OXYGEN LEVEL MEASUREMENTS

In order to ensure that culture conditions remained anoxic, oxygen measurements were done using the Ocean Optics Fospor-A1300 Oxygen probe and the NeoFox Viewer program after initial (Day 0) and second day of flushing (D1), as well as at timepoints Day 8 and Day 13.

2.3.3 GLUCOSE DEPENDENCY EXPERIMENTS

WT-MSCs (P18-23) at $8.5x10^3$ cells/cm^2 were seeded into 35mm tissue culture plates in triplicates with 2ml of complete α-MEM media supplemented with 10% FBS, 1% Penicillin/Streptomycin and 1% L-Glutamine and grown overnight to allow for attachment. Media was removed, plates were placed into small glass jars and freshly flushed (same method described above in 2.3.1) α-MEM complete media with different concentrations of glucose (0.5, 1 and 4.5g/L) was added to the plates, and rubber stoppers used to seal the glass jars. All samples were flushed with a mixture of 95% N_2 and 5% CO_2 for a total of 15 minutes each, then placed in a 37C incubator with 5% CO_2 to culture for various timepoints: 8, 10, 11 and 13 days.

At each timepoint, supernatants of each condition were collected in 15ml conical tubes and centrifuged at 1500rpm for 11 minutes to remove dead cells. Supernatants were collected and frozen at -20C for later assessment of waste accumulation. All cells were trypsinized using 0.4ml of 0.05% trypsin (Wisent) for 45 seconds to a minute and then 0.1ml of complete α-MEM media containing 60% FBS was used to neutralize the trypsin. Trypan blue exclusion using trypan blue stain (Sigma Life Sciences) was used to determine the number of live and dead cells for each sample.

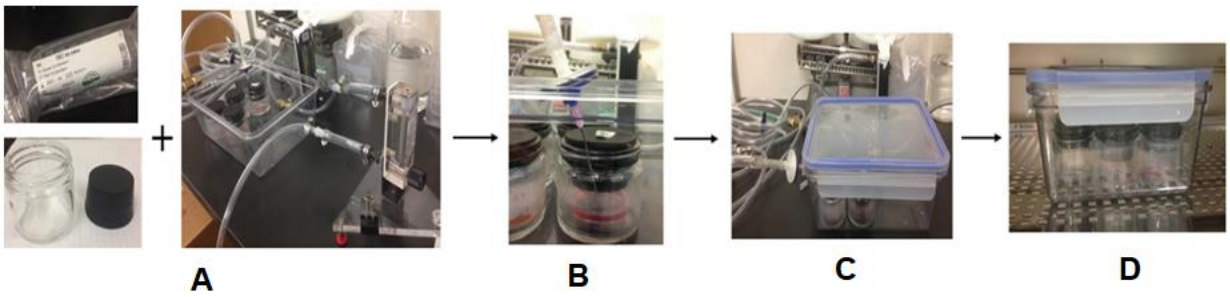

Figure 12. General anoxic culturing system set-up. Figure showing: A) General anoxic culture set-up with the use of 35mm culture plates, glass jars and rubber stoppers, B) Flushing of individual sealed samples with a mixture of CO_2 and N_2 for a total of 15 minutes through the use of a needle, C) Flushing of container to ensure for minimal to no presence of O_2 found within container, and D) Sealed container post-flushing placed in 37C incubator untouched for a predetermined period of time.

2.3.4 TRYPAN BLUE COUNT IMAGING

40ul of Trypan Blue stain was mixed with 40ul of trypsinized cells in each experiment and loaded onto a hemocytometer prior to imaging using an A. KRUSS Optronic (MBI Lab Equipment, Germany) microscope.

2.3.5 CELL DOUBLING TIMES

To determine the effects of 7 day anoxic exposure on the doubling times of WT-MSCs and Epo-MSCs, both cell types were re-plated in 6 well plates and regrown for 7 days in normoxia. At the end of the 7 days, cells were trypsinized, counted and their doubling times determined using the following equation [159]:

$$\text{Doubling time (DT)} = T \times \frac{log(2)}{logN1 - log\,N0},$$

where T represents culture time in hours, N1 represents the total cell number counted at the end of the designated time period, and N0 represents the total cell number initially seeded at the start of the experiment.

2.4 DETERMINATION OF ERYTHROPOIETIN LEVELS

The effects of anoxic culture on the protein secretion abilities of Epo-MSCs was determined by the quantification of mouse EPO levels accumulated in the cell supernatants throughout the 7 days of culture. Quantification of mouse EPO levels found in each of the collected supernatants of Epo-MSCs grown in anoxia and normoxia was performed by ELISA, DuoSet Mouse Erythropoietin ELISA kit, R&D Systems. Standard curves were generated alongside the sample readings, values were normalized (U mEPO/10^6cells/24 hours). All steps taken during the ELISA performance followed the manufacturer's instructions contained within the ELISA kit.

2.5 MSC DIFFERENTIATION

2.5.1 OSTEOGENESIC

In order to determine if the WT-MSCs that were exposed to anoxic conditions as described above, maintain the MSCs osteogenic differentiation ability, these cells were plated in 24 well plates and allowed to reach 100% confluency. Once attained, 0.7ml of differentiation media (complete α-MEM media, 1uM Dexamethasone, 20mM β-glycerolphosphate and 50uM L-Ascorbic acid 2-phosphate), Sigma Aldrich Canada, Oakville, was added to the cells and replaced twice a week for about 3 to 4 weeks until ~50% of calcium deposits were seen under the microscope. Media was then removed, and cells washed with PBS (Wisent). Staining solution (Alizarin Red S from Sigma Aldrich diluted in distilled water with the pH adjusted between 4.1 – 4.3) was added to cells and left for 5 minutes. Staining solution was then removed, and cells were rinsed with distilled water until the water remained clear. PBS was added to prevent drying of the cells while images were generated using a Leica DMIL microscope at 10X magnification.

