Oxygen Concentration Modulates Cardiac Stem Cell Proliferation and Migration

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OXYGEN CONCENTRATION MODULATES CARDIAC STEM CELL
PROLIFERATION AND MIGRATION

By

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A DISSERTATION

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OXYGEN CONCENTRATION MODULATES CARDIAC STEM CELL
PROLIFERATION AND MIGRATION

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Cardiac stem cells (CSCs) are being evaluated for their efficacy in the treatment of heart failure. However, numerous factors impair the exogenously delivered cells’ regenerative capabilities. Hypoxia is one stress that contributes to inadequate tissue repair. Here, we tested the hypothesis that hypoxia impairs cell proliferation, survival, and migration of human CSCs relative to physiological and room air oxygen concentrations. Human endomyocardial biopsy-derived CSCs were isolated, selected for c-Kit expression, and expanded in vitro at room air (21% O₂). To assess the effect on proliferation, survival, and migration, CSCs were transferred to physiologic (5%) or hypoxic (0.5%) O₂ concentrations. Physiologic O₂ levels increased proliferation (P<0.05), but did not affect survival of CSCs. Although similar growth rates were observed in room air and hypoxia, a significant reduction of beta-galactosidase activity (-4203 fluorescent units, P<0.05), p16 protein expression (0.58-fold, P<0.001), mitochondrial content (0.18-fold, P<0.001), and activation of a pro-survival phenotype in hypoxia suggests that transition from high (21%) to low (0.5%) O₂ reduces senescence and promotes quiescence. Additionally, physiologic O₂ levels increased migration (P<0.05) compared to room air and hypoxia, and treatment with mesenchymal stem cell conditioned media rescued CSC migration under hypoxia to levels comparable to
physiologic O₂ migration (2-fold, P<0.05 relative to CSC media control). Furthermore, we confirmed a hypoxic induction of a quiescent phenotype in mCSCs and identified c-Myc as a regulatory stress sensor downregulated upon hypoxic stress. Induction of quiescence was associated with a decrease in the expression of c-Myc through mechanisms involved in protein degradation and subsequent downregulation of Sirt1 and upregulation of the cell cycle blocker p21. Our finding that physiologic O₂ concentration is optimal for in vitro parameters of CSC biology suggests that standard room air may diminish cell regenerative potential and that hypoxic stress induces quiescence through modulation of c-Myc. Therefore, modulating pathways downstream of c-Myc may re-activate their regenerative potential under ischemic conditions. This study provides novel insights into the modulatory effects of O₂ concentration on CSC biology and has important implications for refining stem cell therapies.
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Abbreviations

β-gal- Beta-galactosidase
CSC- Cardiac stem cell
EMB- Endo-myocardial biopsies
ESC- Embryonic stem cell
FACs- Fluorescence-activated cell sorting
FOXO- Forkhead box O
hCSC- Human Cardiac stem cell
HIF1α- Hypoxia inducible factor-1
HSC- Hematopoietic stem cell
KD- Knockdown
mCSC- Murine Cardiac stem cell
MFI- Mean Fluorescent Intensity
MSC- Mesenchymal stem cell
O₂- Oxygen
ROS- Reactive oxygen species
SDF1- Stromal cell-derived factor-1
Sirt1- Sirtuin 1
Chapter 1: Introduction

1.1: Stem Cells

For decades, stem cell research has inspired innovation and controversy. What was once considered science fiction has become a scientific reality. Limb and organ regeneration remains one of the greatest medical challenges that many believed to be impossible. However, with advances in biomedical technologies combined with a greater understanding of stem cell biology, medical advancements have offered solutions to some of our societies most complex diseases.

Stem cells are a population of non-differentiated, clonogenic, and self-renewing cells with the ability to generate into virtually any cell type in the body. Embryonic stem cells (ESCs) are the most primitive form of stem cells. First appearing in the inner cell mass of the blastocyst, ESCs have the pluripotent ability to differentiate into any of the three embryonic germ layers (ectoderm, mesoderm, and endoderm). These stem cells have been critical for the advancement of our understanding of development and the molecular signaling cascades involved in cell lineage commitment. However, due to limited availability, ethical concerns, and government restrictions, human embryonic stem cells research has been limited. It was not until the discovery of adult stem cell populations in the bone marrow that stem cell research was able to reach the clinic and be studied for the treatment of immunodeficiency disorders. This finding has lead way for a revolution in regenerative medicine.
1.2 Types of Adult Stem Cells

The discovery that many of our organs contain reservoirs of adult stem cells capable of tissue regeneration and repair has become the backbone of stem cell therapy. Populations of adult stem cells have been characterized in numerous tissues, most notably, the brain, bone marrow, lungs, kidney, and heart\textsuperscript{3-7}. Adult stem cells are multipotent stem cells, capable of asymmetrical cell division and differentiation into subsets of cell lineages. These cells reside in stem cell niches that preserve the cells’ ability to replicate indefinitely and allow for the persistence renewal of stem cell pools throughout our lifetime. The term ‘adult stem cell’ is often used interchangeably with “progenitor cells” when referring to a non-differentiated, proliferating cell population\textsuperscript{8}. However, it is important to note that adult stem cells are not progenitor cell. Progenitor cells, although self-renewing to an extent, are more mature cells along the line of lineage differentiation from an uncommitted stem cell state to a fully differentiated mature phenotype. Thus, adult stem cells are populations of uncommitted cells that give rise to progenitor cells that further divide and differentiate.

Mesenchymal stem cells (MSCs) are a well-characterized population of adult stem cells found in multiple tissues including the bone marrow, adipose tissue, and the liver. MSCs are multi-potent and give rise to osteoblasts, adipocytes, chondrocytes and other cell types of mesodermal and non mesodermal origin\textsuperscript{7}. In the bone marrow, MSCs are a critical component in bone homeostasis and adipose development. Additionally, these cells play supportive roles for the maintenance of other adult stem cell populations such as the hematopoietic stem cells (HSCs). HSCs are the stem cell reserves for continuous blood and immune cell production\textsuperscript{9}. Whereas in the brain, neural stem cells
migrate along the SVZ enabling the replenishment of mature neurons throughout the hippocampus and cortex. Even more recently, adult stem cells have been found in organs once thought to be terminally differentiated such as the heart and lungs. In particular, cardiac stem cells (CSCs) are described as a population of endogenous adult stem cells that can differentiate into mature cardiomyocytes, endothelial cells, and smooth muscle cells in the heart. In general, the existence of tissue specific adult stem cells has become a well-received scientific fact. However the precise nature of rare populations of adult stem cells, such as CSCs, has provoked buzz and controversy as to their biological role and validity as a genuine stem cell population.

1.3 Purpose of Adult Stem Cells

Adult stem cell populations, although rare and limited in number, are thought to play vital roles in tissue homeostasis and repair. Upon specific molecular signaling, quiescent and immature adult stem cells are activated and recruited from within the stem cell niche. These cells migrate into tissue or through the blood stream to site of injury for homeostatic repair. HSCs are classic examples of stem cells required for the daily replenishment of damaged or exhausted cells. Acting as a continuous surveillance system, HSCs are constantly being mobilized to and from the bone marrow by natural, everyday stimulus such as the circadian rhythm. HSC surveillance contributes to functional immune health and blood. Additionally, circulating chemotactic cytokines or inflammatory proteins released from an area of injury travel through the blood stream and signal HSCs to mobilize and differentiate. Specifically, the upregulation of the chemokine stromal cell-derived factor-1 (SDF-1) is a major recruitment factor that
facilitates the mobilization and maturation of HSCs. Similar recruitment strategies are used when targeting other adult stem cell populations. MSCs and immature endothelial progenitor cells are recruited from the bone marrow by injury associated secretion of SDF-1, Nitric Oxide, and VEGF. Whereas in the brain, neural stem cells migration is regulated by receptor signaling from within the sub-ventricular zone by ephrin/Eph-mediated pathways and plays a direct role in adult neurogenesis. Together, adult stem cell pools allow for the constant replacement and maintenance of our bodies every day wears and tears.

1.4 Stem Cells in Aging and Disease

The described role in tissue homeostatic suggests a major role of adult stem cells in organism aging. As we age, the pools of regenerative stem cells shrink. In the bone marrow, the accumulation of exhausted HSCs leads to impairments in immunity and loss of stem cell reserves. This effect results in an increased occurrence of bone marrow failure, and anemia. In the brain, the decline of functional neural stem cells in rodents is associated with decreased neurogenesis and the onset of progressive parkinsonian disease. Together, compartmental decline in functional stem cells may contribute to the progressive loss of tissue functionality and propensity for repair.

The age associated functional decline of adult stem cells is regulated by both cell-intrinsic and environmental factors. The additive effect of progressive reactive oxygen species (ROS) generation associated with aged cells leads to the accumulation of mitochondrial and nuclear DNA damage. Inability to sufficiently repair ROS mediated DNA or macromolecule damage leads to the induction of abnormal cell
proliferation or cell death pathways and apoptosis\textsuperscript{23}. Aged stem cells often lose replicative function due to a decline in genomic stability and the induction of a senescent phenotype, an effect that is largely due to diminished telomerase activity and the shortening of protective telomeres\textsuperscript{24}.

ROS and other accumulative toxic stress, such as peroxynitrite or oxidized glutathione, can lead to alteration in gene transcription and protein function. The class III histone deacetylase protein Sirtuin1 (Sirt1) and Forkhead Box O (FOXO) transcription factors are examples of important mediators of cellular function in aging that are regulated by these redox stresses. Acting as NAD\textsuperscript{+} dependent histone deacetylase, Sirt1 regulates FOXO mediated transcriptional actions and has direct consequence on cellular proliferation, apoptosis, and DNA damage repair\textsuperscript{25, 26}. Initially characterized as a life-span, longevity factor in yeast, Sirt1 targets and removed acetylation residues on both histone and non-histone proteins in the cytoplasm and nucleus. Sirt1 confers protection against cell cycle arrest by deacetylation of cell cycle regulators P53 and retinoblastoma. This effect alters protein post-translational modification of P53 and retinoblastoma, resulting in functional changes that impairs their function as cell cycle inhibitor proteins\textsuperscript{27, 28}. In the setting of stress, Sirt1 modulates these targets to initiate protective and cellular adaptive pathways such as DNA damage repair and mitochondrial biogenesis\textsuperscript{29, 30}. Thus, the dysregulation of Sirt1 signaling in age has been shown to contribute to an increased sensitivity to ROS, increased induction of senescence, and a decline in cellular longevity\textsuperscript{31}. Changes in redox status are able to regulate proteins like sirtuins at multiple levels, including the oxidation/reduction of post-translational modifications, interaction
with co-regulators, aggregation of damaged protein, and the elevation of protein targeted degradation pathways\textsuperscript{32,33}.

Cell-extrinsic and environmental factors including local and systematically produced cytokines and hormones as well as tissue perfusion and inflammation status impact stem cell populations. As organisms age, cytokine profiles change and systematic inflammation increases with a greater circulation of pro-inflammatory molecules such as Nf-kB, TGF-b, and TNF-a\textsuperscript{34-36}. These factors can target stem cells and impede regenerative response. Similarly, the development of ischemic or diseased tissue contributes to a decline in tissue repair via a loss of oxygen content, nutrient availability, and overall induction of cell death. The combination of hypoxia and serum-deprivation has been shown to be a deadly combination for MSCs by stimulation of apoptotic pathways\textsuperscript{37}. Whereas, inflammatory signaling from within an injury can initiate various stem cell responses depending upon the particular inflammatory milieu\textsuperscript{38}. Overall, the collective effect of environmental factors triggers the accumulation of a cellular inflammatory and senescent phenotype, hindering the function adult stem cell populations.

1.5 Regulation of the Adult Stem Cells in the Niche

The role of the adult stem cell niche is to protect the integrity and functionality of stem cell populations. A healthy stem cell niche is able to preserve the regenerative and self-renewing potential of the stem cells as we become susceptible to age and other disease related stresses. Signaling mechanisms within the niche works to regulate the stemness/differentiation balance while maintaining the stem cell regenerative potential.
To achieve this, stem cell niches are composed of various cell types and microstructures. One such cell type found in the stem cell niche are stromal support cells\textsuperscript{39}. In the bone marrow, MSCs play a stromal support role for HSCs, interconnecting to surrounding cells via N-cadherin gap junction proteins and expressing high levels of HSC maintenance proteins such as c-kit ligand and interleukins\textsuperscript{40}. Similarly, the formation of extracellular matrix protein is a critical component for establishment of niche structure and organization\textsuperscript{41}. Stem cell niches also include blood vessels and neural inputs that deliver systematic signals. These inputs are critical for the delivery of recruitment and communication of physiological cues\textsuperscript{39}.

Furthermore, oxygen concentration and the formation of an oxygen concentration gradient via the proximity of stem cell pools to vasculature is an important regulator of stem cell maintenance\textsuperscript{42}. As in the bone marrow, oxygen concentration can vary from 1-6\% oxygen\textsuperscript{43,44}. Hypoxic, or lower oxygen, niches favor stem cell quiescence, allowing the continuation of slow-cycling proliferation rates free of free radical ROS\textsuperscript{44,45}. Quiescence, unlike senescence, is a form of reversible cell cycle arrest and is the niche’s primary mechanism for the preservation of multi-potency, self-renewing, functioning stem cells\textsuperscript{46}. While both senescence and quiescence negatively regulate cell cycle progression, growth arrest via senescent mechanism is permanent and is often caused by uncontrolled proliferation, inflammation, or DNA damage. In many cases, the accumulation of senescent and exhausted stem cells is though to play a primary role in stem cell niche aging and loss of function\textsuperscript{47}.

An important mechanism in which the stem cell niche prevents growth arrest by senescence is thorough cellular and genetic adaptation to growth in hypoxic
environments. A decrease in mitochondrial metabolism and increase in glycolysis is an important process in the adaptation to quiescence\textsuperscript{48}. Mitochondrial respiration is a source of internal ROS. Thus, a switch to glycolysis as an alternative source of ATP production is one adaptation mechanism to prevent ROS buildup and stress induced senescence\textsuperscript{49, 50}. Mitochondrial biogenesis and increased respiration is also linked to cell differentiation. An increase in oxygen concentration and mitochondrial respiration promotes the development of greater populations of matured, committed progenitor cells\textsuperscript{42}.

