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PRIMING CARDIOVASCULAR STEM CELLS FOR TRANSPLANTATION USING SHORT- TERM HYPOXIA

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PRIMING CARDIOVASCULAR STEM CELLS FOR TRANSPLANTATION
USING SHORT-TERM HYPOXIA

A Thesis
Presented to the
Faculty of
California State University,
San Bernardino

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
in
Biology

by
Ivan Hernandez
June 2016

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ABSTRACT

Conventional medical treatments fail to address the underlying problems associated with the damage inflicted by a coronary event. Thus, the long-term prognosis of patients admitted for heart failure is disheartening, with reported survival rates of 25 percent. Recent advances in stem cell research highlight the potential benefits of autologous stem cell transplantation for stimulating repair in heart tissue. However, a majority of those suffering from cardiovascular diseases are older adults whose autologous cells no longer possess optimum functional capacity. Additional work is needed to identify the optimal cell types or conditions that will promote cardiovascular regeneration across all age groups. A pretreatment, such as short-term hypoxia, and concurrent implementation of a novel progenitor, such as those that co-express Isl-1 and c-Kit, may enhance the results reported in clinical trials completed to date. However, the effects of short-term hypoxia in this novel cell type are unknown and warrant investigation *in vitro*.

Cloned adult and neonatal Isl-1+ c-Kit+ human cardiovascular progenitor cells were characterized and expanded for study. Populations from both age groups were preconditioned using short-term hypoxia (1% O₂ for six hours) and, to identify shifts in gene expression, compared to their respective control (21% O₂ at 37 °C) via qRT-PCR. Flow cytometry and western blot analysis was utilized to measure phosphorylation of Akt. Progression through the cell cycle was also

analyzed by flow cytometry. Cellular function was evaluated by the use of a TUNEL assay and Transwell® invasion assay.

Hypoxia-mediated alterations of a genetic or functional nature in Isl-1+ c-Kit+ human cardiac progenitors are clearly age-dependent. Although both age groups accrued benefit, the neonatal progenitors procured significantly greater improvements. Short-term hypoxia significantly elevated Akt phosphorylation in neonatal Isl-1+ c-Kit+ human cardiac progenitors. Benefits afforded to both age groups by hypoxic pretreatment included significant upregulation of pro-survival transcripts, and enhanced invasion capabilities *in vitro*.

Therefore, prior to transplantation, hypoxic preconditioning may improve the ability of transplanted stem cells to home towards damaged areas of the heart and support cardiac regeneration *in vivo*.

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CHAPTER ONE

INTRODUCTION

Background

Etiology and Global Impact of Cardiovascular Diseases

Cardiovascular diseases (CVDs) encompass a suite of diseases that affect the heart and its vessels, the most common disease being coronary artery disease (CAD). CAD is typified by atherogenesis (plaque accretion) and subsequent lesion formation, both stenotic and non-stenotic, within the coronary artery. Stenotic lesions innately restrict blood flow by growing towards the lumen, but tend to have smaller lipid cores and thick fibrous caps that are not very susceptible to rupture (Libby & Theroux, 2005). On the other hand, a non-stenotic lesion develops abluminally and, therefore, does not inherently affect coronary circulation until the constitutive lipid-rich core and thin fibrous cap are disturbed, initiating coronary thrombosis and occlusion (Libby & Theroux, 2005).

Despite our comprehensive understanding regarding the etiology of CVDs, they remain the number one cause of death across the globe – accounting for approximately one out of every three deaths worldwide (Deaton et al., 2011; Mozaffarian et al., 2015). Additionally, patients hospitalized for heart failure typically develop permanent scarring of the heart and significant loss of cardiovascular function. Traditional treatments fail to address this issue and, as a