2.5.2 ADIPOGENIC

WT-MSCs, previously exposed to the anoxic conditions as described above, were plated in 24 well plates and allowed to reach 100% confluency. Then, 0.7mL of adipogenic differentiation media (complete α-MEM media, 0.5uM Dexamethasone, 0.5uM isobutylmethylxanthine, 50uM indomethacin (Sigma Aldrich Canada) and 1.2ug/ml insulin), was added to the cells and replaced twice a week for 2-3 weeks until lipid droplets were observed within the WT-MSCs. The media was then removed, and cells fixed with paraformaldehyde (from Sigma Aldrich Canada, 4% in PBS) for 1 hour at room temperature. Paraformaldehyde was then removed and lipid droplets were stained with a

solution of 3 parts Oil Red O (0.5% Oil Red-O stock in isopropyl alcohol centrifuged at 1500rpm for 15 minutes to remove precipitate) and 2 parts distilled 0.45um filtered water for 20 minutes at room temperature. Staining solution was removed, then plates rinsed twice with distilled water, and PBS added to cells to prevent drying. Plates were stored at 37C until cell imaging was performed using a Leica DMIL microscope at 40X magnification.

2.6 GLUCOSE AND LACTATE ANALYSIS

Measurements of glucose and L-lactate levels within cell supernatants (conditioned media) were performed in order to determine the level of glucose consumption and corresponding production of lactate by WT-MSCs and Epo-MSCs in oxygen dependency experiments, and the level of glucose consumption and corresponding lactate production by WT-MSCs in glucose dependency experiments. Glucose and L-lactate concentration readings (mmol/L) were performed using a YSI 2300 STAT PLUS Glucose and L-lactate Analyzer (YSI Life Sciences, Yellow Springs, OH, USA) on all the conditioned media collected for each of the samples from both the oxygen and glucose dependency experiments. Prior to analysis, samples were stored at -20C. Duplicate readings were performed for each sample.

2.7 STATISTICAL ANALYSIS

Unless indicated otherwise, triplicate samples and measurements were performed for each experiment and the obtained results used for the calculation of the mean and standard deviation. One-way ANOVAs with Tukey multiple comparison tests was performed for the oxygen dependency experiments along with a two-tailed t-test for the quantification of mEPO production. Two-way ANOVA with Tukey post hoc tests were performed for the four timepoints in the glucose dependency experiments. All statistical analyses were conducted using GraphPad Prism and Microsoft Excel. Values of $p<0.05$ were reported as statistically significant.

CHAPTER 3: RESULTS

3.1 EFFECTS OF OXYGEN ON MSC CELL VIABILITY AND FUNCTION

When WT-MSCs and Epo-MSCs were cultured at 37C in anoxia for 7 days, no significant differences (N=15, P>0.05) in viability were observed compared to their normoxic controls (Figure 11A). However, a significant decrease (P<0.01) in cell number was noted for both cell types after 7 days of culture in anoxia in comparison with normoxic controls (Figure 11B). Between the two cell types grown in anoxia, Epo-MSCs showed a significantly higher total cell number count compared to WT-MSCs ($11.6x10^4$±4.6 vs. $7.1x10^4$±3.8, respectively). Statistical analysis was done through one-way ANOVAs.

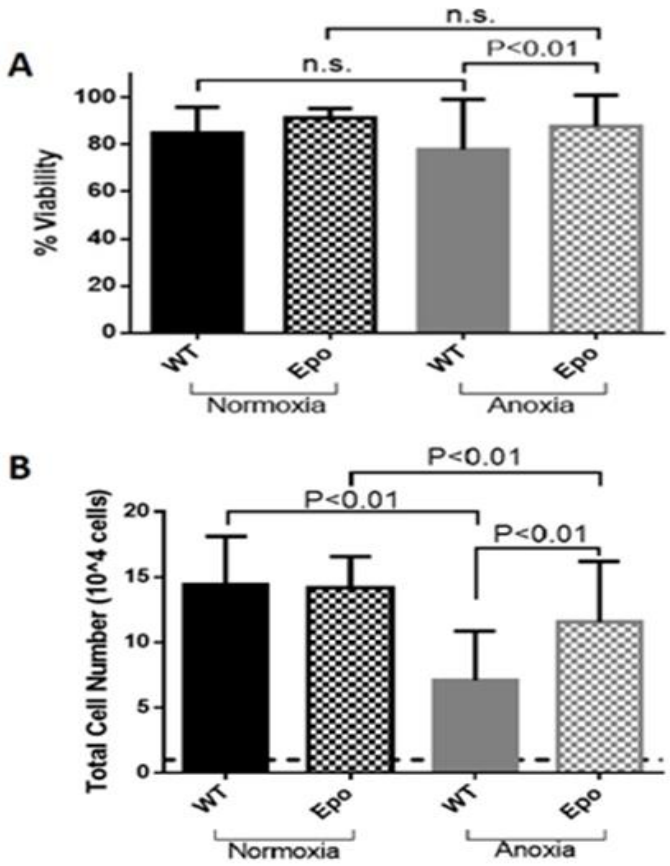

Figure 13. Effects of 7-day anoxic culture on WT-MSCs and Epo-MSCs. Effects of 7-day anoxic culture of C57BL/6 mouse bone marrow-derived WT-MSCs and Epo-MSCs compared to normoxic controls after plating at 850 cells/cm^2. Mean ± standard deviation of A) percentage of cell viability (%), and B) total cell number (containing both live and dead cell counts).