Numerous differentiation and stem cell maintenance factors, such as Oct4, telomerase, and c-Myc, are under the direct regulation of oxygen concentration via hypoxia inducible factor-1a (HIF1a)\textsuperscript{51, 52}. The proto-oncogene, transcription factor c-Myc is commonly known for its’ regulation of cellular processes such as proliferation, differentiation and survival of many cell types\textsuperscript{53, 54}. Two downstream targets in particular, Sirt1 and P21, have been shown to regulate the preservation of cell cycle progression in the presence of environmental stress\textsuperscript{55, 56}. c-Myc is an essential gene for the preservation of stem cell pluripotency and self-renewal\textsuperscript{57-59}. Additionally, research by Florea et al has shown that c-Myc is essential to prevent the accumulation of replicative cellular senescence\textsuperscript{60}.

1.6 Plasticity of Adult Stem Cells and Stem Cell Therapy

After the discovery of residential stem cell populations within adult tissue, researchers began to isolate, expand, and evaluate adult stem cells for their therapeutic regenerative potential. Currently, adult stem cells are being studied for interventional use in a wide range of degenerative and chronic disease. In particular, stem cell therapy has
become an attractive therapeutic option for treatment of diseases involving chronic inflammation, fibrosis, or tissue necrosis.

Adult stem cells such as MSCs and CSCs, possess' unique cellular attributes that make them promising candidates for cellular therapy. Adult stem cell plasticity and versatility is a major contributor to tissue renewal. Upon transplantation into the heart, in various models of heart failure, these stem cells are capable of differentiation and functional integration into various cell types including cardiomyocytes, smooth muscle cells, and endothelial cells. Concurrently, this creation of newly formed cardiomyocytes, in addition to scar size reduction, has been linked to the overall improvement of cardiac function. Cell fusion, although rare, also occurs as a mechanism suggestive of cellular repair. Although the precise rate at which these fusions occur and the exact contribution to regenerative function in unknown, it is hypothesized that stem cell fusion may promote cardiomyocyte cell cycle reentry or repopulation of damaged myocardium. More recently, stem cell transplantation has been revealed to contribute towards organ rejuvenation through the reestablishment of the stem cell niche. Intra-myocardial delivery of MSCs promotes the resurgence of endogenous CSCs in the heart, either by direct interactions via gap junction protein connexin 43 or by paracrine singling. In fact, the re-stimulation of endogenous CSC likely contributes to the vast appearance of newly proliferating cardiomyocytes.

In addition to direct tissue integration, transplanted stem cells have a number of paracrine and signaling effects on the tissue. MSCs in particular, are known to produce and secrete a number of beneficial cytokines that produce anti-inflammatory, anti-fibrotic, pro-migratory, and pro-proliferative effects. In fact, MSCs have even been
viewed as nothing but a “bag of drugs” or an “injury drug store” capable of delivering a rejuvenating cocktail of molecules and proteins that can reverse aging and disease\textsuperscript{64}. Many claim that the primary mechanism of stem cell mediated regeneration is via this delivery of ectopic proteins and signaling molecules. For example, the secretion of pro-angiogenic factor VEGF and the anti-fibrotic MMPs are two paracrine factors identified as vital components of MSC mediated regeneration\textsuperscript{65}. However, it is important to emphasis that stem cells can secrete a number of cellular compounds aside from proteins, including microRNAs and micro vesicles exosomes\textsuperscript{66}. Thus, it is argued that the regenerative potential of stem cells cannot be replaced with a select cocktail of growth factors. The cellular components of stem cells are themselves powerful delivery units that posses the ability to not only differentiate and replace damaged cells, but stimulate and promote regeneration of surrounding tissues and systems.

Stem cell therapy is currently being tested at the University of Miami and other institutions for the treatment of a vast array of ailments. MSCs and CSCs have been used to treat health failure and left ventricular dysfunction. While phase 1/2 clinical trials such as POSEIDON\textsuperscript{67}, TAC-HF\textsuperscript{68}, and SCIPIO\textsuperscript{69} are primarily done to establish safety and efficacy of cell treatment, treatment with either MSCs or CSCs has promoted left ventricular remodeling and elevated ejection fraction. These results, although limited, have sparked interest into the investigation of the use of various cell types, cell preparations, and delivery strategies for treatment of a once incurable disease.
1.7 **Factors Limiting Stem Cell Effectiveness**

Although stem cell therapies are promising, numerous intrinsic and environmental stresses diminish cellular therapeutic potential. As previously mentioned, stem cells residing within aged or diseased tissues become compromised via induction of senescence, ROS mediated damage, and genomic instability. Additionally, in autologous cell therapy, stem cells are isolated from tissues in which age and disease potentially impose serious limitations. Research has identified a signature of aged versus young cells that may contribute to compromised function. Aging induces a number of changes including alterations in histone structure, gene expression, and secretome prolife that jointly influence cell differentiation, survival, and regenerative potential. Importantly, studies are being done to highlight and test the differences between autologous vs. allogeneic cell donors. These results have offered insight in the potential impact of aging on the development and potency of a stem cell product.

Cell preparation is another factor that impacts stem cell regenerative potential. Prior to transplantation, stem cells must be isolated and expanded in current Good Manufacturing Practice (cGMP) facilities under artificial, *in vitro* cell culture conditions. Everything from the cell culture media, flask growing conditions, to cell technician expertise has potential to impact cell performance upon therapeutic use. Particularly in settings of autologous therapy, individual patient comorbidities, genetic variations, and lifestyles will result in unique stem cell lines. While protocols are followed to ensure consistent isolation and cell line establishment, unavoidable variations in cell growth rate, accumulative senescence, and tissue viability greatly impact feasibility of cell preparation.
Lastly, the condition and environment of the cell recipient is a vital factor in the survival, integration, and subsequent proliferation of transplanted cells. From stem cell tracking studies, it has been shown that the vast majority of cells are lost due to necrosis or apoptosis soon after transplantation\textsuperscript{72}. This effect is likely due to hostile, toxic environments of the cell destination. In settings of elevated glucose concentrations, such as with diabetic patients, MSC proliferation is impaired with an increased induction of apoptosis\textsuperscript{73}. Similarly, ischemic damage, such as an injury from a myocardial infarction, results in massive cell death, inflammation, scar formation, and lost perfusion. Although the precise role ischemic injury on particular stem cell regeneration is not fully elucidated, it is hypothesized that the transfer of a stem cell product to an ischemic environment would largely hinder the regenerative potential.

1.8 Enhancing Stem Cell Function

As culture conditions, intrinsic stem cell variations, and overall regenerative limitations impose a risk to the therapeutic effectives of stem cell therapy, research has focused on methods for the enhancement of stem cell function upon transplantation.

As described in an editorial written by myself (Bellio et al. “Is the regulation of SIRT1 by miRNA-34a the Key to Mesenchymal Stem Cell Survival, ATM 2016) manipulation of stem cells by gene editing and the manipulation of protein expression via genetic editing and targeted microRNA (miRNA) technologies has become a promising avenue for the enhancement of cellular regenerative potential\textsuperscript{74}. The delivery of genetically modified MSCs to ischemic hearts in pre-clinical studies has advanced our
understanding of which genes could potentially improve the efficacy MSC therapy. Overexpression of genes such as extracellular superoxide dismutase (ecSOD) and vascular endothelial growth factor (VEGF) in MSCs has improved survival and tissue-repair when cells are injected into sites of ischemic injury\textsuperscript{75,76}. Similarly, modification of miRNAs via lenti-viral induced expression or silencing has made it possible to target entire pathways rather than individual genes. Delivery of MSCs transduced to overexpress miRNA-126 enhanced ischemic angiogenesis through the activation of AKT and ERK regulated signaling pathways\textsuperscript{77}. Together, these studies have provoked further investigation to establish the optimal gene targeting systems that will optimize MSC therapeutic applications.

In an intriguing study published in *Stem Cell Research and Therapy*, Zhang et al. demonstrated a novel role for miRNA-34a in the regulation of apoptosis and senescence by MSCs via the silent information regulator 1 (Sirt1)-mediated pathways\textsuperscript{78}. As a well-studied miRNA, the downregulation of miRNA-34a promotes proliferation, increases stress resistance, and promotes cell migration in a variety of cell types. These outcomes are particularly appealing to a cell-therapy application where cell survival and tissue integration is at risk. The therapeutic advantages of modifying miRNA-34a expression are further showcased through the feasibility of specific alteration using oligonucleotide mimetic or inhibition. Particularly in cancer therapy, induced expression of miRNA-34a safely reduced tumor growth and survival\textsuperscript{79}. In this context, the authors describe that MSC apoptosis and decline of cell proliferation correlates with miRNA-34a upregulation. This response serves as a promising therapeutic target in MSCs because recent studies have demonstrated a consistent role of miRNA-34a in cell cycle progression, particularly
due to its regulation of genes such as p53, c-kit, Sirt1, and Notch\textsuperscript{80}. Based on these findings, the authors formulated and tested the hypothesis that overexpression of miRNA-34a exacerbates hypoxia and serum starvation-induced apoptosis and senescence in MSCs.

The combination of hypoxia and serum starvation is a common inducer of MSC apoptosis, and is a reliable model to test molecular mechanisms that may be altered upon cell injection into hypoxic tissue\textsuperscript{37}. Zhang et al. used this model to test the potency of miRNA-34a overexpression and silencing on cell apoptosis via the detection of Annexin-V positive cells. As expected, those MSCs engineered to overexpress miRNA-34a overexpression exhibited a greater amount of apoptosis upon hypoxic serum starvation conditions, while silencing miRNA-34a significantly reduced this effect. These findings were then expanded upon in order to identify the key molecular players involved in the miRNA-34a response.

Sirt1 is an established and key target of miRNA-34a due to its endogenous regulation of anti-apoptotic and pro-proliferative pathways. Acting as a NAD-dependent protein deacetylase, Sirt1 has been described as a longevity factor, targeting transcription factors such as forkhead box proteins (FOX) and p53, while tightly regulating cellular resistance to oxidative damage\textsuperscript{81}. However, it had not been previously shown whether miRNA-34a manipulation and subsequent regulation of Sirt1 affects MSC biology. The authors used Sirt1 gene silencing paired with miRNA-34a inhibition to test the involvement of Sirt1 in the miRNA-34a-mediated apoptotic response. The authors demonstrated that miRNA-34a regulates Sirt1 and the downstream pro-apoptotic factor FOXO3a. Moreover, silencing Sirt1 expression abolished the anti-apoptotic effects of
inhibiting miRNA-34a. This result was confirmed by the alteration of apoptotic markers caspase-3, poly ADP ribose polymerase (PARP), and mitochondrial cytochrome C. Lastly, markers of DNA damage and senescence decreased with miRNA-34a inhibition, providing further evidence for the multifaceted effects of miRNA-34a in MSCs.

Although these findings serve as a promising first step toward establishing a genetic strategy designed to effectively preserve the viability of MSCs delivered into the ischemic myocardium, many questions are still left to be resolved. The broad range of RNA targets of miRNA-34a may be beneficial or problematic, and the long-term effect of miRNA-34a inhibition on MSCs has not been explored. The goal of MSC therapy is for transplanted MSCs to provide lasting reparative effects on myocardial structure and function by engrafting into the myocardium. miRNA-34a overexpression vectors are currently being investigated in translational models of cancer to halt unregulated cell proliferation and cancer metastasis\textsuperscript{82}. Whether the permanent inhibition of miRNA-34a in MSCs is a safe option that does not pose tumorigenic risk needs to be determined. Additionally, genes that influence MSC differentiation and angiogenesis, two important factors for the regenerative response, are known targets of miRNA-34a. Expression of platelet-derived growth factor receptor (PDGFR), which is down regulated by miRNA-34a, plays a significant role in MSC mediated vasculogenesis\textsuperscript{83, 84}. It would be important to investigate how the regulation of multiple pathways by miRNA-34a would impact the in vivo differentiation and tissue integration of MSCs. Lastly, the experiments performed by Zhang et al. were all done in 5\% oxygen, a level that is hypoxic compared to standard laboratory culture conditions (21\% oxygen) but not compared to the physiological oxygen levels in the mammalian heart. It has been reported that ischemia in the heart
could decrease oxygen levels from a physiologic 5% to a low of 1-3% oxygen\textsuperscript{85}. To confirm the effectiveness of miRNA-34a silencing in ischemia, similar experiments would need to be conducted at lower oxygen tensions.

In summary, Zhang et al have presented a novel and promising genetic targeting strategy that could enhance the effectiveness of MSC therapy in ischemic heart disease. Their findings demonstrate that miRNA-34a inhibition results in greater viability of MSCs in an in vitro model of hypoxia and serum starvation, warranting further investigation in an in vivo translational model.

In addition to the employment of genetic strategies to rejuvenate or enhance stem cell functions, the manipulation of laboratory cell growth conditions provides a simpler and alternative approach for enhancing stem cell properties. As stated, cell culture media, particularly the composition of growth factors, serum percentage, and presence of other stimulatory compounds, influences stem cell viability and therefore therapeutic potential. In particular, hypoxic preconditioning can enhance survival, differentiation, and angiogenic pathways by exposing stem cells to an oxygen-deprived environment prior to transplantation\textsuperscript{86,87}. Hypoxic preconditioning works by exposing stem cells to decreased levels of oxygen and promoting the activation of HIF-1\textalpha regulated pathways. These pathways result in increased expression of pro-angiogenic factor VEGF, pro-survival pathway AKT, and a number of other metabolic adaptive mechanisms\textsuperscript{88}. It has been shown that the activation of these pathways enhances the function of stem cells when transplanted in ischemic injuries. However, hypoxic conditioning protocols vary in the degree of hypoxia and length of preconditioning. Research supports the beneficial effect
of short-term hypoxic preconditioning yet suggests a reversal effect after long-term exposure\textsuperscript{87}.