result, the long-term outlook of these patients is abysmal, with five-year survival rates lower than that of most cancer patients (Stewart, MacIntyre, Hole, Capewell, & McMurray, 2001). Public health efforts to curb the prevalence of CVDs have been largely focused on raising awareness of the many risk factors that are strongly correlated to the development of CVDs. Indeed, CVDs are highly preventable. Eliminating major CVD risk factors, which are mainly a result of poor lifestyle behaviors, results in up to a 90 percent lower lifetime CVD risk (McGill, McMahan, & Gidding, 2008). Unsurprisingly, as public awareness and use of evidence-based medical therapies for secondary prevention increased, the number of deaths in the United States attributed to CVDs (from 2001 to 2011) decreased by 30.8 percent, yet remained alarmingly high at approximately 600,000 deaths per year (Mozaffarian et al., 2015). Therefore, while much progress has been made through raising of public awareness, the extreme burden of CVDs on worldwide human health will likely continue to persist lest novel treatments are developed that can repair the damaged caused by infarction and significantly lessen the associated mortality.

Cardiovascular Stem Cell-based Therapy for the Injured Heart

Although cardiac stem cells were known to be prevalent within the neonate heart, the notion that these cells steadily diminish and senesce into adulthood was widely accepted and, thus, the adult human heart was designated a terminally differentiated organ with no regenerative capabilities. With little evidence to suggest otherwise, early clinical trials for the treatment of myocardial

injuries primarily focused on bone marrow-derived cells for stimulating cardiovascular repair. However, the discovery that the adult human heart indeed houses populations of stem cells that support its regeneration (Beltrami et al., 2003), prompted investigators to shift their focus onto transplantation of cardiac progenitor cells (CPCs) for the treatment of myocardial infarction.

The potential benefits of endogenous CPC transplantation as a means of stimulating repair in heart tissue are currently under investigation with encouraging results in clinical trials (Bolli et al., 2011; Gerbin & Murry, 2015; Makkar et al., 2012). Phase I clinical research studies completed to date, examining the safety and feasibility of CPC transplantation, have noted little to no adverse effects resulting from cardiovascular infusion of stem cells, and, thus, the procedure is considered safe by clinicians. Most notably, investigators have reported significant reductions in total scar tissue and significant improvements in left ventricular ejection fraction – progressing even after 12 months post-op (Bolli et al., 2011; Makkar et al., 2012). However, it has been demonstrated that, although transplanted CPCs alleviate some cardiovascular dysfunction, they fail to engraft and differentiate into new myocardium (Hong et al., 2014). Therefore, it is believed that the observed reductions in scar size and improvements in cardiac function – typically measured using left ventricular ejection fraction – are a result of paracrine signaling stemming from growth factors secreted by newly transplanted cells (Barile et al., 2014). Furthermore, autologous stem cells

derived from adult patients are known to lack the functional potency of their neonatal counterparts (T. I. Fuentes et al., 2013).

Due to the correlation between incidence of CVDs and age, older adults, by way of sheer numbers, are the group most in need of cell-based cardiac therapy. If adult autologous stem cells are unable to impart significant functional benefits after transplantation, then the overall application of autologous stem cell-based therapies for treatment of cardiovascular injury may be drastically limited to treating neonatal heart diseases. Even so, stem cell-based therapies for the treatment of congenital heart diseases (CHDs) are of significant interest to clinicians. While the number of afflicted patients is relatively small in comparison to those suffering from adult CVD, CHDs occur in roughly one percent of live births worldwide and CHD incidence has increased in recent decades (Marelli, Mackie, Ionescu-Iltu, Rahme, & Pilote, 2007). Indeed, the mortality rate for CHDs is declining and more of those born with CHD are surviving into adulthood. However, data gathered between 2007 and 2010 reveals that CHD-related mortality for neonates is still a cause for concern at 10.1%. In the year 2011 alone, mortality related to CHD in the U.S. was 4900 (Mozaffarian et al., 2015). Thus, CHDs remain responsible for more deaths than any other congenital birth defect. All too often, neonates suffering from a particularly severe heart malformation are in need of a heart transplant. Complete organ transplants are costly, invasive, and, most importantly, limited by the availability of donor organs. Hence, there has been a growing interest in stem cell-based therapies for

treating CHDs. Clinical trials completed to date have established the safety of stem cell transplantation for the treatment of hypoplastic left heart syndrome and other CHDs (Ishigami et al., 2015). However, much like the clinical trials that established the safety of stem cell therapy for CVDs in adults, further work is needed to improve post-operative outcomes.