No significant difference (N=20, P>0.05) was seen in the amount of Epo produced over the 7 day culturing period between anoxic and normoxic conditions (31.6±8.7 vs. 39.7±18.9, respectively) (Figure 12).

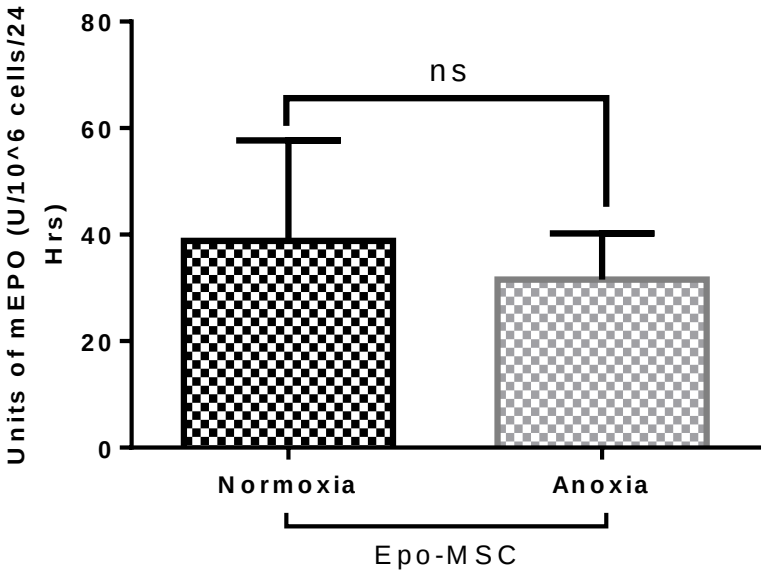

Figure 14. Effects of 7-day anoxia on Epo protein production by Epo-MSCs. Quantification (mean ± standard deviation) of Epo production by C57BL/6 mouse bone marrow-derived Epo-MSCs after 7-day culture in anoxic and normoxic conditions. Not shown is the negative control indicating negligible EPO levels found within the supernatants of WT-MSCs.

Culture in the absence of oxygen for 7 days significantly increased the doubling time of both WT-MSCs and Epo-MSCs (N=9, p<0.001), from 32.3 hours to 73.3 hours ± 15.3, and 27.2 hours to 115 hours ± 19, respectively when compared to their normoxic controls, when these cells were subsequently regrown in normoxia for 7 days (Figure 13).

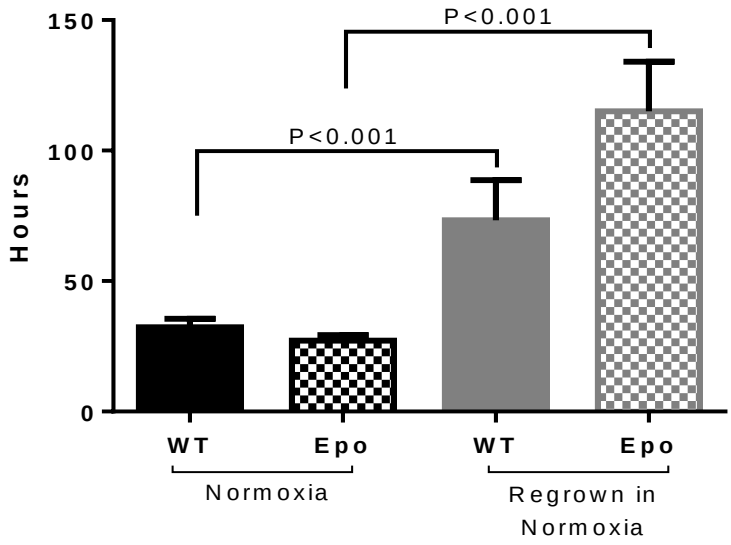

Figure 15. Effects of 7-day anoxic exposure on MSC doubling times when regrown in normoxia for 7 days. Effects of 7-day anoxic exposure on WT-MSCs and Epo-MSCs doubling times regrown in normoxia for 7 days. Mean ± standard deviation of doubling times for both WT-MSCs and Epo-MSCs grown in normoxia (control) without prior anoxic exposure and regrown in normoxia for a total of 7 days (168 hours) after anoxic exposure.

3.2 EFFECTS OF GLUCOSE ON MSC VIABILITY IN ANOXIA

Since cell viability was unaffected by oxygen, we sought to evaluate whether supply of nutrients could affect cell viability. WT-MSCs and Epo-MSCs were initially plated at $8.5x10^3$ cells/cm^2 and cultured from 8 to 13 days in anoxic culture with either regular (1g/L) or high (4.5g/L) glucose media. Regular glucose levels media is used in the majority of cell culture experiments. While there was no significant difference (N=11, P>0.05) in

cell number, viability in high glucose media was greater when compared to regular glucose conditions, with a significant difference found at day 13 (N=11, P<0.001, 68.5%±25 vs. 30.9±28.8, respectively, Figure 14A). Two-way ANOVA analysis was used to determine any statistical significances between timepoints and the software Graphpad Prism 6 was used to generate Figure 14B with dotted lines indicating the initial cell plating number.