1.9 Objective and Significance of Study

As previously stated, stem cells used in clinical trials for treatment of heart failure undergo isolation and ex-vivo expansion in cGMP facilities under a standard 21% oxygen (O\textsubscript{2})-rich environment prior to intracoronary or intra-myocardial delivery. Once transplanted, stem cells face a shift towards O\textsubscript{2} concentrations that instantly drop to 5% in healthy myocardium and as low as 0-1% in an ischemic site\textsuperscript{85}. To date, the culture of stem cells in an artificial, hyperoxic (21%) O\textsubscript{2} concentration is known to impair stemness and cell quality\textsuperscript{89, 90}. CSCs are currently one of the primary stem cell candidates for treatment of heart failure. However, it is unknown how O\textsubscript{2} concentration regulates CSC regeneration and the precise molecular mechanisms governing the ability of CSCs to survive, proliferate, and migrate in a physiologic or hypoxic myocardial microenvironment. O\textsubscript{2} concentration is a critical component in stem cell niche formation and differentiation. After an ischemic injury, the loss of vascular perfusion and accumulation of massive cell death renders the myocardium incapable of self-regeneration and formation of toxic environment. This damage skews the microenvironment of the heart towards the development of scar tissue, progressive cardiac hypertrophy, and eventually heart failure. Thus, the impact of ischemic O\textsubscript{2} on CSCs is not fully elucidated.

The \textbf{objective} of this dissertation is to identify basic \textit{in vitro} effects of O\textsubscript{2} concentration on parameters of CSC biology that impact the therapeutic effectiveness of
stem cell therapy. Additionally, we investigated the molecular signaling pathways that regulate CSC regenerative potential in hypoxia. We hypothesized that hypoxic (0.5%) \(O_2\) concentration impairs proliferation, survival, and migration of human CSCs compared to physiological (5%) and room air (21%) \(O_2\) concentrations. Furthermore, we hypothesized that hypoxic induced impairment in CSC regenerative function is mediated by a down regulation in Sirt1 and c-Myc mediated pathways. Together, our findings provide a novel insight into potential improvements in cell preparation techniques and regulatory mechanisms that affect survival and integration post transplantation into the injured myocardium.
Chapter 2: Cardiac Stem Cells

2.1 Cardiac Stem Cell Overview

For decades, the heart has been considered a static organ incapable of regeneration and repair. After cardiac development, cardiomyocytes exit the cell cycle and enter a permanent state of growth arrest. Cardiac hypertrophy, due to the elongation or thickening of already existing cardiomyocytes, is thought to be the primary mechanism of cardiac growth. However, it wasn’t until the analysis of post partum hearts from patients of cardiac hypertrophy and heart failure that researchers determined it was unlikely for a heart to achieve a mass of greater than 500g without the development of new cardiomyocytes. It was later determined that the observed increase in cardiomyocyte volume was too large to be achieved by hypertrophy alone and must be derived from either cardiomyocytes reentry into the cell cycle or the formation of genuine new cardiomyocytes. This concept that the heart contains the ability to generate new cardiomyocytes became particularly appealing for regenerative medicine. If researchers could determine the mechanism in which new cardiomyocytes were formed, then medicine could be developed to target and enhance cardiomyocyte production to repair and replace damage myocardium.

Like in the bone marrow, the formation of new cells would require the presence of a progenitor cell/ stem cell population. With this in mind, it was discovered that the adult human heart contained non-differentiated, c-Kit receptor positive (CD177) cells interconnected with cardiomyocytes and cardiac fibroblasts via gap junction protein connexin43 and adhesion molecules such as integrin. These cells, negative for markers of hematopoietic lineage (CD45), negative for endothelial progenitor markers (CD34,
CD31), and negative for other lineage specific markers, have been confirmed to meet all
criteria of bona fide stem cells. Cardiac derived c-Kit positive cells are clonogenic, multi-
potent, and possess the ability to contribute to the formation of new tissue when
transplanted in models of myocardial infarction. The integration and differentiation into
endothelial, smooth muscle, and cardiomyocytes was confirmed through the exogenous
labeling of cardiac derived c-Kit+ CSCs with BrdU or GFP labeling\(^6\).

Since the discovery of CSC, numerous debates and controversies have developed
conserving the cells’ origin, the extent of contribution to cardiac turn over, and the
validity of considering them actual stem cells. CSCs were first characterized as an
endogenous population of self-renewing stem cells that persist within myocardial stem
cell niches throughout life. However, the developmental identity of these cells has been
largely unknown. c-Kit expression is a classic marker of hematopoietic stem cells and
progeny; therefore, it was originally suspected that c-Kit positive cells in the heart might
be deposits of hematopoietic derived progenitor cells circulating through the blood
stream. Importantly, the absence of CD45 and CD34 expression counteracts this.
Currently, much of what we are leaning about CSC biology and endogenous function is
being done with Cre recombinase reporter genes driven by the endogenous c-Kit gene
promoter. These models allow for specific labeling of c-Kit+ cells via Cre recombination
and induced expression of GFP+ expression in only c-Kit expressing cells. Initial results
of these experiments suggest that the percentage of cardiomyocytes derived from c-Kit+
cells is so low, that these cells cannot be considered a physiological reserve for cardiac
turn over. Instead, the vast majority of c-Kit derived cells in the heart are actually
endothelial or endothelial progenitors\(^10,\,93\). This finding counteracted the concept of CSCs
being pools of stem cells contributing to ongoing cardiac repair. Nonetheless, it has also been shown using the CreER\textsuperscript{T2} mediated recombination system that CSC are indeed a population of endogenous cardiomyogenic stem cells that migrate into the heart as neural crest derived cells and contribute to the production of new cardiomyocytes\textsuperscript{94}. The different conclusions over the extent of CSC contributed cardiomyocyte turn over are largely due to the use of different Cre recombinase reporter systems and different experimental designs, specifically the time of tamoxifen recombination and sensitivity of the Cre Reporter. It is argued that these models do not accurately predict the differentiation capabilities of CSCs since these models rely on the assumption that detection of c-Kit reporter in cardiomyocytes translates to the differentiation capacity of CSCs\textsuperscript{11}. In fact, in vivo factors such as the activity of bone morphogenetic protein signaling greatly impact the differentiation capacity of CSC. Thus, the true differentiation capacity of CSCs has been tested in vitro using the Cre recombination system and induced pluripotent stem cell modeling to eliminate these in vivo factors. These experiments conclude that c-Kit+ CSCs are in fact bona fide stem cells with cardiomyogenic potential\textsuperscript{94}.

### 2.2 Use of Cardiac Stem Cells in the Clinic

Despite current controversy and ambiguity regarding the role of CSCs in the heart, preclinical and clinical trials have tested the potency and efficacy of exogenously delivered CSCs in the treatment of heart failure. Preclinical models have tested the effect of CSCs alone or in combination with MSCs in porcine models of myocardial infarction\textsuperscript{95}. The clinical trial SCIPIO tested the administration of CSCs to patients with
ischemic cardiomyopathy. Results of SCIPIO indicate that autologous CSCs are safe and effective in promoting an elevation in left ventricular ejection fraction and a reduction in infract size up to 1 year. With no reported adverse side effects, further testing is warranted. However, as previously stated, numerous factors continue to limit CSC mediated regeneration. Thus, both clinical and basic science research is needed to explore means of enhancing therapeutic potential of exogenously delivered CSCs.

2.3 Production of Human and Murine Cardiac Stem Cell Lines

The production of primary human and murine CSC lines involves the isolation and expansion of c-Kit+ cells from endo-myocardial biopsies (EMB). In short, digested and dissociated EMBs are plated in cell culture where outgrowth cells are expanded. Cell expansions are obtained and CSCs are purified via magnetic cell section for positive c-Kit (CD117) cell surface expression. CSCs are further expanded, cryopreserved, and characterized via flow cytometry analysis prior to use for experiments.

The production and maintenance of human and murine CSCs follow similar isolation and expansion protocols, however there are major differences in culture media composition. Human EMBs and CSCs are maintained in HAM’s F-12 Media (GIBCO) containing 10% FBS (Atlanta), 10 ng/ml FGF, 5 mU/ml EPO, 0.2 mM glutathione, and 1% penicillin-streptomycin. Whereas murine EMBs and CSCs are maintained in HAM’S F12 media (Lonza) supplemented with 10% FBS, 10ng/mL mouse bFGF, 20ng/mL mouse EGF, 10ng/mL mouse leukemia inhibitory factor, 1% insulin- transferrin-selenite supplement, and 1% penicillin/streptomycin. In this study, human EBMs were extracted from patience with dilated cardiomyopathy and
mouse EMBs were obtained from healthy, adult C57BL/6 mice. Upon retrieval of myocardial samples, EMBs are further minced into small pieces in 1 ml of sterile culture media. Minced EMBs are then transferred to 5 ml collagenase type II (1035U/ml diluted in F12 media) and incubated for 20 minutes at 37°C. The digested solution is then pipetted up and down several times before tissues are allowed to settle at the bottom of collection tube. The supernatant is removed, and tissues undergo 2 more rounds of collagenase digestions. After each digestion tissues are plated in culture media. Additionally, supernatants can be collected, washed, and plated in culture media, as CSCs may be present in these fractions. Cell cultures are performed under standard laboratory room O₂ conditions (21% O₂) in an incubator with 5% CO₂ at 37°C. EMBs are allowed to attach for 3 days prior to initial media change followed by subsequent media changes every 2-3 days until cell out growths reach flask confluence of 80%. Upon confluence, cells are wash with 1XPBS and incubated with 0.05% trypsin until cell detachment. Trypsin is neutralized using culture media and cells are then collected, counted, and plated at a seed density of 2,500-7,000 cells / cm². Cells are further expanded until 80% confluence, trypsinized, and collected for Miltenyi magnetic selection. Prior to magnetic selection, it is important to de-gas the Miltenyi Buffer solution as to prevent bubbles from clogging the magnetic column. De-gassing simply requires the removal of bubbles by tapping the buffer bottle to bring bubbles to the top of solution and applying an active vacuum hose on top of bottle for 45 minutes. 3 ml of collected cells are passed through a Miltenyi MACs Pre-Separation filter to remove cell clumps, re-suspend in 5 ml of Miltenyi buffer, and pelleted via centrifugation. Cell pellets are re-suspended with 300ul of Miltenyi buffer per 1x10⁸ total cells and incubated with 100ul of FcR Blocking Reagent and 100ul of CD117 (c-Kit) Miltenyi
Micro Beads for 15 minutes at 4°C. Next, cells are washed 2 times with cold Miltenyi buffer before re-suspension in 500ul of Miltenyi Buffer. The suspension is then past through the Miltenyi MACs separation column assembled in the magnetic field. Flow through solution is collected and stored as the negative fraction, as these are the CD117 negative cells, while positive cells remain attached to the column and are washed 3 times with Miltenyi Buffer. After the final wash, the MACs separation column is removed from the magnetic field, 1ml of Miltenyi Buffer is added and a plunger is used to flush the selected cells out of the column. Purified cells are counted, plated, and further expanded to establish CSC/CD117+ cell lines.

2.4 Characterization of Cardiac Stem Cell Lines

After further cell expansion, CSC lines are characterized by flow cytometry to confirm the enrichment of CSCs. CSCs in culture are trypsinized and washed with fluorescence-activated cell sorting (FACS) buffer before cell surface staining with CD45 (BD Pharmingen, 555482), CD31 (Biolegend, 303106), and CD117 (eBioscience, 17-1171) antibodies. Matching fluorescent isotype controls are used as negative staining controls. The hematopoietic LUVA mast cell line is used as a positive CD45 and CD117 control while human umbilical vein endothelial cells (HUVECS) are used as a positive CD31 control. After surface antibody staining, cells were washed again with FACS buffer and fixed with cytofix/cytoperm buffer (BD Biosciences). Cells are then incubated again with CD117 antibody to ensure staining of total cell surface and internalized CD117 expression. After this final antibody incubation, cells are washed with FACS buffer and fixed with 2% paraformaldehyde. Data is collected using BD Aria II LSRII flow cytometry (Biorad) and analyzed with
FloJo software in the Sylvester Comprehensive Cancer Center Flow Cytometry Core Facility.

This characterization process is essential for confirming the identity of purified cells used in subsequent experiments. CSCs must be positive for c-Kit and negative for hematopoietic marker CD45 and endothelial marker CD31. Flow cytometry analysis of our purified CSCs confirmed successfully isolation (Figure 1). These cells are CD45-, CD31-, and CD117+. CD117+ surface staining of CSCs is generally lower than that of permeabilized CD117+ staining, here we show a 43.8% surface stain compared to a 91.6% permeabilized stain. This difference is likely due to a frequent internalization of the c-Kit receptor. Permeabilization of the staining ensures that CSCs expressing the c-Kit receptor are analyzed regardless of the localization of c-Kit. In all human CSC studies, experiments were repeated using CSC cell lines isolated from a minimum of 3 individual patients. Each cell line was characterized for c-Kit expression prior to use (Table 1).

2.5 Challenges and limitations of Cardiac Stem Cell Cultures

Several challenges persist while attempting to establish permanent CSC cell lines for research and clinical purposes. First, CSCs are heterogeneous in nature and possess various propensities for lineage deviations and signaling response. Thus, CSC clones may vary in sensitivity to stress, experimental conditions, or regenerative capacity96. This challenge is further complicated by human donor variability induced by comorbidities, genetics, age, and cellular senescence. Cell morphology, proliferation rate, and senescence are a common example of variability observed across various donors (Figure 2).
Table 1: Percentage of isolated hCSCs from various donors: Representative percentage of hCSC population, c-Kit positive, isolated from three separate donors.