The effectiveness of cell-based treatments for the heart may be significantly improved by use of a novel cell type or pretreatment method, such as hypoxic exposure, that alters the function of adult-derived CPCs to mirror that of the more capable neonatal stem cells. Any benefits afforded to adult CPCs would also likely be procured by neonatal CPCs. However, additional work is needed to identify these optimal cell types or conditions that will promote autologous stem cell-mediated cardiovascular regeneration in the injured adult and neonatal human heart. To date, several potential preconditioning methods have been evaluated for their ability to augment cellular function. For instance, it has been demonstrated that pretreatment with growth factors enhances the therapeutic efficacy of mesenchymal stem cells for myocardial infarction (Hahn et al., 2008). Additionally, cobalt protoporphyrin pretreatment protects human embryonic stem cell-derived cardiomyocytes from oxidative stress (Luo et al., 2014). However, the benefits afforded by any one preconditioning method may vary depending upon the characteristics of the cell type chosen for therapy. Interestingly, there is a growing body of evidence in support of a master heart progenitor that gives rise to all the cells in the developed adult heart (Kattman,

Huber, & Keller, 2006; Laugwitz, Moretti, Caron, Nakano, & Chien, 2007; Wu et al., 2006). The discovery, characterization, and subsequent implementation of this primordial heart cell would undoubtedly lead to improved patient outcomes following cell therapy. To date, several populations of resident CPCs have been identified within the human heart and are promising candidates for use in future studies (T. Fuentes & Kearns-Jonker, 2013).

Optimizing Cell Type

CPCs are capable of self-renewal, expansion, and differentiation into all three major cell types of the heart (Bu et al., 2009; Moretti et al., 2006). The Isl-1+ c-Kit+ population represents one type of CPC that was initially found exclusively within fetal progenitor populations (Simpson et al., 2012). However, Isl-1+ c-Kit+ hCPCs were subsequently identified within endogenous progenitor populations isolated from human neonatal and adult cardiac tissue, as well as from sheep cardiac tissue, as reported by the Kearns-Jonker laboratory at Loma Linda (T. I. Fuentes et al., 2013; Hou et al., 2012). Isl-1 is a transcription factor that is required early in development for the survival, proliferation, and migration of CPCs into the primordial heart and, as a result, the developed heart is largely a product of Isl-1+ cells (Cai et al., 2003). Specifically, Isl-1+ CPCs play a critical role in early heart formation by contributing to the outflow tract, right atrium, right ventricle, and septum (Cai et al., 2003; Dodou, Verzi, Anderson, Xu, & Black, 2004; Yang et al., 2013). One of the most widely studied progenitor cell types, however, is the c-Kit+ CPC. While current clinical trials using c-Kit+ cells show

promise, the role of c-Kit+ cells in the development and regeneration of the heart remains controversial (Cheng et al., 2014; Ferreira-Martins et al., 2012; Kubo et al., 2008; van Berlo et al., 2014; Zaruba, Soonpaa, Reuter, & Field, 2010). Isl-1+ c-Kit+ CPC populations, by virtue of their distinct protein fingerprint, may be inherently superior to those CPC populations expressing only Isl-1 or only c-Kit, and may be better suited for transplantation into the hypoxic heart. Moreover, this double-positive CPC population may react more strongly to preconditioning methods (aimed at enhancing cell function) when compared to single-positive CPCs. Thus, in order to surpass the results attributed to paracrine effects, and reach the desired levels of cardiovascular repair by direct engraftment, further investigation of Isl-1+ c-Kit+ CPCs is warranted.