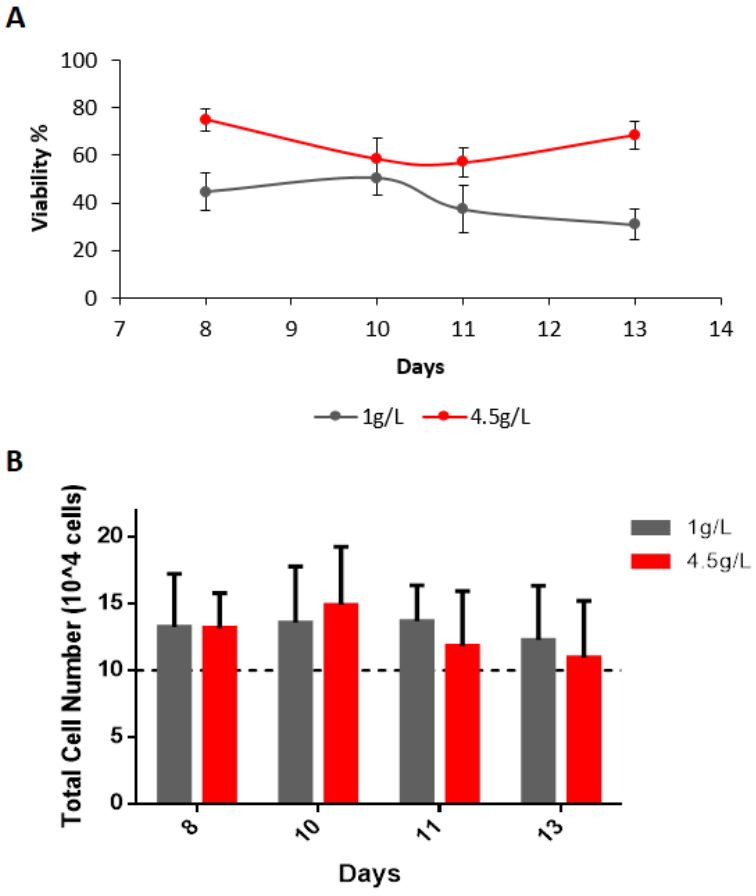

Figure 16. Effects of glucose concentration on MSCs up to 13-days in anoxic culture. Effects of high glucose concentration (4.5g/L) media when compared to regular (1g/L) glucose media C57BL/6 mouse bone marrow-derived WT-MSCs cell viability plated at $8.5x10^3$ cells/cm^2 in anoxic conditions for a total of 13 days. Mean ± standard deviation of A) cell viability (%) and B) Total cell number (live plus dead cells) at days 8, 10, 11 and 13, no significance was found (P>0.05).

3.3 OSTEOGENIC AND ADIPOGENIC DIFFERENTIATION OF WT-MSC POST EXPOSURE TO ANOXIA

WT-MSCs cultured in anoxic conditions were then exposed to osteogenic and adipogenic differentiation-inducing media, Alizarin Red S and Oil Red O staining specific for calcium deposits and lipid droplets, respectively were performed to determine if cells maintained their mesenchymal stem cell differentiation abilities after anoxic culture. Positive stain results were obtained (Figure 15-16) indicating that WT-MSCs previously cultured in anoxia maintained these differentiation abilities. Similar data were observed after WT-MSCs were cultured in anoxia and high (4.5g/L) glucose media. Both osteo- and adipo- genic differentiation occurred (data not shown).

OSTEOGENIC DIFFERENTIATION

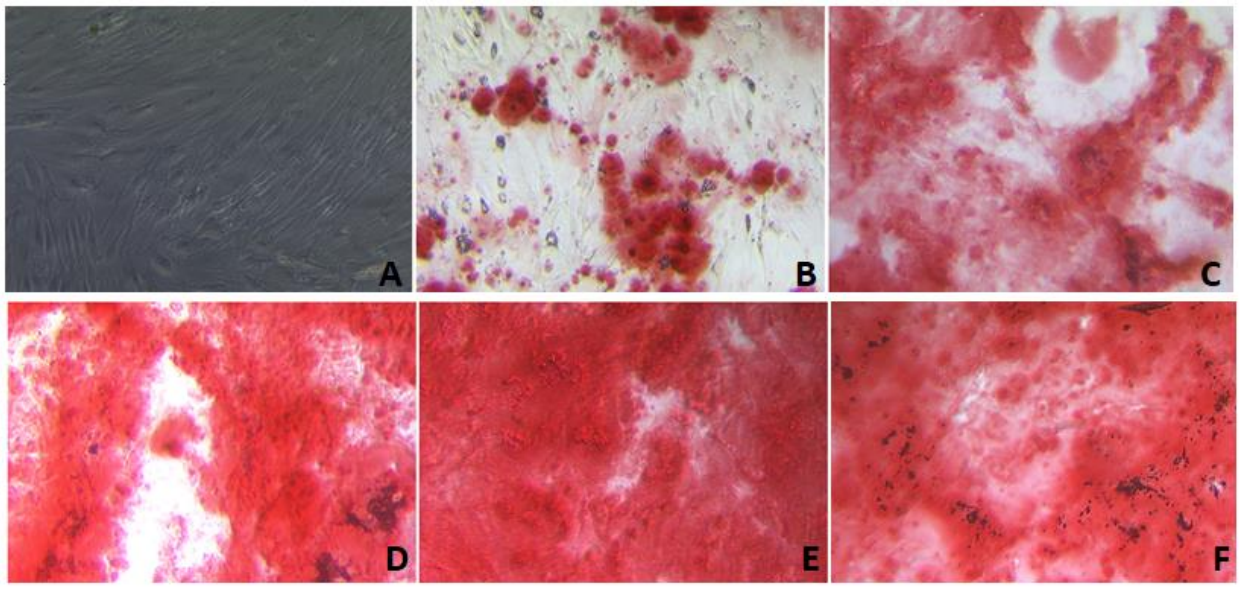

Figure 17. Osteogenic differentiation of WT-MSCs after up to 13-days of anoxic exposure in regular (1 g/L) glucose medium. Images stained with Alizarin Red S solution were taken using Leica DMIL microscope at 10X magnification: A) negative control (WT-MSCs not exposed to osteogenic differentiation – inducing media), B) positive control (Day 0, WT-MSCs only exposed to normoxia), WT-MSCs previously exposed to anoxia for C) 8, D) 10, E) 11, and F) 13 days.