<table>
<thead>
<tr>
<th>Donor</th>
<th>c-kit Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 1</td>
<td>91.6%</td>
</tr>
<tr>
<td>Donor 2</td>
<td>98.9%</td>
</tr>
<tr>
<td>Donor 3</td>
<td>95.4%</td>
</tr>
</tbody>
</table>

Figure 1: CSC characterization: Flow cytometry analysis of hCSC cell line. (A) LUVA stained CD45, (B) hCSC stained CD45, (C) HUVEC stained CD31, (D) hCSC stained CD31, (E) LUVA stained CD117, (F) hCSC surface stained CD117, (G) hCSC permeabilized CD117 stained.
Figure 2: hCSC line morphologies: Representative images of hCSC morphology isolated from four individual donors. Donors 1 and 2 represent homogenous and fast growing cell morphology while Donors 3 and 4 contain more heterogeneous, slow proliferating, and senescent cells. Magnification 4X.
Chapter 3: Methods

3.1 Cell Culture

As previously mentioned, human and murine CSCs were maintained in respective medias. For human, HAM’s F-12 Media (GIBCO) containing 10% FBS (Atlanta), 10 ng/ml FGF, 5 mU/ml EPO, 0.2 mM glutathione, and 1% Penicillin-Streptomycin and for murine, HAM’S F12 media (Lonza) supplemented with 10% FBS, 10ng/mL bFGF, 20ng/mL EGF, 10ng/mL mouse leukemia inhibitory factor, 1% insulin- transferrin-selenite supplement, and 1% penicillin/streptomycin. Cells from three to four donors were used in all experiments for up to 10 passages. Various O₂ conditions were maintained using the thermo Scientific Heracell 150i incubator at 90% N₂, 5% CO₂, 37 degrees C for physiological O₂ concentration (5% O₂) and the Coy Laboratory hypoxia chamber at 94.5% N₂, 5% CO₂, 37 degrees C for hypoxic O₂ levels (0.5% O₂). LUVA cells (Kerafast) were maintained in suspension cultures containing StemPro-34 SFM (Gibco), 2mM L-glutamine, 1% penicillin/streptomycin, and 0.1mg/ml primocin. HUVECS were maintained in EGM-2 (Lonza) bullet kit media.

3.2 Flow Cytometry Analysis

CSCs in culture were trypsinized and washed with fluorescence-activated cell sorting (FACS) buffer before staining with CD45 (BD Pharmingen, 555482), CD31 (Biolegend, 303106), and CD117 (eBioscience, 17-1171) antibodies. After surface antibody staining, cells were washed again with FACS buffer and fixed with cytofix/cytoperm buffer (BD Biosciences). Cells were then incubated again with CD117 antibody to ensure staining of total cell surface and internalized CD117 expression. After this final antibody incubation,
cells were washed with FACS buffer and fixed with 2% paraformaldehyde. Data was collected using BD Aria II LSRII flow cytometry (Biorad) and analyzed with FloJo software in the Sylvester Comprehensive Cancer Center Flow Cytometry Core Facility.

3.3 Protein Expression and Stability Analysis with Western Blotting

Protein extracts were quantified by standard Bradford assay to ensure equivalent protein loading onto Criterion tris-HCl gels (Biorad) and transferred to a nitrocellulose membrane in a sodium bicarbonate transfer buffer. Membranes were blocked with 5% milk in TBST (1X tris-buffered saline/0.1% Tween) for 1 hour, followed by overnight incubation with the following primary antibodies: Sirt1 (1:2000, Abcam ab110304), P16 (1:1000, Abcam ab108349), c-Myc (Cell Signaling, #5605), P21 (Santa Cruz Biotechnology, #SC-756), AKT (Cell Signaling, #9272), phospho-AKT (Ser473) (Cell Signaling, #9271), Tubulin (Cell Signaling, #2146), and Actin (1:1000, Santa Cruz sc-1616). The blots were then washed 3 times with TBST buffer followed by incubation with HRP-conjugated secondary antibody. Blots were imaged using Super Signal Chemiluminescent substrate (Thermo) and the Chemi Doc XRS imagining system (Biorad).

3.4 Gene Expression Analysis

Total RNA was isolated from cell and tissue lysates using the RNeasy mini plus kit (Qiagen) according to manufacturers instructions. In c-Myc inhibitor experiments, RNA was collected from CSCs exposed to c-Myc inhibitor for 24 and 48 hours. RNA was then converted to cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems) prior to analysis by quantitative PCR (qPCR). qPCR reactions
were done using Taqman Universal Master Mix (Applied Biosystems) and Taqman gene expression probes (Thermo) in an iQ5 real-time PCR detection system (Biorad). Samples were run in duplicate and normalized to the housekeeping gene GAPDH. Gene expression changes were calculated using the 2−ΔΔCt method. The probes used for gene expression analysis are as follows: SDF-1 (human) Hs03676656_mH, CXCR4 (human) Hs00607978_s1, SDF-1 (mouse) Mm00445553_m1, CXCR4 (mouse) Mm01996749_s1, Sirt1 (mouse) Mm01168521_m1, c-Myc (mouse) Mm00487804_01, and P21 (mouse) Mm04205640_g1.

3.5 Proliferation Assays

hCSCs were seeded at a density of 1.0x10^5 onto 100mmX20mm cell culture dishes and counted with the TC10 automated cell counter (Biorad) after 24, 72, and 96 hours of growth in various oxygen conditions. Doubling rates were then calculated using the equation: 

\[
\frac{96 \text{ hours} \times \log(2)}{(\log(\text{Endpoint}) - \log(\text{Startpoint}))}
\]

mCSCs were plated at an initial density of 1 x 10^5 cells in 60 mm culture dishes, allowed to attach and placed under room (21% O_2) and hypoxia (0.5% O_2) conditions the same day. The number of cells was computed daily using an automated cell counter (Biorad). For quiescence experiments, cells were cultured for 3 days in hypoxia and then transferred to room O_2 for 3 more days. Cell numbers were computed every 3 days. Additionally, BrdU incorporation analysis was used to confirm the effect of O_2 on mCSC proliferation. mCSCs were plated an initial density of 1 x 10^5 cells in 60 mm culture dishes and grown in room or hypoxia for 48 hours. Cells were then collected and plated at 3 x 10^3 cells per 96 well, allowed to attach, and
BrdU was added to the media for 16 hours of incubation, for a total growth period of 72 hours. After the overnight BrdU incubation, the cells were fixed and stained for BrdU using the BrdU cell proliferation kit (Millipore) according to manufactures instructions. The effect of c-Myc inhibition on mCSC growth was determined by staining cells with 1µM of CellTrace Far Red Cell Proliferation Dye (Life Technologies) followed by treatment with 40µM the c-Myc inhibitor 10058-F4 (Sigma) or vehicle control (DMSO). After a period of 3 days, cells were harvested and analyzed by flow cytometry for changes in mean fluorescent intensity (dye dilution is an indication of proliferation) using a BD Aria II LSRII cell sorter.

3.6 Lenti-virus Transduction

Human SIRT1 p-GIPZ silencing plasmid (Dharmacon) and CMV-MT-DsRED-Puro vectors were transfected, along with lenti-viral packaging plasmids VSVG and Gagpol (Addgene), into 293T cells using Lipofectamine 2000 (Life Technologies). The pLV-CMV-MT-DsRED-Puro was created by PCR amplifying the Mito-DsRED fragment using the pDsRed2-Mito plasmid (Clontech) as template, and then the MT-DsRED fragment was sub cloned in place of the GFP fragment into the pLenti-CMV-GFP-Puro plasmid (Addgene 17448) at XbaI and SalI sites. Lenti-viral particles were concentrated through precipitation from culture supernatants using PEG-IT (System Biosciences). Lenti-virus concentration was then quantified with the QuickTiter Lenti-virus Titer Kit (Cell Bio Labs). CSCs were seeded 24 hours before being transduced at a ratio of 1.0x10^10 LPS/ 2.00x10^5 cells/100mm dish for 5 hours in serum free F12 medium. After this time, complete media was added. Efficiency of transduction was determined by
identification of GFP or DsRed-positive cells by fluorescence microscopy. Cells carrying viral particles were selected by addition of 10µg/ml puromycin.

### 3.7 Beta-Gal Senescence Assay

Replicative senescence was determined by the fluorometric cellular senescence associated (SA)-beta galactosidase activity assay (Cell Bio Labs). Cells were harvested after 24 and 96 hours of growth and protein lysates were prepared according to manufacture instructions. Sample concentration was determined by a standard Bradford assay, and 20 ug of protein were used to detect beta-galactosidase activity in a reaction of 3 hours. Fluorometric intensity was then measured using the Spectra Max M5 plate reader (Molecular Devices).

### 3.8 Glycolysis Assay

The measurement of cellular glycolysis was performed using the Glycolysis Cell-Based Assay Kit (Cayman Chemical), according to the manufactures instructions. In short, CSCs were grown in various O₂ concentrations for 96 hours, collected, and re-plated at a density of 1x10⁴ cells/well in a 96 well plate. After cell attachment, culture media was replaced with F12 media containing 1% FBS for overnight incubation. Supernatants were collected and the concentration of L-Lactate was determined by measuring absorbance of reaction samples using the Spectra Max M5 plate reader (Molecular Devices).
3.9 Apoptosis Detection

Apoptotic and necrotic cells were identified by live staining with Annexin V, Pacific Blue conjugate, (Life Technologies) and propidium iodide (Life Technologies). Briefly, 1.0 X 10^5 cells were plated onto 60 mmX15 mm cell culture dishes and incubated in serum-free F-12 media overnight in various O_2 conditions. Apoptosis was induced by treatment with 500 µM hydrogen peroxide. After 3 hours, cells were trypsinized, washed twice with phosphate-buffered saline (PBS) and stained with 5 µL of Annexin V conjugate and 1µg/ml propidium iodine in 1X binding buffer (Life technologies). Data was collected using BD Aria II LSRII flow cytometry (Biorad) and analyzed with FloJo software.

3.10 Cytotoxicity Detection

The cytotoxicity detection kit (Roche) was used to detect hypoxic induced cell death via the abundance of supernatant lactate dehydrogenase according to manufacturing instructions. In short, 1.0 X 10^3 CSCs were plated in a 96-well plate in triplicates and allowed to grow in 21% or 0.5% O_2 for 72 hours. Phenol red free media was used to minimize background absorbance of LDH. The reaction cocktail was mixed with cell supernatants and incubated for 1 hour followed by measuring absorbance at 490nm.

3.11 Transwell Migration

The ability of CSCs to migrate was determined by using 0.8 µM pore size transwell inserts (Falcon) on 24 well plates. Human MSCs and CSCs were seeded at a density of 1x10^5 cells onto a 24-well plate overnight followed by media replacement with starvation
media (serum free F12 media containing 0.1% BSA). Starvation media alone was used as the control. Transwells with 100ul of starvation media containing 1x10^5 CSCs were placed onto the prepared wells and migration was allowed for 24 hours. After completion of the assay, the transwells were removed and cleaned of excess media and cells with a cotton-tipped applicator. The bottom of the transwells were then fixed with 70% ethanol and stained with 0.2% crystal violet for 5-10 minutes. Stained membranes were then washed and allowed to dry before imagining on a Nikon Eclipse light microscope. A minimum of three pictures was taken of different fields within each transwell replicate at 4X magnification using a Nikon Digital Sight camera. Cell migration was determined by counting the number of crystal violet stained cells on the underside of the transwell apparatus. The level of cell migration under starvation media in room air served as the standard control for all experiments.

3.12  c-Kit-IRG Transgenic Mice and Migration Assay

The cKit^{CreERT2/+}, IRG, Isl1-nLacZ, Wnt1-Cre, tdTomato, Wnt1::Flpe and RC::Fela mice that were employed in this study, have been previously described^{94}. To induce CreER^{T2}-mediated recombination and specific GFP labeling of c-Kit+ cells, tamoxifen (Sigma) was prepared by resuspension in peanut oil (Sigma) at a final concentration of 20 mg/ml by shaking overnight at 37°C. Tamoxifen was then administered via single subcutaneous injections (50 μl/injection) to two to four day old neonatal pups. One-day post injections, hearts were harvested, washed in ice-cold HBSS and cleared of unwanted tissues under a stereomicroscope (VistaVision). These hearts were further processed in a tissue-cultured hood following additional washing steps with DMEM (GIBCO). Heart explants were
created by mincing neonatal hearts into ~2-3 mm³ fragments and digested in a solution of DMEM/F12 (GIBCO), 20% FBS, 1% penicillin/streptomycin and 200 units/ml Collagenase-Type II solution (Worthington) at 37°C. Following two washes with DMEM to remove residual enzyme, digested explants were hand-picked under sterile conditions with a micropipette, and cultured individually upon gelatin-coated 24-well plates. Samples were fed every other day for the one-week culture period with medium consisting of DMEM/F12, 15% FBS (Atlas), 1% penicillin/streptomycin (GIBCO), 1% β-mercaptoethanol (GIBCO), 1000 units/ml recombinant mouse LIF (Millipore), 1 ng/ml recombinant mouse bFGF (Peprotech), 100 ng/ml recombinant murine SCF (Peprotech), and 0.1 mM nonessential amino acids (GIBCO). The number of GFP⁺ cells was counted from pictures taken after one-week of culture based on GFP epifluorescence, under a fluorescent microscope (Olympus IX81).

3.13 Statistical Analysis

The results are expressed as mean ± standard error of the mean (SEM). A p value <0.05 was considered significant. Differences between groups were examined for statistical significance using Student’s t-test or analysis of variance (ANOVA), with Newman-Keuls multiple comparisons test where appropriate for post hoc analyses. Differences within groups are examined by 2 way ANOVA and the Newman-Keuls multiple comparison post hoc test. Experiments were repeated a minimum of three separate times using cells from three or more different donors.
Chapter 4: Physiologic and Hypoxic Oxygen Concentration Differentially Regulate Human c-Kit+ Cardiac Stem Cells

4.1 Human Cardiac Stem Cell Proliferation is Enhanced in Physiologic Oxygen Concentration

To elucidate the effect of O₂ transition on hCSC proliferation, a standard growth curve analysis of 0.5x10⁵ cells was performed for 96 hours. hCSCs grown at room air were transferred to either physiologic (5%) or hypoxic (0.5%) O₂ concentrations⁹⁷. The recorded cell counts demonstrated increased cell proliferation under physiologic O₂ conditions at 72 hours as compared to room air and hypoxia (4.4x10⁵±0.4 cells in physiologic, 3.1x10⁵±0.3 cells in room, and 2.8x10⁵±0.2 cell in hypoxia, P<0.01). Cell proliferation continued to increase at physiologic O₂ over 96 hours as compared to room air or hypoxia (7.6x10⁵±0.6 cells in physiologic, 5.0x10⁵±0.3 cells in room, and 4.3x10⁵±0.4 cell in hypoxia, P<0.001) (Figure 3A).