Hypoxic Preconditioning

Using other models, previous studies have established that hypoxia treatment can boost cellular function through intracellular signaling pathways (Filippi et al., 2014; Hu et al., 2014; Studer et al., 2000; van Oorschot, Smits, Pardali, Doevendans, & Goumans, 2011; Yan et al., 2012). However, the effects of low oxygen tension on Isl-1+ c-Kit+ hCPC function have yet to be elucidated. Nonetheless, it is well understood that the partial pressure of oxygen in the tissues where CPCs reside, is much lower than in atmospheric air. Theoretically, the maximum amount of oxygen that can be bound by hemoglobin in the alveolar capillaries is approximately 21 percent by volume (mL O₂/100 mL blood) or 159 torr. However, because the rate at which hemoglobin binds and releases oxygen

is limiting, the maximum amount that can be bound per milliliter of blood is much lower, approximately 105 torr. Additionally, Fick's law of diffusion applies at every point of gas exchange, thus further limiting the amount of oxygen that makes its way to the deep tissues of the heart per unit of time. Because of these limitations, by the time oxygen reaches the heart, its partial pressure can be as low as 30 torr (Ivanovic, 2009). Furthermore, the heart houses small microenvironments, or niches, that support cardiac stem cells and play vital roles in the regulation of stem cell function (Li & Xie, 2005). While the tissues of the heart may experience a gradient of partial pressures, the innermost stem cell niches of the heart and their constituent CPCs are consistently hypoxic – with oxygen levels as low as 1.0 percent, approximately 7.6 torr (Kimura & Sadek, 2012; Sanada et al., 2014; Tan et al., 2016).

Accordingly, the impact of short-term hypoxia (six hours at 1.0 percent O₂) on the *in vitro* biology of clonal Isl-1+ c-Kit+ hCPCs must be evaluated for pertinent information that will help optimize future transplant studies within animal models. Historically, in other cell types, Protein kinase B (Akt) expression increases in response to short-term hypoxia (Beitner-Johnson, Rust, Hsieh, & Millhorn, 2001) and is well-known to play vital roles in numerous cell functions including cell survival, proliferation, and chemotaxis (Manning & Cantley, 2007). An improvement in just one of these cellular processes may have a substantial effect on the overall efficacy of stem cell transplantation for cardiovascular therapy. Therefore, the hypothesis that short-term hypoxia upregulates Akt

phosphorylation in Isl-1+ c-Kit+ hCPCs and is correlated with enhanced cell function *in vitro* was tested. The combined benefits of using other progenitor cell types, such as those characterized by Isl-1, and pretreatment to prepare these transplanted cells for the hypoxic environment of the damaged heart, may allow for improved cardiac regeneration *in vivo*.

Definition of Terms

CVD: cardiovascular disease; CAD: coronary artery disease; CPC: cardiac progenitor cell; CHD: congenital heart disease; MEM NEAA: Minimum essential medium non-essential amino acids; DPBS: Dulbecco's phosphate-buffered saline; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; SDF-1 α : Stromal cell-derived factor-1 α ; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TUNEL: Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α ; HSP: Heat shock protein; MW: Molecular weight marker; ROS: Reactive oxygen species.

CHAPTER TWO

MATERIALS AND METHODS

In Vivo Components and Experiments

Isolation and Culture of Isl-1+ c-Kit+ hCPCs

The Institutional Review Board of Loma Linda University approved the protocol for use of tissue that was discarded during cardiovascular surgery, without identifiable private information, for this study with a waiver of informed consent. Isl-1+ c-Kit+ hCPCs were isolated from cardiac tissue as previously described (T. I. Fuentes et al., 2013). Briefly, atrial tissue was cut into small clumps (approximately 1.0 mm³) then enzymatically digested using collagenase (Roche, Indianapolis, IN) at a working concentration of 1.0 mg/mL. The resulting solution was then passed through a 40- μ m cell strainer. Cells were then cloned in a 96-well plate by limiting dilution to a final concentration of 0.8 cells per well to create populations for expansion. Twelve hCPC clones, derived from six distinct donor samples, were used in this study. Clonal hCPC cultures were supplemented with growth media comprised of 10% fetal bovine serum (Thermo Scientific, Waltham, MA), 100 μ g/mL Penicillin-Streptomycin (Life Technologies, Carlsbad, CA), 1.0% minimum essential medium non-essential amino acids solution (MEM NEAA, Cat no. 11120052, Life Technologies, Carlsbad, CA), and

20% endothelial cell growth media (Lonza, Basel, Switzerland) in Medium 199 (Life Technologies, Carlsbad, CA).