ADIPOGENIC DIFFERENTIATION

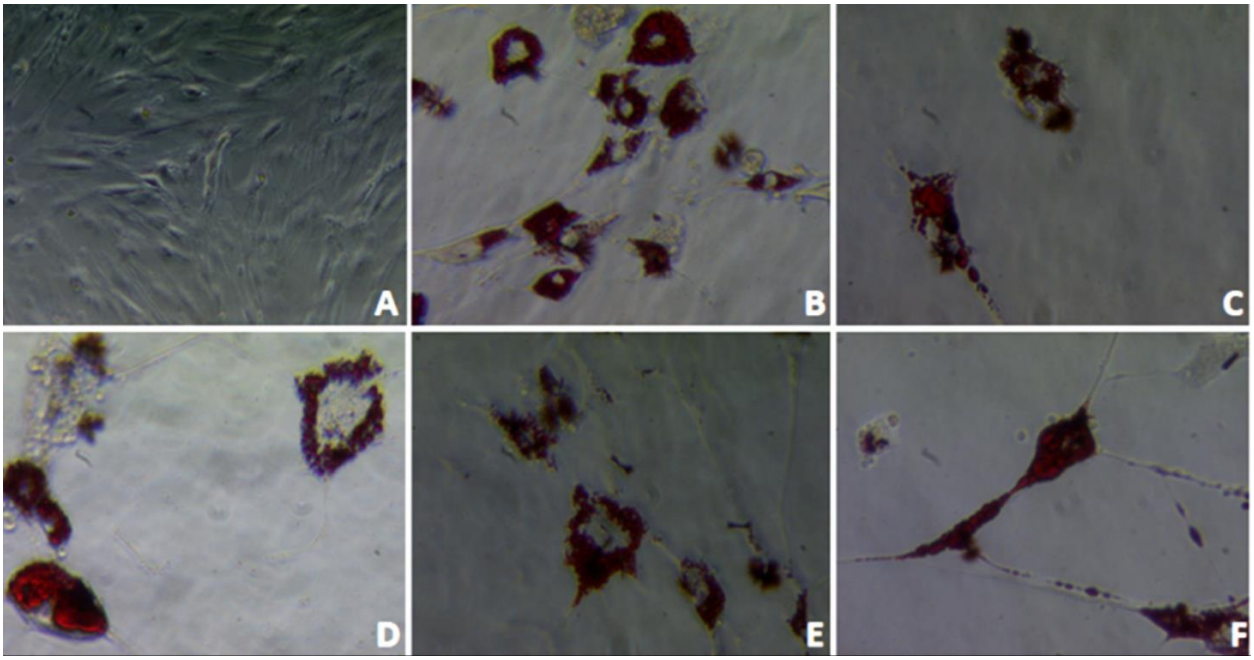

Figure 18. Adipogenic differentiation of WT-MSCs after up to 13-days of anoxic exposure to regular (1 g/L) glucose medium. Images stained with Oil Red O were taken using Leica DMIL microscope at 40X magnification: A) negative control (10X, WT-MSCs not exposed to osteogenic differentiation – inducing media), B) positive control (Day 0, WT-MSCs only exposed to normoxia), WT-MSCs previously exposed to anoxia for C) 8, D) 10, E) 11, and F) 13 days.

3.4 ANALYSIS OF LACTATE PRODUCTION

To determine the levels of respiration (aerobic and anaerobic) that occur in normoxic and anoxic conditions, glucose consumption and lactate production were measured to assess for the metabolic shift in cellular respiration. As seen in Figure 17A, there was a significant increase ($P<0.001$) in lactate content found within the media of anoxic culturing conditions when compared to normoxic control conditions for both WT-MSCs (3.8mmol/L ± 42.7 vs. 8.7mmol/L ± 4.2, N=15) and Epo-MSCs (2.8mmol/L ± 0.4 vs. 10.1mmol/L ± 2.9 respectively, N=15) after 7 days of exposure. The opposite effect, where original glucose concentrations show a decrease due to its consumption by MSCs

was observed between normoxic and anoxic conditions for WT-MSCs (4.3mmol/L ± 1.1 vs. 2.2mmol/L ± 2, respectively) and Epo-MSCs (4.2mmol/L ± 0.5 vs 1.4mmol/L ± 1.2, respectively).

A significant increase in lactate concentration measurements was found for regular glucose media conditions, as shown in Figure 17B between days 8 and 13 ($N=20$, $P<0.01$, 10.97±0.75 vs. 13.32±0.75, respectively), as well as between days 10 and 13 ($N=20$, $P<0.01$, 10.72±0.75 vs. 13.32±0.75, respectively). Between the other time points, no significant difference was found ($P>0.05$). High glucose conditions in Figure 17C showed no significant differences ($P>0.05$) of lactate and glucose concentrations detected between timepoints.