Figure 3: hCSC proliferation is enhanced in Physiological O₂: (A) Growth curve analysis of hCSCs grown in room, physiologic, and hypoxia O₂ concentration over 96 hours. (B) The calculated cell doubling time of hCSCs grown in room, physiologic, and hypoxia O₂ concentration (n=7). Data is presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001
However, there was no significant difference in proliferation when hCSCs were grown in room air or hypoxia. The calculated doubling time of hCSCs grown in physiological O$_2$ decreased relative to both room air and hypoxic O$_2$ concentrations (23.4±0.7 hours in physiologic, 27.6±1 hours in room, and 29.1± 1.2 hours in hypoxia, P<0.05) (Figure 3B).

Sirtuin1 (Sirt1) is a regulator of cell proliferation that plays a key role in cellular responses to environmental stressors$^{27-29, 98}$. To test the involvement of Sirt1 in the O$_2$ mediated effect on cell proliferation, Sirt1 protein expression was measured at the 96-hour time point. Consistent with the growth curve data, Sirt1 was up regulated in hCSCs cultured at physiological O$_2$ compared to room air (1.375±0.9-fold, P<0.01) and hypoxia O$_2$ concentration (Figure 4). There was no change in SIRT1 expression after growth in hypoxia relative to room O$_2$ (0.85±0.9-fold, P>0.05).

**Figure 4: Sirt1 protein expression increases in hCSC cultured at physiologic O$_2$:**
(A) Western blot analysis of Sirt1 protein expression in hCSC lysates collected after 96 hours of culture in room, physiologic, and hypoxia O$_2$. (B) Densitometry analysis of Sirt1 protein expression relative to the actin loading control (n=13). Data is presented as mean ± SEM and normalized to the room control. **P<0.01, ****P<0.0001.
The requirement of Sirt1 in hCSC proliferation was confirmed by lenti-viral transduction of specific siRNA gene knockdown (KD) under room air conditions. Sirt1 KD significantly decreased the number of hCSCs grown over 96 hours (4.35x10^5±0.3 scrambled control cells and 1.75x10^5±0.2 Sirt1 KD cells, P<0.0001) and increased the doubling time (23.3±0.3 hours for scrambled control and 34.17±0.3 hours for Sirt1 KD, P<0.0001) (Figure 5).

**Figure 5: Sirt1 is required for the preservation of hCSC proliferation:** (A) Growth curve analysis of hCSCs transduced with Sirt1 KD siRNA or the scrambles siRNA control. (B) The calculated cell doubling time of hCSCs transduced with Sirt1 KD siRNA or the scrambles siRNA control. (C) Western blot analysis of Sirt1 protein in hCSCs transduced with Sirt1 KD siRNA or the scrambles siRNA control (n=3). Data is presented as mean ± SEM. ****P<0.0001
4.2 Hypoxia Decreases Senescence and Mitochondrial Content and Increases Glycolysis.

To determine if the decreased hCSC proliferation observed in hypoxia and room air, relative to physiologic O₂, was due to senescence, we tested the expression of the senescence-associated markers beta-galactosidase (β-Gal) and P16INK4a. Detection of β-Gal activity in cell lysates decreased within 96 hours of growth in both physiologic and hypoxic O₂ concentrations but remained unchanged at room air (-2,924±984 fluorescent units in physiologic, P<0.05, -4203±1065 fluorescent units in hypoxia, P<0.05, and -139±155 fluorescent units in room air, P>0.05). Additionally, the level of β-Gal activity at 96 hours relative to room air was significantly lower only in the hypoxia treated group (12272±727 fluorescent units in hypoxia and 15502±841 fluorescent units in room air, P<0.05) (Figure 6).

![Figure 6: Hypoxia decreases β-Gal activity in hCSCs](image)

**Figure 6: Hypoxia decreases β-Gal activity in hCSCs:** The recorded β-Gal activity in hCSC protein lysates after 24 and 96 hours of culture in room, physiologic, and hypoxia O₂ (n=3). Data is presented as mean ± SEM. +P<0.05 within group, *P<0.05 between room control.
Similarly, P16INK4a expression was downregulated after 96 hours of growth in hypoxia (0.58±0.1-fold, P<0.001) but not in physiological oxygen (0.96±0.02-fold, P>0.05) (Figure 7) compared to room air. These findings suggest that the impaired cell proliferation effect observed in room and hypoxic O₂ concentration, relative to physiologic O₂ concentration, is under the regulation of different mechanisms.

Mitochondria are key regulators of stem cell fate commitment, stem cell maintenance, and redox balance. We tested the effect of different O₂ concentrations on hCSC mitochondrial content by transducing hCSCs with a lenti-viral CMV-MT-DsRed-Puro vector for specific and prolonged fluorescent labeling of the mitochondria (Figure 8A). Alterations in total mitochondrial content after 24 and 96 hours of exposure to physiologic and hypoxic O₂ were assessed by flow cytometry analysis of the DsRed

**Figure 7: P16 protein expression deceases in hypoxia:** Western blot analysis of P16 protein expression in hCSC lysates collected after 96 hours of culture in room, physiologic, and hypoxia O₂ and densitometry analysis of P16 protein expression relative to the actin loading control (n=6). Data is presented as mean ± SEM and normalized to the room control. ***P<0.001
mean fluorescent intensity (MFI). We found that hypoxic O$_2$ reduced the total MFI of mitochondria after 24 hours (0.75±0.1-fold, P<0.05), with the greatest reduction at 96 hours (0.18±0.03-fold, P<0.0001), as compared to the room O$_2$ group. The transfer from room air to physiological O$_2$ did not significantly decrease mitochondrial MFI after 24 hours (0.94±0.05-fold, P>0.05), but did have a decreasing effect over the 96 hours time course (0.6±0.2-fold, P<0.05) (Figure 8 B-D). Together these results confirm a role of O$_2$ in the maintenance and abundance of total mitochondria.

Figure 8: Total mitochondrial content decreases in hypoxia: (A) Fluorescent images of hCSCs transduced with CMV-MT-DsRed-Puro vector after 96 hours of culture in room, physiologic, and hypoxia O$_2$. (B) The flow cytometry analysis of the MFI of pDSRed2-Mitro transduced hCSCs after 24 hours of culture in O$_2$ concentrations. (C) The flow cytometry analysis of the mean fluorescent intensity of transduced hCSCs after 96 hours of culture in O$_2$ concentrations. (D) The calculated fold change in MFI (n=3). Data is presented as mean ± SEM and normalized to the room control. *P<0.05, **P<0.01, ****P<0.0001
hCSC anaerobic metabolism was also measured by recording glycolytic activity via supernatant L-lactate detection. The concentration of supernatant L-lactate was elevated after growth in both physiological (1.23±0.08 mM, P<0.01) and hypoxic (1.13±0.09 mM P<0.05) O₂ concentrations relative to room air (0.9±0.05 mM), demonstrating the expected increase in glycolysis activity and metabolic adaptation to decreased O₂ concentrations (Figure 9).

![Figure 9: Physiologic and hypoxia O₂ concentrations upregulate L-Lactate production](image)

*Figure 9: Physiologic and hypoxia O₂ concentrations upregulate L-Lactate production: The concentration of L-Lactate detected in hCSC supernatants collected after 96 hour cultures in room, physiologic, and hypoxia O₂ concentration (n=6). Data is presented as mean ± SEM. *P<0.05, **P<0.01*

### 4.3 Human Cardiac Stem Cell Sensitivity to Oxidative Stress is Similar in all O₂ Concentrations

Cell survival post transplantation may be the most critical contributor to successful cell therapy. To test the impact of O₂ concentration on cell sensitivity to apoptosis, hCSCs from various donors were cultured in room air, physiologic, and hypoxic O₂ concentrations, stimulated with the pro-apoptotic agent hydrogen peroxide, and analyzed for the induction of apoptosis via positive Annexin V staining. Flow
cytometry analysis recorded the percentage of early apoptotic cells by gating cells based on Annexin V positivity and propidium iodine (PI) negativity. Late apoptotic or necrotic cells were identified as Annexin V positive and PI positive cells (Figure 10A). A similar percentage of apoptotic hCSCs resulted in all O₂ concentrations, relative to the full media control, 3 hours post hydrogen peroxide stress (4.9±0.8% in full media control, 15.89±0.9% in room O₂, 16.24±1.1% in physiologic O₂, and 20.82±2.9% in hypoxic O₂) (Figure 10B). Additionally, O₂ concentration did not influence the hydrogen peroxide-induced necrosis (3.58±0.7% in full media control, 11.49±2.3% in room O₂, 13.34±2.7% in physiologic O₂, and 11.78±2.5% in hypoxic O₂) (Figure 10C).

Figure 10: O₂ concentration does not affect hCSC apoptosis sensitivity.
(A) Flow cytometry analysis of Annexin V and PI stained hCSCs 3 hours post hydrogen peroxide stress in room, physiologic, and hypoxia O₂ concentrations. (B) The percentage of early apoptotic (AnnexinV+/PI-) in all treatments. (C) The percentage of late apoptotic/necrotic (AnnexinV+/PI+) in all treatments (n=9). Data is presented as mean ± SEM.
4.4 Human Cardiac Stem Cell Migration is Enhanced in Physiological Oxygen Concentration

To study the effect of O$_2$ concentrations on hCSC migration, a transwell migration assay was used. Positive cell migration was recorded by counting crystal violet stained cells on the underside of the trans-well apparatus. Under starvation media conditions, there was a basal level of hCSC migration within 24 hours that became enhanced under physiological O$_2$ conditions (2.40±0.13-Fold, P<0.0001) and unchanged in hypoxia (1.11±0.15-fold, P>0.05) relative to room O$_2$ concentration (Figure 11 A, C). MSCs promote chemotactic and proliferative responses in CSC via the SDF1/CXCR4 pathway\textsuperscript{102}. Therefore, to investigate the potential use of MSCs to enhance CSC migration under hypoxic and room air conditions, CSCs were exposed to stimulus from human MSCs or hCSCs under similar starvation media conditions. Upon addition of the human MSCs, the migration of hCSCs was enhanced in hypoxic O$_2$ to a level comparable to physiologic migration (Figure 11 B, D). A similar trend for MSC-improved migration was found in room air. There was no effect found with the addition of hCSCs. RNA analysis of pro-migratory genes showed that the transfer of CSCs to hypoxia caused a significant reduction in both SDF-1 (0.57±0.09-fold, P<0.01) and CXCR4 (0.52±0.12-fold, P<0.05) gene transcripts relative to room air O$_2$. However, there was no significant difference in SDF-1 (0.99±0.11-fold, P>0.05) or CXCR4 (0.71±0.14-fold, P>0.05) expression in hCSCs in the physiological O$_2$ group relative to room air O$_2$ (Figure 12).
Figure 11: Physiological O₂ enhances hCSC migration. (A) Representative images of transwell migrated hCSCs stained with crystal violet under room, physiologic, and hypoxia O₂ concentrations. (B) Representative images of migrated hCSC in hypoxia with CSC or MSC stimulus. (C) The fold change of migratory hCSCs under various O₂ concentrations. (D) The fold change of migratory hCSCs under various O₂ concentrations with CSC or MSC stimulus (n=4). Data is presented as mean ± SEM and as fold change to room control. *P<0.05

Figure 12: Hypoxia downregulates migratory genes in hCSCs: (A) RNA expression of SDF-1 after 24 hour culture in room, physiologic and hypoxia O₂ concentrations. (B) RNA expression of CXCR4 after 24 hour culture in room, physiologic and hypoxia O₂ concentrations (n=4). Data is represented as fold change relative to the room air control after normalization to the housekeeping gene GAPDH Data is presented as mean ± SEM. *P<0.05
4.5  c-Kit+ Migration from Murine Neonatal Explants is Enhanced in Physiologic Oxygen

These findings were further confirmed via the analysis of c-Kit+/GFP+ CSC migration from neonatal murine cardiac explants. The murine model used ensures specific and accurate GFP labeling of c-Kit positive cells within the tissue after tamoxifen treatment. Migration of GFP-positive cells was then monitored over a one-week culture period and a significantly elevated c-Kit+ CSC migration was observed in cultures grown in physiological $O_2$ (20.0±1.3 cells, $P<0.05$) compared to room air (2.22±1.3 cells) and hypoxic $O_2$ (5.33±2.92 cells)(Figure 13 A, B).

Figure 13: $O_2$-dependent effect of CSC migration from neonatal murine explants: (A) Representative images of migrated GFP+/c-Kit+ CSCs from neonatal murine explants after a one week culture in room, physiologic, or hypoxia $O_2$ concentrations. (B) The total number of migratory cells in each $O_2$ concentration (n=9). Data is presented as mean ± SEM. *$P<0.05$
Gene expression analysis also showed a decrease in SDF-1 mRNA in hypoxic samples (0.47±0.09-fold, P<0.001) relative to room air, but no significant difference in CXCR4 expression (1.3±0.45-fold, P>0.05) (Figure 14). Gene expression of SDF-1 and CXCR4 were also similar between room air and physiological O₂ groups (1.10±0.13-fold and 1.81±0.50-fold, respectively, P>0.05).

**Figure 14:** Hypoxia downregulated SDF-1 in murine neonatal explants: (A) RNA expression of SDF-1 after 1 week of culture in room, physiologic and hypoxia O₂ concentrations (n=9). (B) RNA expression of CXCR4 after 1 week of culture in room, physiologic and hypoxia O₂ concentrations (n=9). Data is represented as fold change relative to the room air control after normalization to the housekeeping gene GAPDH. Data is presented as mean ± SEM. ***P<0.001, ****P<0.0001
Chapter 5: Hypoxia Induced Quiescence is Associated with Decreased SIRT1 and c-Myc Protein Stability in Murine Cardiac Stem Cells

5.1 Hypoxic Stress Reduces Murine Cardiac Stem Cell Proliferation

mCSCs were used to investigate potential mechanisms of hypoxic regulation of CSC induced proliferation and quiescence. First, to replicate findings in hCSCs, mCSC were grown in room, physiologic, or hypoxic O₂. Significant reduction in mCSC proliferation was observed in hypoxic O₂ over 96 hours. Cell counts were decreased in hypoxia (4.38x10⁵±0.72 cells/ml, P<0.0001) relative to both room (13.31x10⁵±1.64 cells/ml) and physiological (13.45x10⁵±3.77 cells/ml) (Figure 15A). There was no difference in mCSC growth between physiologic and room O₂ conditions. Analysis of BrdU incorporation after 72 hours of O₂ exposure confirms this result, BrdU incorporation decreased in hypoxia relative to room air (0.519±0.1-fold, P<0.01) while remaining unchanged in physiologic O₂ (1.27±0.11-fold) (Figure 15B).