Hypoxic Preconditioning

The day before hypoxic pretreatment, Isl-1+ c-Kit+ hCPCs received fresh culture media and, if necessary, were passaged to achieve 80% confluency within 24 hours. Experimental hCPCs were then placed in a Heracell™ 150 tri-gas incubator (Thermo Scientific, Waltham, MA) set to 1.0% O₂, 5.0% CO₂, and 94% N₂ for six hours at 37 °C. Control hCPC conditions were 21% O₂, 5.0% CO₂, and 74% N₂ at 37 °C. Cells were then immediately processed for analysis to avoid prolonged exposure to normoxic conditions.

Transwell® Invasion Assay

Cultrex® basement membrane extract (Trevigen, Gaithersburg, MD) was applied to the upper chamber of a Corning HTS Transwell® plate (8.0-µm pore size, Venlo, Limburg). Isl-1+ c-Kit+ hCPCs were suspended in starvation media composed of 98.5% Iscove's Modified Dulbecco's Medium with GlutaMAX™ (Life Technologies, Carlsbad, CA), 1.0% insulin-transferrin-selenium (Life Technologies, Carlsbad, CA), and 0.5% fetal bovine serum (Thermo Scientific, Waltham, MA) then plated onto the coated wells at a density of 50,000 cells per well. Stromal cell-derived factor-1α (SDF-1α, Life Technologies, Carlsbad, CA), a chemoattractant, was diluted with growth media to a final concentration of 100 ng/mL and administered to the lower chamber. After 48 hours of incubation at 37 °C, the cells in the lower chamber were dissociated, stained with calcein AM (BD

Biosciences, San Jose, CA), and analyzed using an FLx800™ microplate fluorescence reader (BioTek Instruments, Winooski, VT).

In Vitro Assays

Quantitative Reverse Transcription PCR

Isl-1+ c-Kit+ hCPCs were washed with Dulbecco's Phosphate-Buffered Saline (DPBS), then lysed using TRIzol® reagent (Life Technologies, Carlsbad, CA). Total RNA was isolated using the RNeasy® Mini Kit (Qiagen, Venlo, Limburg) and cDNA was prepared with superscript III (Life Technologies, Carlsbad, CA). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in triplicate using Go-Taq® qPCR Mastermix (Promega, Madison, WI). Measurements were recorded using the iCycler iQ™5 PCR Thermal Cycler (Bio-Rad, Hercules, CA). Cycler settings were set to 94 °C for 10 minutes, 94 °C for 15 seconds, 52 - 56 °C (depending on the primer) for 60 seconds, and 72 °C for 30 seconds for a total of 45 cycles. Human primers were created using the National Center for Biotechnology Information Primer-BLAST program as listed in Table 1. Relative gene expression data was analyzed using the comparative C_T method (Schmittgen & Livak, 2008).

Table 1. Primer Sequences Used for qRT-PCR.

PRIMER	SEQUENCE
ACTNB FWD	TTT GAA TGA GCC TTC GTC CCC
ACTNB REV	GGT CTC AAG TCA GTG TAC AGG TAA GC
BCL2 FWD	TGC ACC TGA CGC CCT TCA C
BCL2 REV	AGA CAG CCA GGA GAA ATC AAA CAG
T (Brachyury) FWD	ACT GGA TGA AGG CTC CCG TCT CCT T
T (Brachyury) REV	CCA AGG CTG GAC CAA TTG TCA TGG G
HMOX1 FWD	CTC TCG AGC GTC CTC A
HMOX1 REV	TTG AGC ACC TGG CCC CCA GA
HSP40 FWD	TTT TCG GAG GGT CCA ACC CCT
HSP40 REV	TCT TGT TTG AGG CGG GAT GGC C
HSP70 FWD	TGA CCA AGA TGA AGG AGA TCG
HSP70 REV	GTC AAA GAT GAG CAC GTT GC
HSP90 FWD	GGA TTT GAG GGG AAG A
HSP90 REV	TGA GCT TTC ATG ATT C
POU5F1 (Oct-4) FWD	AAC CTG GAG TTT GTG CCA GGG TTT
POU5F1 (Oct-4) REV	TGA ACT TCA CCT TCC CTC CAA CCA
PDGFRA FWD	GCG CAA TCT GGA CAC TGG GA
PDGFRA REV	ATG GGG TAC TGC CAG CTC AC
PIK3CA FWD	AAC AAT GCC TCC ACG ACC AT
PIK3CA REV	TCA CGG TTG CCT ACT GGT TC
RELA FWD	GCG AGA GGA GCA CAG ATA CC
RELA REV	GGG GTT GTT GTT GGT CTG GA