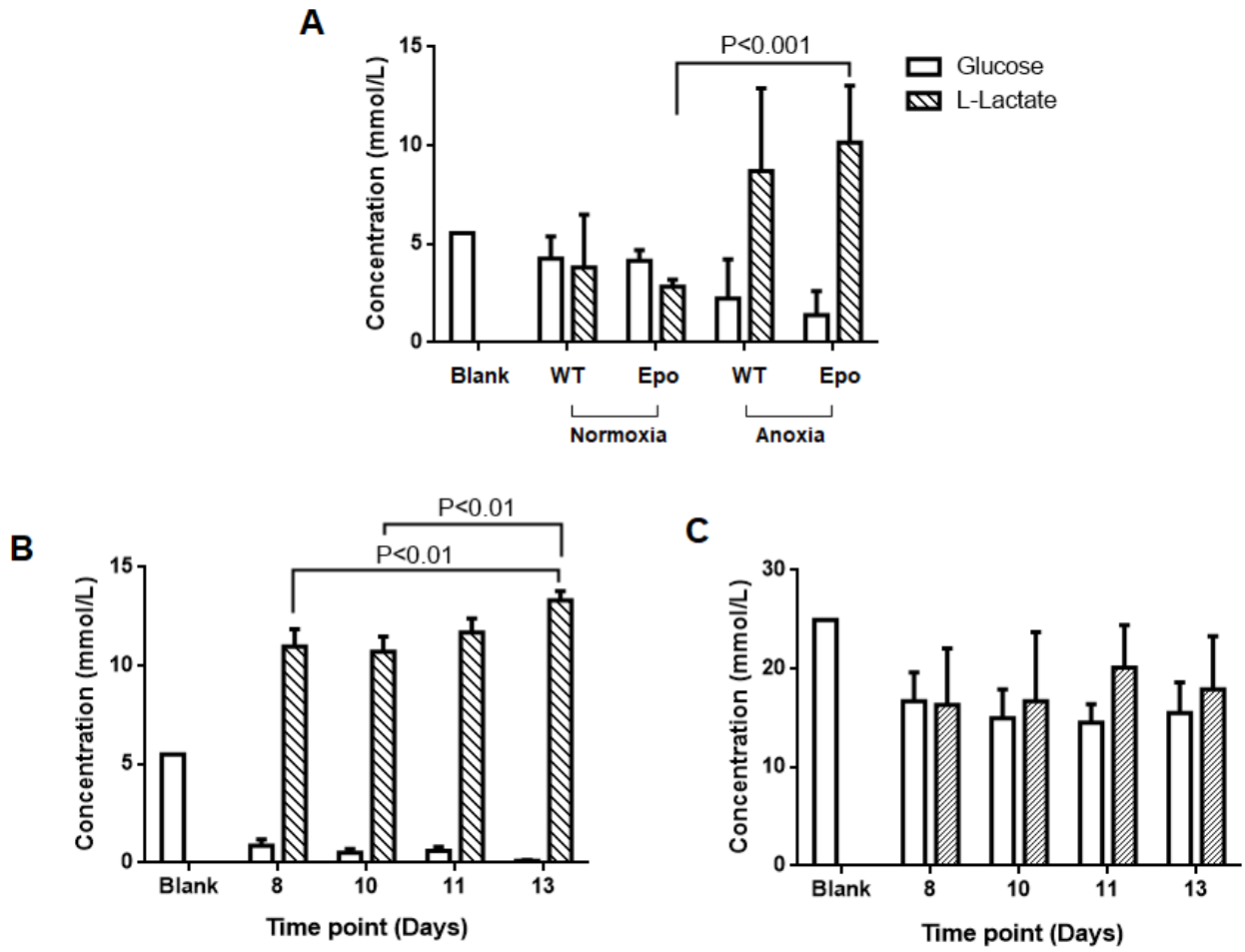

Figure 19. Analysis of average Glucose and L-Lactate concentration readings within collected conditioned media. Average levels of Glucose and L-Lactate in mmol/L detected within the collected supernatants (conditioned media) of A) WT-MSCs and Epo- MSCs after 7 days of culture in both normoxic (21% Oxygen) and anoxic (<1% Oxygen) culturing conditions, N=15 B) WT-MSCs after 8, 10, 11 and 13 days of culture in anoxic culturing conditions with complete α-MEM regular (1g/L) glucose media, N=20 and C) WT-MSCs after 8, 10, 11 and 13 days of culture in anoxic culturing conditions in high (4.5g/L) glucose media, N=20.

CHAPTER 4: DISCUSSION

The main objective of this study was to determine if MSCs have the capacity to survive in anoxic environments through a metabolic switch from aerobic to anaerobic respiration in an *in vitro* setting.

4.1 ANAEROBIC RESPIRATION AND CELL SURVIVAL OF MSCs

One of the greatest indictors of anaerobic respiration is the presence of lactate converted from glucose [143], [160]. In anoxic conditions where oxygen supply is limited, glucose becomes the main source of energy for cells and enters the glycolysis pathway to produce pyruvate. Due to lack of oxygen, pyruvate molecules are unable to undergo oxidative phosphorylation within the mitochondria through the TCA cycle [147]. As a result, pyruvate molecules undergo a fermentation process by LDH-A which produce lactate to prevent accumulation of pyruvate within cells. This continuous cycle contributes to the build-up of lactate levels found within the collected cell supernatants at the end of the experimental timepoints [147], [153]. Similar to results reported by Deschepper et al. 2011 who used sheep bone marrow-derived MSCs, where they observed gradual accumulation of lactate in hypoxic conditions, while regular conditions had low levels of lactate accumulated [160], conversion of glucose present within our MSC culture media into lactate was present with a significant difference seen between normoxic (control) and anoxic conditions (Fig 17A). These findings show that when MSCs are placed in conditions without oxygen, they have the ability to metabolically switch from aerobic respiration – originally relying on oxygen to maintain their metabolic activity, to anaerobic respiration where glucose is the main energy provider for cells to help maintain all their cellular functions. These interpretations are made because the by-product of anaerobic

respiration is lactate by Lactate dehydrogenase A [147]. With the increased lactate concentrations detected in the collected supernatants (conditioned media) of MSCs grown in anoxic conditions compared those of MSCs in normoxic conditions, this shows that anaerobic respiration is occurring.