Figure 15: Hypoxia impairs mCSC proliferation: (A) Growth curve analysis of mCSCs grown under room, physiological, and hypoxic O₂ concentrations over a 96 hour culture (n=3). (B) The calculated fold change of incorporated BrdU in mCSC grown under room, physiological, and hypoxic O₂ concentrations for 72 hours (n=4). Data is represented as mean ±SEM and fold change is normalized to the room control. **P<0.01, ****P<0.0001
5.2 Hypoxia Induces a Quiescent Phenotype in Murine Cardiac Stem Cells

In general, cell types respond to stress by accumulating senescence, undergoing apoptosis, or initiating quiescence. The quantification of senescence-associated β-gal activity in mCSC protein lysates after 96 hours of exposure to hypoxia shows that reduction in mCSC proliferation is not associated with the accumulation of senescence. β-gal activity was similar after 24 hours in room O\textsubscript{2} (8095±594 fluorescent units) and hypoxic O\textsubscript{2} (7836±460 fluorescent units), however was higher after 96 hours in room O\textsubscript{2} (11152±427 fluorescent units, P<0.05) compared to hypoxic O\textsubscript{2} (8353±738 fluorescent units) (Figure 16A). Alternatively, we considered quiescence as a potential cause of proliferation impairment in hypoxia. Thus, mCSCs were cultured under hypoxia for 3 days and then transferred back to control room O\textsubscript{2} conditions for an additional 3 days. Our results show reversible growth impairment after 3 days of hypoxic stress. Cell number after reoxygenation at day 6 was higher (27.11x10\textsuperscript{5}±2.5 cells, P<0.0001) than continuous hypoxia (20.78x10\textsuperscript{5}±1.47 cells), however both reoxygenation and hypoxia groups remained lower than the room O\textsubscript{2} control (37.85x10\textsuperscript{5}±2.73 cells, P<0.0001) (Figure 16B).

Importantly, we show that the reduction in mCSC growth is not associated with increased induction of cell death. Surprisingly, hypoxia treatment significantly reduced the accumulation of cytotoxicity and necrotic cell death by 27.06±4.31% (P<0.01), as shown by reduced detection of accumulated supernatant lactate dehydrogenase over 72 hours (Figure 17A). Similarly, we recorded a significant induction of the pro-survival AKT pathway in hypoxia. Western blot analysis shows an elevation of phospho-AKT
(Ser473) after 5 and 10 minutes of media stimulation of starved cells in hypoxia relative to normoxic control (Figure 17B,C).

Figure 16: Hypoxia promotes a quiescence phenotype in mCSCs: (A) The fluorescence of senescence-associated β-gal activity in mCSC after 24 and 96 hours of culture in room and hypoxia O₂ concentration (n=4). (B) The 6-day growth curve analysis of mCSCs cultured under room, continuous hypoxia, and reoxygenation treatments (n=7). Data is represented as mean ±SEM. *P<0.05 between groups, +P<0.05 between groups.
Figure 17: Hypoxia promotes a pro-survival phenotype in mCSCs: (A) The measured absorbance level of LDH detection in mCSC supernatants after 72 hours of growth in room and Hypoxia O$_2$ (n=3). (B) Western blot analysis of P-AKT and total-AKT 5 and 10 minutes after serum starvation and media stimulation in room and hypoxia O$_2$. Tubulin is used as a protein loading control (n=5). (C) Densitometry analysis of the ratio of P-AKT to total-AKT at observed time points in room and hypoxia. Data is represented as mean ±SEM. *P<0.05, **P<0.01
5.3 Hypoxic Induced Impairment in Murine Cardiac Stem Cell Proliferation is Associated with Reduced Sirt1 and c-Myc Expression

In order to establish a mechanism of hypoxia induced quiescence in mCSCs, we investigated the expression levels of two proteins involved in cell cycle regulation and stress response, Sirt1 and c-Myc. Time-course analysis of Sirt1 in mCSC exposed to hypoxic stress revealed a downregulation of Sirt1 protein expression after 72 hours (0.57±0.1-fold, P<0.05). This effect persisted up to 96 hours (0.53±0.01-fold, P<0.001) (Figure 18A). Specific Sirt1 KD confirmed the role of Sirt1 in the preservation of mCSC proliferation. mCSC transduced with the Sirt1 KD lenti-virus had decreased incorporation of BrdU (0.66±0.8-fold, P<0.05) relative to the NS scrambled control (1.00±0.05-fold). Western Blot analysis confirms the successful KD of Sirt1 (Figure 18B,C).

![Figure 18: Hypoxia downregulates Sirt1 expression in mCSCs:](image)

(A) Western blot of a time course analysis of Sirt1 expression over 96 hours of culture in room or hypoxia O2. Actin is used as a protein loading control (n=4). (B) Western blot analysis of Sirt1 expression post transduction with NS scrambled control or Sirt1 KD. (C) Analysis of BrdU incorporation in mCSCs transduced with NS scrambled control or Sirt1 KD (n=3). Data is represented as mean ±SEM. *P<0.05
A similar time course analysis of c-Myc expression showed a significant and persistent decline after 48 hours in hypoxia (Figure 19A). Treatment of mCSCs with the specific c-Myc inhibitor 10058-F4 confirms c-Myc as another mediator of sustainable proliferation. c-Myc inhibited mCSCs had significantly reduced proliferation as demonstrated by a higher concentration of CellTrace proliferation dye fluorescence compared to vehicle control (1007.25±194.02 MFI vs. 439.75±104.93 MFI, P<0.05) (Figure 19B,C).

Figure 19: Hypoxia downregulates c-Myc expression in mCSCs: (A) Western blot of a time course analysis of c-Myc expression over 96 hours of culture in room or hypoxia O₂. Actin is used as a protein loading control (n=4). (B) CellTrace proliferation assessment by flow cytometry of baseline mCSCs and mCSCs after 72 hours of treatment with c-Myc inhibitor or DMSO control (n=4). (C) The recorded MFI of CellTrace dyed mCSCs after 72 hours of treatment with c-Myc inhibitor or DMSO control. Data is represented as mean ±SEM. *P<0.05
Further analysis of the change in Sirt1 and c-Myc expression over time confirms a high correlation of protein expression with change in cell growth. Graphical depiction of the fold change in protein expression and the fold change in proliferation rate over time in hypoxia shows a similar pattern of downregulation (Figure 20). A linear regression of these two parameters confirms a significant positive correlation of c-Myc and a near significant correlation of Sirt1 protein with cell growth in hypoxia (r=0.986, P<0.01 for c-Myc correlations and r=0.857, P=0.074 for Sirt1).

Figure 20: Hypoxic induced change in SIRT1 and c-Myc expression correlates with change in cell proliferation: (A) The correlation of SIRT1 expression change with mCSC proliferation change in hypoxia (n=4). (B) The correlation of c-Myc expression change with mCSC proliferation change in hypoxia (n=4). Drawn lines represent linear regression calculated for protein and proliferation changes over time. R-values were calculated from correlation of protein change to proliferation change.
Although distinct in cellular function, Sirt1 and c-Myc interact with each other and target similar downstream protein and gene targets. Thus as expected, we found that downstream molecular targets of Sirt1 and c-Myc are altered in response to hypoxic stress. As a histone deacetylase, Sirt1 targets and reduces acetylation residues on both histones and transcription factor proteins. We found that exposure to only the hypoxic 0.5% O₂ concentration increased the ratio of histone H3 acetylation at Lysine 9 relative to the loading control actin (1.44 ±0.11, P<0.05) (Figure 21A). Histone H3 lys9 is a published target of Sirt1\(^{103}\). This effect was not seen after transition to the other test oxygen concentrations, 10% and 5% O₂ (0.78 ±0.12 and 0.77 ±0.16 respectively). Furthermore, the ratio of acetylated P53 to total P53, a transcription factor target of Sirt1, was increased after 72 hours of exposure to hypoxia relative to room O₂ control (0.24 ±0.006 vs. 0.15 ±0.02 respectively, P<0.001) (Figure 21B).

**Figure 21: Hypoxia increased acetylation of Sirt1 targets:** (A) Western blot analysis of acH3 (Lys9) after 24 hours in 21%, 10%, 5%, and 0.5% O₂ and the calculated ratio of expression to the loading control Actin (n=3). (B) Western Blot analysis of acP53, total p53, and the calculated ratio of acP53 to total P53 after 72 hours of culture in room and hypoxia O₂ (n=4). Data is represented as mean ±SEM. *P<0.05, **P<0.01
Lastly, we analyzed expression levels of P21, a well-characterized mediator of cell cycle progression and downstream target of Sirt1 and c-Myc. Protein expression analysis revealed an increase (149.30±41.33%, P<0.01) of P21 expression in hypoxia (Figure 22A). Treatment of mCSC with the c-Myc inhibitor confirmed P21 as a downstream target of c-Myc. Expression of P21 mRNA increased (1.91±0.27-fold, P<0.05) 24 hours post inhibitor treatment (Figure 22B). Additionally, we found Sirt1 mRNA expression to be under regulation of c-Myc activity as treatment with the c-Myc inhibitor significantly downregulated Sirt1 mRNA (0.84±0.05-fold, P<0.05) after 48 hours (Figure 22C).

**Figure 22: Hypoxia O₂ concentration increases expression of the c-Myc target P21:** (A) Western blot analysis of P21 expression in mCSCs after 96 hours of culture in room and hypoxia O₂ and the densitometry analysis of the ratio of P21 expression relative to the loading control actin (n=5). (B) The expression of P21 mRNA 24 hours post c-Myc inhibitor treatment relative to the DMSO treated control (n=4). (C) The expression of Sirt1 mRNA 48 hours post c-Myc inhibitor treatment relative to the DMSO treated control (n=4). Data is represented as mean ±SEM. *P<0.05, **P<0.01, ***P<0.001
5.4 Hypoxic Stress Decreases c-Myc and Sirt1 Protein Stability

Analysis of c-Myc mRNA abundance showed that the hypoxia induced decrease in c-Myc protein expression was not associated with changes in c-Myc gene transcription (Figure 23A). Thus, we investigated c-Myc protein stability by exposing mCSC to hypoxic stress for 48 hours, the time point in which c-Myc is significantly down regulated, and treating with the protein synthesis inhibitor cycloheximide. Our results show that hypoxic stress accelerated c-Myc protein degradation (Figure 23B). After 30 minutes of cycloheximide treatment, the total expression of c-Myc decreased by 60.79±5.48% (P<0.05) in hypoxia compared to 47.75±11.58% in room oxygen (Figure 23C).

Figure 23: c-Myc protein stability is impaired in hypoxia: (A) Time course analysis of c-Myc mRNA and protein expression in hypoxia relative to room O₂ control. (B) Western blot analysis showing c-Myc protein degradation up to 50 minutes post cyclohexamidine treatment in room and hypoxic O₂. (C) c-Myc degradation rate under room (closed circles) and hypoxia (open circles) relative to baseline (n=9). Data is represented as mean ±SEM. *P<0.05
A similar analysis was done regarding the hypoxia-induced decline in Sirt1 expression. Conversely, we found both Sirt1 mRNA and protein stability to be negatively effected at the 72 hour time point of hypoxic stress. SIRT1 mRNA was downregulated in hypoxia after 72 hours in hypoxia (0.44±0.001-fold, P<0.05) (Figure 24 A). Similarly, the treatment of mCSC with cyclohexamide after 72 hours, the time point in which SIRT1 expression is significantly downregulated, showed an accelerated degradation of SIRT1 protein over the 8 hour test period in hypoxia relative to the room O2 control (0.5±0.05-fold in hypoxia vs. 1.2±0.15-fold in room O2 control, P<0.05) (Figure 24 B, C).

**Figure 24: SIRT1 mRNA and protein stability is impaired in hypoxia:** A: The fold change of Sirt1 mRNA in hypoxia relative to room O2 control (n=4). B: Western Blot analysis of Sirt1 expression post cyclohexamide treatment in room and hypoxic O2 (n=4). C: SIRT1 degradation rate under room (closed circles) and hypoxia (open circles) relative to baseline, prior to cycloheximide treatment (n=6). Data is represented as mean ±SEM. *P<0.05
Chapter 6: Discussion: Oxygen Concentration Modulates CSC Function

The primary findings of this study demonstrate a major role of O$_2$ concentration in the regulation of CSC biology, particularly in the balance of stem cell senescence and quiescence. This effect has downstream effects on CSC mechanisms of proliferation and cell migration. In stem cell based therapies, cell products are first isolated and expanded from tissues in a hyperoxic (21% O$_2$), \textit{in vitro} culture environment prior to transplantation back into tissues$^{104}$. Although donor age, patient health status, and feasibility of cell maintenance in culture are known to influence the potency of delivered cells, minimal information has been reported concerning the effect of lower O$_2$ concentrations on human CSC regenerative potential$^{105-107}$. The objective of this study was to identify basic \textit{in vitro} effects of O$_2$ concentration on parameters of CSC biology and identify novel mechanism that impact the therapeutic effectiveness of stem cell therapy. Thus, our result that the transfer of human CSCs to physiologic (5%) O$_2$ provides superior benefit to their proliferative and migratory ability provides novel insight into potential improvements in cell preparation techniques and regulatory mechanisms that affect survival and cellular integration post transplantation into the injured myocardium. Secondarily, our results suggest that 5% O$_2$ is an optimal \textit{in vitro} condition for CSC cell preparation and may enhance bioprocessing of potent CSC cell lines.