Western Blotting

Protein immunoblots for Akt and phosphorylated Akt were prepared using protein from normoxic hCPCs and hCPCs exposed to 6 hours of hypoxia (1.0% O₂). Additionally, blots for β -Actin and p-Akt were prepared using samples obtained from untreated normoxic controls, normoxic hCPCs treated with stromal cell-derived factor-1 α (SDF-1 α), and hCPCs treated with both SDF-1 α and hypoxia. Following 18 hours of serum deprivation, hCPCs were stimulated using

10 µg/mL of SDF-1 α (Biolegend, San Diego, CA). Hypoxic groups were exposed to low oxygen conditions during the final six hours of the 18-hour starvation period. All protein lysates were loaded into a 12% Tris-glycine pre-cast gel (Thermo Scientific, Waltham, MA), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). After blocking with 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween-20 (TBST), membranes were labeled with mouse anti-human β -Actin antibody (2F1-1) (1:500 dilution, Biolegend, San Diego, CA), rabbit anti-human Akt monoclonal antibody (pan) (C67E7) (1:500 dilution Cell Signaling Technology, Danvers, MA), or rabbit anti-human Phospho-Akt monoclonal antibody (S473) (D9E) (1:300 dilution, Cell Signaling Technology, Danvers, MA) overnight at 4 °C with agitation. The following day, membranes were washed and labeled using either an IRDye® 680RD-conjugated goat anti-mouse antibody (1:5000 dilution, LI-COR, Lincoln, NE) or an IRDye® 800CW-conjugated goat anti-rabbit antibody (1:5000 dilution, LI-COR, Lincoln, NE) in 5.0% BSA in TBST for 60 minutes at room temperature. Final protein levels were visualized using an Odyssey® infrared imaging system (LI-COR, Lincoln, NE) model 9120. Resulting protein bands were analyzed using ImageJ software.

Flow Cytometry

Progenitor cell populations were fluorescently labeled with antibodies as recommended by their respective manufacturers then analyzed using a MACSQuant® analyzer (Miltenyi Biotec, Auburn, CA). Quantification of data was performed using FlowJo software (Ashland, OR). Small particulate matter, dead cells, and gas-bubbles were excluded from final analysis using forward-scatter and side-scatter data.

Antibodies Used in Cytometry Experiments

Antibodies used for cytometric analysis include: Anti-Isl-1 (1H9) mouse monoclonal antibody, (1:50 dilution, Abcam, Cambridge, MA), Anti-c-Kit Rat IgG2b Kappa monoclonal antibody (2B8) conjugated to Dylight 650 (0.5 mg/mL, Novus Biologicals, Littleton, CO), Anti-Akt phospho (Serine 473) rabbit Ig polyclonal antibody (0.23 mg/mL, Biolegend, San Diego, CA), Fluorescein-anti-BrdU (PRB-1) monoclonal antibody (1:20 dilution, Phoenix Flow Systems, San Diego, CA), FITC goat anti-mouse IgG polyclonal antibody (1:25 dilution, Southern Biotech, Birmingham, AL), PE goat anti-mouse IgG polyclonal antibody (1:100 dilution, Southern Biotech, Birmingham, AL), and FITC goat anti-rabbit IgG polyclonal antibody (1:50 dilution, BD Biosciences, San Jose, CA).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