To further indicate the possibility for MSCs to adapt to anoxic conditions through the metabolic switch from aerobic to anaerobic respiration, we observed, as depicted in Figure 17B and 17C, differences between MSCs grown in regular (1g/L) and high (4.5g/L) glucose media and the large amount of lactate produced over the growth period of 13 days when initial readings show no presence of lactate prior to exposure to anoxic environments (environments to which the MSCs are exposed to with less than 1% dissolved oxygen found within the media that they are cultured in). This ability that MSCs posses which allow for them to undergo a metabolic switch from aerobic to anaerobic respiration in times when oxygen levels are low or non-existent provides for a survival advantage over cells that do not possess this ability. By being able to switch to anaerobic respiration, this allows for MSCs to not only have a higher rate of survival, but this ability becomes useful for tissue engineering scientists when they search for ways to optimize the survival of cells embedded within scaffolds. This may allow for scientists to increase the use of MSCs in tissue engineering techniques to try to overcome the current limitation of tissue necrosis and hypoxia in cells within large tissue engineered constructs post transplantation. Previous literature also reports similar lactate findings when MSCs are exposed to hypoxic (2-6% oxygen) conditions [104]. The accumulation of lactate we observed for both regular (1g/L) and high (4.5g/L) glucose conditions at the end of the 13

day timepoint did not exceed the maximum reported cytotoxic lactate concentration limit of 40mmol/L reported in the literature [160]. From the various studies performed prior to our experiments, it appears that this phenomenon of metabolically switching between aerobic and anaerobic respiration is species-independent as our results are based on mouse MSCs, whereas other studies (mentioned above) used MSCs that originated from other sources such as sheep [160] and humans [104].

While this study did not look at the activity of Hypoxia-inducible factor-1 (Hif-1); a key transcription factor in cellular metabolism, previous studies have shown links between Hif-1 and its ability to activate promoters in glycolytic genes within cells which allow for their adaptation to hypoxic environments [161]–[163].

Although oxygen is essential for the survival of cells due to its role as an important component within various metabolic pathways responsible for the maintenance of cellular function, glucose also plays a very important role for cell growth and survival [132]–[134], [142].

Due to the importance of these two main factors that contribute to cell survival and our results which showed the successful survival of MSCs in anoxic conditions for 7 days (Figure 11), we wanted to determine whether varying the glucose nutrient available to MSCs in culture would help extend their ability to survive for an extended period of time (a total of 13 days in our case). The presence of extended cell viability can be seen when high (4.5g/L) glucose media was supplied to MSCs as opposed to MSCs exposed to

regular (1g/L) glucose media as shown in Figure 14a. These results concur with findings obtained in previous experiments done in hypoxic conditions [104], [160], [161].

Interestingly, the cell viability percentage for MSCs grown in regular glucose media (1g/L) for both the oxygen and glucose experiments (Figures 11a and 14a, respectively) showed varying percentages (77.9%±21.3 vs. 44.6%±7.9, respectively). A likely reason for this is a difference in the experimental set-up, where for the oxygen experiments, 850 cells/cm^2 were initially seeded, whereas for the glucose experiments 8.5x10^3 cells/cm^2 were initially seeded. This change was implemented with the objective to obtain an earlier timepoint at which there would be 100% cell death through the increase in cell confluency. Although even with the increase in cell seeding amount, after 13 days, there was still roughly 30% cell viability in the regular glucose media condition as opposed to the 0% that we were hoping to see. The increased initial seeding value of the glucose experiments will allow for the cells to reach 100% confluency much earlier than the cells seeded at a lower initial value in the oxygen experiments. Due to this, it could cause the MSCs to begin dying at earlier timepoints, which may explain the varying percentages as mentioned earlier. This can be verified by the total cell number counts obtained for both the oxygen experiments at day 7 in Figure 11b, and the glucose experiments at day 8 in Figure 14b (7.08x10^4 cells vs. 1.3x10^5 cells, respectively) where the total cell number count was much higher for glucose experiments as opposed to the oxygen experiments. As a result, the decrease in cell viability percentages of the glucose experiments at day 8 when compared to the cell viability percentages of the oxygen experiments at day 7 can be explained by the increase in cell confluency.

Similar to our obtained results shown in Figure 14b, Moya et al., 2018, also reports a lower total cell number count in near-anoxia as opposed to normoxic (control) conditions for bone marrow-derived human mesenchymal stem cells (hMSCs) when cells were exposed up to 14 days. These findings show that while oxygen is not necessarily the deciding factor on MSC survival, it does affect MSCs through the increase in their doubling times after exposure to anoxia.

Since in all our experiments MSCs were able to survive for up to 13 days without oxygen, the most important factor that ultimately determines whether MSCs survival can be extended in anoxic environments will be the presence of glucose for MSCs to use as an alternative energy source. With the knowledge that glucose is the most important source of energy for extended MSC survival in unfavourable anoxic conditions, this finding is very useful for future scaffold designs where MSCs will be incorporated into the constructs. While the lack of vascularization should not have a hugely negative affect on MSCs embedded within these scaffolds, as long as future constructs can incorporate a mechanism that can slowly release or deliver glucose to the embedded cells, this will provide for a possible alternative solution to the main limitation that scientists currently have for tissue engineered constructs that contain cells embedded within scaffolds. Also, as these results only pertain to MSCs, this can also act as a good indicator that MSCs may hold a lot of potential to help solve current limitations as the possible best type of cells to use for embedment within scaffolds. From here, scientists can also focus more on the use of MSCs for tissue engineering applications as opposed to other types of cells

due to their ability to differentiate into multiple cell types and especially for their numerous paracrine effects.