6.1 Oxygen Concentration Regulates CSC Proliferation

O$_2$ has been a well-established regulator of stem cell proliferation from within and outside the stem cell niche$^{42,108}$. Typically, hypoxic stem cell niches promote reversible
cell cycle arrest or quiescence while maintaining a non-differentiated state\textsuperscript{109}. However, to what degree hypoxia has an effect on cell proliferation and survival seems to vary based on cell type and origin\textsuperscript{108}. Mild hypoxic conditions that mimic the natural physiologic environment, 3-5\% O\textsubscript{2}, has proven to provide superior benefits for the culture of various stem cell types. Interestingly, the culture of MSCs under a physiologic 3\% O\textsubscript{2} concentration increases the rate of self-renewal while significantly decreasing rates of spontaneous differentiation, apoptosis, and senescence\textsuperscript{88}. These results have guided further research into the use of hypoxia as a preconditioning tool to further enhance stem cell function prior to transplantation into experimental models of ischemic cardiomyopathies\textsuperscript{86,110-112}. However, in most pre-clinical and clinical trials, stem cells prepared for therapy are expanded at a level of O\textsubscript{2} far greater than that of the physiological tissue. Thus, transplantation introduces various degrees of hypoxic stress depending upon tissue viability and perfusion. Therefore, we tested the effect of cell transition to two different degrees of hypoxia relative to standard laboratory room air that are comparable to a physiological and ischemic/hypoxic environment\textsuperscript{85}.

A limited number of studies have addressed the impact of O\textsubscript{2} concentration on exogenously isolated CSCs. To date, these studies report consistent findings with the previously known effects of hypoxic cultures on stem cells. The culture of CSCs at moderate hypoxia, O\textsubscript{2} > 2\%, is beneficially to cell proliferation and stem cell maintenance\textsuperscript{113, 114}. However, these early studies do not address issues that are highlighted in our study. CSC cell therapy currently uses autologous cell products where cell lines are developed from individual patient. Thus, it is important to study CSCs derived from ill, elderly patients as opposed to neonatal derived CSCs, as these are the
cells being used in clinical trials. Moreover, most studies focus on only one O\textsubscript{2} concentration in which is considered hypoxia. It is important to note that most “hypoxic” studies are done at levels of O\textsubscript{2} more closely to that of “physiologic” O\textsubscript{2}. Although an O\textsubscript{2} concentration of 2-5% may be hypoxic compared to room air conditions, it is actually physiological growth conditions for the normal healthy heart. Thus, CSC function in 2-5% O\textsubscript{2} may be an adaptation back to physiologic O\textsubscript{2} where as O\textsubscript{2} less than 1% mimics a response to that of ischemic stress\textsuperscript{85}. In this study, we test two levels of decreased O\textsubscript{2} concentration to mimic both cardiac physiologic oxygen and ischemic stress.

Studies have investigated the effect of hypoxic niche formation on CSC function \textit{in vivo}. As the heart ages, microenvironments regulating stem cell niches change and intracellular oxygen concentration decreases. This decrease in oxygen tension promotes CSCs to preserve and accumulate a slow-dividing, uncommitted stem cell phenotype that can be reversed via the treatment of growth factors such as SCF. Interestingly, this effect reduced the number of senescent CSCs without altering mitochondrial volume, a major player in stem cell fate regulation\textsuperscript{115}. In all, it is suggested that the diminished regenerative potential of the aged heart is in part due to this accumulation of in-active CSCs. Fortunately, the development of a quiescent phenotype, rather than senescent, allows for the possibility of cell cycle re-entry and stem cell activation. This is particularly good news for stem cell therapies designed to re-stimulate the endogenous population of CSC in the heart. However, it is important to note that this published study does not offer any molecular targets or pathways that may be regulated in response to the decrease in oxygen concentration. Thus, the identification of molecular switches that
control the in-active and active states of CSC could potentially lead to further understanding of what is required for CSC stimulation in hypoxic stress.

Our initial experimental findings show an enhanced proliferation rate of those hCSCs grown in physiologic versus hypoxic O₂. However, no significant difference was found comparing hCSCs grown at room air versus hypoxia. Interestingly, there appears to be a “sweet spot” in O₂ concentration gradient that favors cell expansion. This finding is reflective of the concept that the expansions of hCSCs in an unnatural hyperoxic or hypoxic condition are equally stressful. Additionally, this finding is consistent with that of other who found that low O₂ promotes CSC proliferation. However, it is clear that there is a threshold or minimum O₂ concentration that is optimal for hCSC self-renewal. Our hypoxic O₂ concentration proved to be inhibitory to hCSC proliferation relative to physiologic O₂. Combining our observations with those of others, it can be concluded that hCSC self-renewal is optimal at an O₂ concentration of 2%-5%[111, 112]. As O₂ concentration drops below this point, the stimulatory effect of “hypoxia” is lost.

Further insight into the effect of oxygen on CSC proliferation was achieved via the study of decreased O₂ concentration on mCSCs. In contrast to our results with hCSCs, hypoxic O₂ decreased mCSC proliferation relative to both room and physiological O₂. However, there was no change in growth rate between room and physiological O₂. Interestingly, mCSC did not seem to benefit from the transfer from room to physiological O₂, suggesting that growth at room and physiological O₂ concentration are both optimal. The impaired cell growth in hypoxia, however, is consistent in both hCSCs and mCSCs. Together, the cell growth analysis confirms an inhibitory effect of hypoxia on CSC proliferation.
Few studies have tested the long-term effect of hypoxic O₂ (0.5%) on parameters of CSC biology. Typically, models utilizing these low levels of hypoxic O₂ are designed to investigate the effects of hypoxic preconditioning. In these models, CSCs develop beneficial effects after short-term exposure to hypoxia. In fact, this effect has been shown to be time-dependent, as CSC lost the benefits associated with hypoxic preconditioning when hypoxic exposure extended over 12 hours \(^{86}\). In many cases, long-term exposure to hypoxic stress promotes apoptosis and cell death in stem cells \(^{37,87}\).

Our study shows that hypoxia imposes a growth restricting stress upon CSCs that is independent from the induction of cell death. Thus, to characterize hypoxic growth impairment, we tested for the expression of senescence-associated markers. Notably, although cell proliferation rate was similarly decreased in room air and hypoxia relative to physiologic O₂, the differences in senescence marker expression suggest that O₂ concentration regulates hCSC growth through different pathways. The elevated levels β-galactosidase activity and P16INK4a in room O₂ indicate a greater population of replicative senescence CSCs, while a decrease in these markers suggests a decrease of a senescent phenotype in hypoxia. Similarly, mCSC growth restriction in hypoxia was not paired with an increased in β-galactosidase activity. Therefore, it can be concluded that hypoxia does not causes CSC senescence. Alternatively, hypoxic exposure promoted phenotypes associated with cellular quiescence.

### 6.2 Hypoxia Promotes a Cardiac Stem Cell Quiescent Phenotype

Evidence that growth in hypoxia leads to a quiescent phenotype is shown by the measurement of mitochondrial content and glycolytic activity. As the respiration centers
of the cell, an increase in mitochondrial biogenesis and elevated rate of oxygen consumption has been linked to stem cell differentiation, reprogramming, and ROS induced replicative senescence. On the other hand, decreased levels correlate with increased stemness and self-renewal\textsuperscript{115,116}. Thus, our observation that hypoxic O\textsubscript{2} concentration promotes decreased mitochondrial content, elevated glycolysis, and decreased senescence-associates markers in hCSCs, relative to the room air and physiological oxygen groups, provides strong evidence of hypoxia-induced quiescence.

Supplementary findings with mCSC further demonstrate a quiescent induced growth arrest. Unlike senescence, quiescent growth arrest is reversible and permits re-cell cycle re-entry. As expected, we found hypoxic induced growth arrest to be reversible upon re-oxygenation back to room oxygen. Quiescence also works to preserve stem cell function through the activation of survival pathways\textsuperscript{117}. In addition to a decrease in cytotoxicity induced cell death, as measured by supernatant LDH detection, we found that hypoxia activated the pro-survival AKT pathway. The increased phosphorylation of AKT and subsequent activation of survival pathways has been shown to be a critical aspect of hypoxic induced effects on stem cells\textsuperscript{87,88,110}. Together, these finding concurs with previously published data concerning hypoxic regulation of stem cell biology. The hypoxia induced reduction in mitochondrial respiration, switch to glycolysis, and activation of AKT survival pathways in CSCs may serve as an adaptive mechanism that allows the preservation of a slow growth state, free of oxidative stress damage, and ultimately reducing replicative senescence in low O\textsubscript{2}. 
6.3 Hypoxia does not Increase Cardiac Stem Cell Sensitivity to Hydrogen Peroxide

An immediate obstacle that CSCs transplanted into the heart must overcome is survival from oxidative damage\textsuperscript{72,118}. The ability of CSCs to resist cell death is a critical component of stem cell therapy that needs to be improved\textsuperscript{119}. It has been reported by Hong et al that the vast majority of intracoronary infused CSCs do not survive in the heart longer than 24 hours\textsuperscript{72}. Therefore, we tested the short-term effect of transfer to lower O\textsubscript{2} concentration on cell apoptosis and survival. As previously stated, we did not find an induction in cell death by simply transferring CSC to hypoxia. Thus, we sought to test the sensitivity of CSCs to free radical stress. We transferred hCSCs to physiologic and hypoxic O\textsubscript{2} concentration and treated with the pro-apoptotic agent hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). Although there was not a consistent effect of O\textsubscript{2} on H\textsubscript{2}O\textsubscript{2}-induced apoptosis and necrosis, those CSCs transferred to hypoxia did have a trend that suggests a possible increase in sensitivity to apoptotic stress. This non-significant result is likely due to a great deal of variation in CSC batch sensitivity to H\textsubscript{2}O\textsubscript{2}. CSC isolated from various donors responded differently to the 3-hour treatment of H\textsubscript{2}O\textsubscript{2}. In some cases, apoptosis induction was high, in others necrosis was high and apoptosis was low, while others had a low apoptotic response (data not shown). Variable responses are common in autologous therapies. Since the CSCs are derived from different patients with different comorbidities and ages, experiments with variability may require the inclusion of a greater number of donor batches.
6.4 Oxygen Concentration Regulates Cardiac Stem Cell Migration

CSCs need to migrate and integrate into the tissue in order to sufficiently contribute to the repair of the injured myocardium\textsuperscript{120}. As expected, we found an $O_2$-dependent regulation of CSC migration. Similar to the effect on cell proliferation, we found physiologic $O_2$ concentration enhanced CSC migration relative to both room air and hypoxic $O_2$ concentrations. Importantly, CSC migration and proliferation is influenced by the secretion and expression of the pro-migratory proteins SDF-1 and CXCR4\textsuperscript{102, 121}. The similar expression levels of SDF-1 and CXCR4 mRNA in CSCs exposed to room air and physiological $O_2$, however, do not offer an explanation for enhanced migration. Rather, it can be hypothesized that similar pathways, perhaps related to senescence, regulate physiological oxygen’s positive effect on proliferation and migration relative to room air\textsuperscript{122}. On the other hand, the placement of CSCs in hypoxia did reduce the transcription levels of SDF-1 and CXCR4, suggesting a potential mechanism underlying the impaired migration at this $O_2$ concentration.

We further sought to rescue the defect in migration of CSCs by introducing conditioned MSC media. MSCs secrete many soluble factors, including SDF-1, and positively regulate CSC proliferation and migration\textsuperscript{62, 102, 123}. MSCs also play a major stromal cell support role in the maintenance and regulation of HSC function in the bone marrow niche. Thus, we hypothesized that the combination of MSC would provide a supportive role that could improve hCSC function in the hypoxic environment. Our result was consistent, indicating that MSCs promote CSC migration under hypoxic conditions. This finding further strengthens the argument that a combination of CSCs with MSCs may provide superior benefit in cell regeneration\textsuperscript{95, 124}. 
Although our findings contrast with that of others who found that hypoxic preconditioning enhances murine CSC migration and tissue integration, there are important differences in study methodology that may account for this discrepancy. In general, studies have reported that short-term activation of HIF-1a-regulated targets by hypoxic preconditioning activates numerous pro-survival and pro-migratory pathways that enhance stem cell function upon transplantation. In our model, we test the migratory ability of standard, room air cultured CSCs over 24 hours of exposure to physiologic or hypoxic O₂. We do find that reducing O₂ concentration below that of room air enhances CSC migration, however this benefit is lost with the extreme low levels that are considered hypoxia (0.5% O₂). We believe that these differences may be due to the studied cell type (murine vs. human). Human CSCs isolated from patients’ endomyocardial biopsies introduce several variability factors, such as cell source conditions and patient co-morbidities. These conditions, as well as species differences, may account for the decreased migratory function in extreme hypoxic stress that this study found in human CSCs compared to studies with murine derived CSCs.

### 6.5 Physiological Oxygen is Optimal for Cardiac Stem Cell Cultures

The finding that physiological O₂ concentration enhances CSC migration was further confirmed using a murine model that utilized the accurate and specific labeling of c-Kit+ CSCs with genetic GFP recombination. With this model, migrating CSCs from cardiac explants are tracked in cell culture. We found a greater number of migratory CSCs moving out of neonatal mouse cardiac explants in physiological O₂ relative to both hypoxia and room air.
This finding, along with our proliferation and senescence results, has direct implications on the process in which CSCs are isolated and expanded from patient endomyocardial biopsies \textit{in vitro}. CSCs are a small population of cells within the heart, making it challenging to isolate and expand a large number of cells in a short period of time prior to transplantation. As primary cell lines, the sensitivity of CSC to develop replicative senescence in room air $O_2$ also imposes problems when cell line sustainability. Our data indicates that culturing endomyocardial explants and isolated CSCs \textit{in vitro} under physiological $O_2$ improves this challenge. Currently, CSC preparations are all done in room air conditions. However, our data proves physiological $O_2$ enhances the number of migratory CSCs from explants. This effect increases the number of cells available for magnetic sorting, reduces senescence, and promotes a more rapid proliferation and expansion. Standardizing CSC preparation to be done in physiological $O_2$ may improve bioprocessing for use in clinical trials.