Isl-1+ c-Kit+ hCPCs were rinsed with DPBS and treated with a solution of 0.5 mM H₂O₂ in culture media for 21 hours to induce cell death (Clément, Ponton, & Pervaiz, 1998). Terminal deoxynucleotidyl transferase deoxyuridine

triphosphate nick-end labeling (TUNEL) assay was performed following the manufacturer's recommendations. Briefly, cells were counted using trypan blue and concentrated to 10^6 cells/mL. The concentrated progenitor cell solution was then re-suspended in 1.0 mL of 1% paraformaldehyde in DPBS, placed on ice for 30 minutes, then reconstituted into 1.0 mL of 70% ethanol for overnight incubation at -20 °C. The next day, the cells were labeled with Br-dUTP (Phoenix Flow Systems, San Diego, CA) and re-suspended in antibody solution containing the Fluorescein anti-BrdU antibody (Phoenix Flow Systems, San Diego, CA). Population analysis was performed using flow cytometry.

Cell Cycle Analysis

Isl-1+ c-Kit+ hCPCs at 80% confluency were trypsinized, counted, and concentrated to 2.5×10^5 cells per 0.3 mL DPBS. Ice-cold 70% ethanol (0.7 mL) was added drop-wise to fix the cells then stored at -20 °C overnight. The following day, cells were incubated at 37 °C for 1 hour with RNase A (0.5 mg/mL, Life Technologies, Carlsbad, CA). Propidium Iodide solution (0.5 mg/mL) was added, and the resulting cell solution was analyzed using a MACSQuant® analyzer (Miltenyi Biotec, Auburn, CA). Cytometer data was quantified using FlowJo software (Ashland, OR).

Statistical Analysis

Data was reported as mean +/- standard error. Error bars were designed using propagation of error and the Cousineau method (Morey, 2008). P values < 0.05 were deemed significant.

CHAPTER THREE

RESULTS

Akt Activation in Isl-1+ c-Kit+ hCPCs

Hypoxic Preconditioning Stimulates the Akt Pathway in Isl-1+ c-Kit+ hCPCs

Akt, a master regulator of numerous genes their corresponding proteins, oversees various critical cell processes such as apoptosis, proliferation, and chemotaxis (Manning & Cantley, 2007). To determine the effect of short-term hypoxia on the Akt pathway within Isl-1+ c-Kit+ hCPCs, clonal populations previously characterized by my laboratory colleagues (T. I. Fuentes et al., 2013), expressing Isl-1, c-Kit, Brachyury, Oct-4, and Platelet-derived growth factor receptor- α (PDGFRA, Fig.1A, B), were selected for experimentation. Enhanced Akt phosphorylation is of main interest for its potential benefit in autologous hCPC-based therapy for myocardial infarction in older adults. To determine whether the hypoxia-mediated activation of Akt is significantly influenced by the age of the cell donor, expression of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (PIK3CA), which is known to directly activate Akt (Baba et al., 2011), was measured via qRT-PCR in both control and experimental cells. Data analysis revealed that pretreated cells, regardless of age group, expressed significantly higher levels of PIK3CA mRNA when compared to non-treated, normoxic, Isl-1+ c-Kit+ hCPCs. Moreover, the data also shows that neonatal

hCPCs more strongly upregulate PIK3CA mRNA than do adult hCPCs and may lead to superior Akt activation in neonatal populations (Fig. 2A). Additionally, antibody labeling of activated Akt was measured by flow cytometry in adult and neonatal hCPC clones (Fig. 2B). Comparative analysis of the flow cytometry data gathered against phosphorylated Akt further highlights the distinction between the hypoxia-mediated activation of Akt in adult and neonatal Isl-1+ c-Kit+ hCPCs. While p-Akt expression indeed trends upwards in preconditioned adult clones, only the neonatal clones procured statistically significant elevations in Akt activation after preconditioning (Fig. 2C). However, the promise of short-term hypoxia preconditioning in adult Isl-1+ c-Kit+ hCPCs is illustrated via protein immunoblots (Fig. 3A). Quantification of the imaged protein from a single representative adult clone shows that phosphorylation of Akt can be promoted by exposure to short-term hypoxia (Fig. 3B). Moreover, serum starved adult Isl-1+ c-Kit+ hCPCs show a significant decrease in phosphorylated Akt after stimulation with SDF-1 α (Fig. 3C, D). However, when serum deprivation is conducted in tandem with short-term hypoxia and followed up with SDF-1 α treatment, adult Isl-1+ c-Kit+ hCPC p-Akt protein levels are rescued to levels at or above normoxic control, but not to a statistically significant degree (Fig. 3D).