4.2 PROTEIN PRODUCTION IN ANOXIA

As a side experiment, MSCs were gene-modified to produce Erythropoietin. These Epo-MSCs were subjected to the same culture conditions as the unmodified MSCs to determine whether the ability for MSCs to produce proteins was affected by the lack of oxygen. As seen in Figure 12, no difference was found in Epo secretion by MSCs between the two environments. These findings contrasted literature where protein synthesis was inhibited in the presence of anoxia [164], [165]. This may be explained by the use of different cell types as well as the differences in our experimental procedures. Horman et al. [164], investigated the protein synthesis ability of rat hepatocytes while Smith et al. [165], studied these similar effects on select organs within crucian carps such as the heart, brain, lungs and liver. In the experiment conducted by Horman et al., hepatocytes were isolated from rat livers and later the cells were exposed to a continuous flow of 95% N_2 and 5% CO_2. Post exposure, cells were then homogenized using an extraction buffer for further analysis of protein synthesis. Smith et al., conducted their experiment by placing crucian carps into two water tanks where one contained regular normoxic conditions through regular aeration of the tank by pumps, while anoxic conditions were created through bubbling nitrogen into the water to remove the dissolved oxygen. After 48h exposure, the carps were sacrificed, and individual organs retrieved and homogenized to obtain protein fractions to determine protein concentrations. Due to the varying experimental protocols and the use of different cell types to perform each

experiment, further research will have to be done in order to verify that with regards to MSCs, anoxia does not affect their protein synthesis abilities, as observed in our experiments when we determined Epo secretion by Epo gene-modified cells cultured in anoxia.

4.3 RETENTION OF STEMNESS AFTER ANOXIC EXPOSURE

As MSCs possess the capacity to survive in anoxic environments, the next objective was to determine whether these harsh environments had an impact on their mesenchymal stem cell ability to differentiate into various cell types.

MSCs are depicted as metabolically flexible with regards to the abilities that they possess to survive in various unfavourable conditions [166]. Low oxygen environments are known to induce close to quiescent, if not full quiescent state for stem cells [137], [167], with findings from Beegle et al., proving that this occurs through their own study findings with the use of human bone marrow-derived MSCs where they noted that exposure to hypoxia caused a decrease in proliferation rates and glucose consumption and corresponding lactate production [168]. Our findings have shown that when MSCs are exposed to anoxia over 13 days and regrown in normoxia, they maintain their potential for adipo- and osteo- genic differentiation. These findings, seen in Figures 15 and 16, agree with previous studies performed *in vitro* using human, rat and sheep bone marrow-derived MSCs in hypoxic conditions where all three studies show successful osteogenic and adipogenic differentiation abilities after 14 days of hypoxic exposure for rat and human bone marrow-derived MSCs, and 12 days of hypoxic exposure for sheep bone

marrow-derived MSCs [160], [166], [168] . While similar to previous studies, our findings demonstrate that even exposure to extreme anoxic conditions does not affect the ability for MSCs to differentiate when they are re-exposed to normoxia. What made our study unique from the previous studies done is that we re-exposed our MSCs to normoxia during the differentiation experiments after they were exposed to anoxia for 8, 10, 11 and 13 days, while other studies performed their differentiation experiments under hypoxic conditions. Our observation is an important finding as it indicates the hardiness of MSCs. Since MSCs maintain their differentiation abilities even after anoxic exposure, this shows that MSCs are good candidates to be incorporated into tissue engineered scaffolds even with the current problem of lack of vascularization that results in hypoxia or tissue necrosis of cells embedded within constructs. Through their abilities to remain unaffected by anoxia and be able to survive and successfully differentiate into the desired cell type, scientists can focus more on the use of MSCs specifically to be embedded within the constructs. These constructs may then possess a higher success rate once transplanted into patients, as they will have higher survival rates compared to other cell types and possess the ability to help with regeneration through their ability to differentiate into desired cell types once transplanted into patients.

4.4 INFLUENCE OF ANOXIA ON MSC CELL DOUBLING TIME

Lastly, after 7 days of anoxic culture, MSCs previously exposed to anoxia showed an increase in cell doubling time as opposed to their normoxic counterparts when they were re-exposed to normoxia for a total of 7 days (Figure 13). When compared to the literature, others have reported the opposite result of a decrease in cell doubling time

when regrown in anoxic conditions, although their cells were previously exposed to hypoxic conditions as opposed to anoxic conditions [160]. While our findings conflict with those reported by Deschepper et al. 2011 [160], our experimental results of MSCs having a longer doubling time for post exposure to anoxia makes sense because in environments with high stress, cells will want to conserve their energy to help with their self preservation [169]. With the lack of oxygen availability; a major energy provider in cellular respiration, the entire metabolic system will slow down due to the limited amounts of oxygen, which will result in a longer doubling time as less energy will be produced to help support the other metabolic pathways found within cells to help with their regular functions.

4.5 CONCLUSION

In summary, MSCs were exposed to anoxia for up to 13 days in regular and high glucose media without any media change in between. MSCs showed the ability to survive as long as there is sufficient glucose present within the media for cells to use, and their stem-like properties remain unaffected post anoxic exposure, regardless of the level of glucose the MSCs were exposed to. While our findings suggest that vasculature may not be necessary for the survival of MSCs for 13 days *in vitro*, this effect where MSCs possess the ability to metabolically switch between aerobic to anaerobic respiration to survive in anoxia can be further optimized through additional experiments, although further research will also need to be done *in vivo* to investigate this finding. These results demonstrate that the survival of mouse bone marrow - derived MSCs in anoxic environments can be significantly affected by controlling oxygen/glucose metabolism – representing a paradigm shift from traditional approaches. The next steps are to: 1) determine the mechanism, 2) optimize this observed effect to produce self sustaining MSCs that can last for several weeks – long enough for the estimated time of ingrowth of vasculature to occur within implants, and 3) evaluate construct performance in animal models of ischemic repair.

CHAPTER 5: REFERENCES

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