\section*{6.6 Summary of the Effect of Oxygen Concentration on Human and Murine Cardiac Stem Cells}

Exposing both hCSCs and mCSCs to hypoxic oxygen promotes the formation of a quiescent phenotype. Together, we observed a decreased in proliferation rate, senescence-associated markers P16 and $\beta$-gal, migration and migratory signaling pathway SDF1/CXCR4, and total mitochondrial content while accompanied by an increased in pro-survival pathways (Figure 25). The optimal CSC functional phenotype (maximal proliferative and migratory phenotype) was promoted by exposure to physiologic $O_2$ for hCSCs and exposure to both physiologic and room $O_2$ for mCSCs. A fundamental difference between the two species of CSCs is that the high room $O_2$ induced similar
adverse effects to hCSC proliferation and migration as hypoxic stress but did not in mCSCs. Based on the molecular survey of senescence associated markers and migratory pathways, room O₂ impedes hCSC function via senescence rather than quiescence. Interestingly, mCSCs are not affected by the inhibitory effect of room O₂. The expansion of stem cells in room O₂ concentrations impairs function via the accumulation of toxic ROS stress. Specifically, MSCs grown at room air O₂ concentration have compromised colony-formation capacity through the promotion of ROS induced senescence\textsuperscript{127,128}. The difference observed at room O₂ are likely due to the differences in CSC sensitivity to the development of senescence. Differences in senescence sensitivity may be attributed to the fact that hCSCs are from sick patients versus “healthy” mice or an inherit difference between species. mCSC may have greater protection against replicative senescence via protein expression of antioxidants or other protective signaling pathways. Although minor differences exist between the two species of CSCs, we can conclude that hypoxic O₂ induces a quiescent phenotype in CSCs and either mCSCs or hCSCs can be used to study a relevant molecular regulation of quiescence in hypoxic stress.
Figure 25: The effect of O\textsubscript{2} concentration on CSC quiescence and senescence: (Top) The summarized data of the effect of O\textsubscript{2} concentration on hCSC function. (Bottom) The summarized data of the effect of O\textsubscript{2} concentration on mCSC function.
Chapter 7: Discussion: Oxygen concentration modulates Sirt1 and c-Myc

A hampered CSC response caused by hypoxia-induced quiescence likely contributes to inadequate cardiac regeneration in an ischemic injury. Although this hypoxic response has been previously suggested in CSCs and is common in other stem cell population, a molecular regulation of this response has yet to be established in CSCs. Identifying the molecular control switch that regulates hypoxia induced quiescence is important when considering potential therapeutics designed to reactivate endogenous CSC regeneration in ischemic disease. In this study, we sought to identify novel regulatory pathways induced by hypoxic stress that promote CSC quiescence and render the stem cells incapable of mounting a regenerative response. We found that the stress response proteins Sirt1 and c-Myc were downregulated in hypoxia via elevated protein degradation. This result points to Sirt1 and c-Myc mediated pathways as novel targets for potential re-stimulation of CSC function in hypoxia.

7.1 Oxygen Concentration Regulates Sirt1 Protein Expression.

We tested the expression level of the cell cycle regulator Sirt1 in CSCs exposed to hypoxic stress. Acting as a NAD+ dependent histone deacetylase, Sirt1 is a well-established target for cellular “longevity” through its role in cellular adaptation to stress and protection against non-reversible cell cycle arrest or senescence. Accordingly, we found that Sirt1 is necessary for the preservation of CSC proliferation and that Sirt1 protein expression is regulated by O2 concentration. In fact, the upregulation of Sirt1 in hCSCs at physiologic O2 suggests a potential mechanism that favors optimal cell proliferation at this O2 concentration. We found a similar downregulation of Sirt1 protein...
expression in CSC exposed to hypoxia. It is important to note that a change in Sirt1 expression tightly correlates with the change in proliferation rate in CSCs. Sirt1 is found to be expressed the highest in highly proliferation CSCs. In settings where proliferation impairment is observed, hypoxic and room O$_2$ for hCSCs and hypoxic O$_2$ for mCSCs, Sirt1 expression is decreased. The association of Sirt1 expression and cell cycle progression has been widely characterized in multiple cell types. Particularly in stem cells, Sirt1 plays a pivotal role in protection against senescence-associated growth arrest and ROS-mediated stress. During stem cell differentiation, Sirt1 expression is often downregulated in response to decreased self-renewal and proliferation$^{129}$. Our time course analysis of Sirt1 expression shows a downregulation of Sirt1 protein after 72 hours of hypoxic stress in mCSCs. The late effect of Sirt1 downregulation post transfer to hypoxic stress suggests that Sirt1 is not the cause but rather a contributor to the sustained proliferation impairment.

7.2 Hypoxia Induces an Increased Acetylation of Sirt1 Targets

The presence or absence of Sirt1 protein is not the only factor that directly dictates a change in Sirt1 mediated signaling. Sirt1 requires the presence of the NAD$^+$ substrate for its deacetylation reaction to occur. Thus, Sirt1 activity must be confirmed by the analysis of the acetylation status of direct Sirt1 protein targets. Sirt1 targets both histones and signaling proteins within the nucleus and cell cytoplasm. We found that histone H3 (lys9) acetylation increased as early as 24 hours post transfer to hypoxia. Histone deacetylation directly effects gene transcription via the silencing of promoter of gene encoding sequencings. A downregulation of Sirt1 activity is likely part of the
hypoxic response to activate the hypoxic gene milieu. This effect is further amplified by the activation and upregulation of histone acetyltransferase P300 in hypoxia cardiomyocytes\textsuperscript{130}. Although the direct effect of Histone H3 (lys9) acetylation on CSC function and gene transcription is unknown, it is used here to establish a dysregulation in Sirt1 mediated histone deacetylation. This effect shows an early downregulation of Sirt1 activity that occurs prior to the downregulation of SIRT1 protein expression.

In addition to histone acetylation, Sirt1 targets and manipulates the function of a number of transcription factors and signaling proteins. The tumor suppressor and apoptosis regulator P53 is a well-characterized transcription factor target of Sirt1 whose function is directly regulated by acetylation. In general, transcription factor proteins contain DNA binding domains or other regulatory regions that are altered upon the addition of acetylation to lysine residues. This alteration of a protein motif can mediate changes in gene targeting or protein complex formation. For example, P53 acetylation promotes protein phosphorylation, subsequent DNA binding, and increased expression of growth inhibitory genes\textsuperscript{131}. Thus, P53 activity and transcriptional activation is expected to increase upon downregulation of Sirt1. We measured the ratio of acetylated P53 to total P53 after 72 hours of exposure to hypoxia and found an increased proportion of acetylated P53. Although this data does not provide concrete evidence for the role of P53 in the CSC hypoxic response, this result does confirms our result that both Sirt1 expression and activity are decreased in hypoxia.
7.3 **Oxygen Concentration Regulates c-Myc Protein Expression.**

The transcription factor c-Myc has been recognized for its role in stem cell proliferation, differentiation, and its function as a stress sensor\textsuperscript{53, 132}. c-Myc protein expression is tightly regulated in response to different injury models including acute myocardial infarction\textsuperscript{133-137}. Therefore, we sought to investigate the role of c-Myc in the hypoxic response. Using time course analysis of c-Myc protein expression, we found 48 hours of exposure to hypoxia significantly induced prolonged downregulation of c-Myc expression in mCSCs. Treating test cells with the specific c-Myc inhibitor 10058-f4 produced a deficiency in sustained proliferation that confirmed the role of c-Myc in the preservation of mCSC proliferation. Additionally, we found that hypoxia induced change in c-Myc expression directly correlated with that hypoxia induced growth impairment. Together, our findings support the notion that c-Myc downregulation promotes the formation of CSC quiescence.

Several reports have shown that c-Myc plays an important role in maintaining the quiescence state of different cell types, including hematopoietic stem/progenitor cells, via decreased c-Myc expression and the downstream upregulation of cell cycle inhibitor p21\textsuperscript{138, 139}. In fact, P21 expression is essential for the maintenance of a cellular quiescent state\textsuperscript{140}. Accordingly, we confirmed that c-Myc inhibition resulted in elevated P21 expression and found an upregulation of P21 protein expression 96 hours after hypoxic stress.
7.4 Hypoxic stress Impairs Sirt1 and c-Myc Protein Stability.

A major contributor to cellular adaptation to hypoxic stress is the activation of hypoxic response genes via the stabilization of the HIF-transcription factor family and changes in histone acetylation. Thus, HIF mediated protein expression changes are often accompanied by alterations in gene transcription and RNA expression. Both c-Myc and Sirt1 are reported targets of HIF-1α stabilization and are subsequently regulated in various conditions of hypoxic stress\textsuperscript{141}. Accordingly, to further characterize the mechanism of hypoxic downregulation of c-Myc and Sirt1 signaling, we tested expression levels of c-Myc and Sirt1 mRNA. Again using time course analysis, we found no change in c-Myc mRNA in hypoxia. This result suggests c-Myc expression is regulated by a HIF-independent mechanism. Aside from genetic regulation, stress activated proteins, such as mitogen-activated kinases, extracellular kinase 1/2 (ERK) or c-JUN N-terminal kinase (JNK), and glycogen synthase kinase 3 (GSK-3β), control c-Myc protein stability and tightly regulate c-Myc expression\textsuperscript{142, 143}. We found that hypoxia promotes c-Myc downregulation by a mechanism that involves elevated protein degradation. Although the precise mechanisms contributing to decreased c-Myc stability remains unknown, it is hypothesized that the alteration of c-Myc post-translational modification leads to an enhanced protein degradation cascade involving ubiquitination. In the context of stem cell quiescence, suppression of c-Myc by increased activity of the E3 ubiquitin ligase Fbxw7 in hypoxia promotes cell cycle dormancy\textsuperscript{144}. Our results fit with this finding that decreased c-Myc stability is a promoting factor in the establishment of a quiescent cell phenotype in hypoxia.
In contrast to c-Myc, we found both Sirt1 mRNA and protein stability to decrease after 72 hours in hypoxia. Interestingly, reports have connected Sirt1 protein expression as a downstream target of c-Myc and have tightly linked the two molecules in the establishment of positive feed back mechanisms that positively regulate cell proliferation\(^{55,145}\). These reports are further supported by our time course analysis that shows a significant downregulation of c-Myc occurring before a downregulation of Sirt1 in addition to a decrease in Sirt1 mRNA in c-Myc inhibitor treated mCSCs. The downregulation of Sirt1 by decreased expression of c-Myc, in part, offers an explanation for hypoxic induced downregulation of Sirt1 signaling. However, the observed defect in Sirt1 protein stability in hypoxia is a novel finding induced by an unknown mechanism. Interestingly, similar to c-Myc, Sirt1 protein stability is impaired upon activation of the stress response protein JNK\(^{146}\). A similar mechanism of elevated protein degradation in response to hypoxic stress is likely responsible for the observed decrease and enhanced depletion of c-Myc and Sirt1 protein.

### 7.5 Proposed Pathway of Hypoxic Induced Quiescence in Cardiac Stem Cells

In summary, we have shown that exposure to hypoxia induces a quiescent phenotype that correlates with decreased c-Myc protein stability and protein expression. The inhibition of c-Myc causes dysregulation of c-Myc downstream protein targets and results in decreased Sirt1 expression and elevated P21 (Figure 26). Together, this response contributes to the development of the impaired proliferation observed in hypoxia. These findings offer a novel and mechanistic explanation for the blunted CSC
regenerative response reported in ischemic injury and outline potential therapeutic protein targets for the rejuvenation of CSC response.

Figure 26: Proposed pathway regulating hypoxia induced quiescence: In physiological O₂, a functional phenotype (proliferative and migratory cell phenotype) is achieved via active c-Myc and Sirt1 signaling and suppression of p21. In hypoxia O₂, c-Myc degradation contributes to a downregulation in Sirt1 signaling and an upregulation of P21.
Chapter 8: Closing Remarks

CSCs have proven to be a promising cell type for use in stem cell therapies designed to treat cardiac disease. However, various constraints limit the therapeutic effectiveness of both the endogenous and exogenously delivered CSCs. This study provides novel insight into the effect of O$_2$ concentration on parameters of CSC function.

The finding that the transfer of CSCs from room to physiological O$_2$ induces beneficial effects on proliferation and migration has implications for both cell therapy and cell bioprocessing. The characterization of a quiescent phenotype upon transfer to a hypoxic environment may in part explain a blunted CSC mediated regenerative response. This finding further validates the transplantation of CSCs into the viable border zone of an ischemic injury, where O$_2$ concentration is at a physiological level. We have previously shown that co-administration of CSCs with MSCs provide superior benefit to CSC cell function and overall cardiac repair$^{62,95}$. Consistent with those findings, we show here that MSCs promote the migration of CSCs in hypoxia, supporting the notion that MSCs may serve as a viable option for reducing hypoxic induced quiescence and promoting CSC regeneration. Furthermore, CSC bioprocessing is currently performed in room air culture conditions prior to cell transplantation. Our finding that physiological O$_2$ concentration increases expansion of c-Kit+ selectable cells from cardiac explants, reduces CSC senescence, and enhances migratory ability suggests that the isolation and expansion of CSCs at physiological O$_2$ concentration may offer a better therapeutic option for cell preparation.

The identification of molecular pathways involved in hypoxic regulation of CSC is essential when considering therapeutic approaches designed to re-activate the
endogenous regenerative potential of the heart. This study identifies c-Myc as a molecular control switch that mediates hypoxia-induced quiescence in CSCs. Our finding that hypoxic stress promotes degradation of c-Myc protein expression and the subsequent downregulation of Sirt1 and upregulation of P21 offers novel molecular targets that may reverse the buffered CSC regenerative response in hypoxia.

Future studies are needed to further strengthen the concepts established here. Particularly, hCSC processing should be done at physiological O$_2$ and tested in a mouse or pig animal model for increased potency, cardiac retention, and overall induction of cardiac repair. Although further characterization of the effect of cell culture expansion in physiological O$_2$ on parameters of CSC differentiation and specific stem cell properties would be interesting, greater value would come from practical experiments that tested the functional benefits achieved from the physiological O$_2$ growth conditions. Additionally, it is important to test the potential use of therapeutic c-Myc and Sirt1 targeting strategies to enhance CSC function in hypoxia. Overexpression or gene activating techniques should be used to reverse the dilatory effects of hypoxia on c-Myc and Sirt1 expression. Alternative strategies could also be created to target P21, as this protein is believed to play a major role in quiescence establishment. Lastly, the findings in this study offer a very basic overview of the mechanistic changes that occur upon exposure of CSCs to a hypoxic environment. Thus, a continuation and further development of the presented concepts is warranted to enhance CSC regeneration in ischemic injuries to ultimately enhance the clinical benefit of CSC therapies.
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