Isl-1+ c-Kit+ hCPC Function After Exposure to Short-Term Hypoxia

Isl-1+ c-Kit+ hCPCs Invade More Readily After Exposure to Short-term Hypoxia

Hypoxia has also been shown to influence cellular motility (Filippi et al., 2014; van Oorschot et al., 2011; Yan et al., 2012). To determine if short-term hypoxia has any effect on Isl-1+ c-Kit+ hCPC motility, and to validate the selection of the six-hour exposure time, the chemotactic response to SDF-1 α amongst non-treated and pretreated hCPCs was compared using a Transwell® invasion assay. A time course experiment was performed in which a representative clone was pretreated with hypoxia for 3 hours, 6 hours, and 18 hours for comparison of invasion capacities with normoxic control. Over the same 48-hour incubation period, the six-hour hypoxia-pretreated group most efficiently invaded through the basement membrane layer and into the receiver well (Fig. 4A). Using the six-hour time point across 10 different biological replicates, a statistically significant difference was noted between non-treated hCPCs and pretreated hCPCs. The hCPCs preconditioned with six hours of hypoxia invaded through the basement membrane extract and transwell pores in significantly greater numbers than the non-treated hCPCs (Fig. 4B). However, when the data is analyzed by age group, it is clear the adult hCPCs do not respond as vigorously to the hypoxia pretreatment. A trend towards enhanced invasion is present, but this trend is not significant. Only in the neonatal hCPCs did we observe a statistically significant hypoxia-mediated improvement in chemotactic response to SDF-1 α (Fig. 4C).

Short-term Hypoxia Triggers a Pro-survival Response in Isl-1+ c-Kit+ hCPCs

In other cell types, hypoxic pretreatment has been shown to improve survival (Hu et al., 2014; Yan et al., 2012). To determine the effect of short-term hypoxia on Isl-1+ c-Kit+ hCPCs, the expression profiles of several heat-shock proteins in control and experimental populations were examined using qRT-PCR. Data analysis confirmed that pretreated hCPCs indeed express higher levels of select heat-shock protein (HSP) mRNAs (Fig. 5A, B). However, only HSP70 is significantly upregulated by hypoxic preconditioning and only in neonatal clones (Fig. 5B). Subsequent electrophoresis of PCR products confirmed the upregulation of HSP70 in neonatal Isl-1+ c-Kit+ hCPCs via hypoxia preconditioning (Fig. 5C). Further gene expression analysis indicated that short-term hypoxia not only induces a stress response but also significantly upregulates the transcription of genes associated with cell survival (Fig. 6A, B). However, only the neonatal group exhibited a significant change – an approximate twofold increase in the RELA gene transcript, known for its role in the regulation of apoptosis (Beg & Baltimore, 1996). If the data for both age groups is pooled, a threefold increase of Hemoxygenase 1 (HMOX1) is also noted ($p = 0.04$, $n = 6$). HMOX1 plays a role in protecting the cells from oxidative damage (Poss & Tonegawa, 1997). Within a six-hour timeframe, we found that the hypoxia-induced modifications to the pro-survival gene program resulted in fewer apoptotic cells in response to oxidative stress (Fig. 7A) However, quantification of results obtained from three independent adult clones revealed

little change in apoptotic response (Fig. 7B). TUNEL assay results from three individual neonatal hCPC populations reveal a downward trend in the number of apoptotic cells in response to H₂O₂ (Fig. 7C).

Hypoxia-pretreated Isl-1+ c-Kit+ hCPCs Remain Undifferentiated

To evaluate the effects of short-term hypoxia on differentiation in Isl-1+ c-Kit+ hCPCs, the expression of several differentiation markers in control and experimental hCPCs was evaluated using qRT-PCR. Quantification of data revealed no significant difference between pretreated and non-treated adult hCPCs in two out of three markers examined (Fig. 8A). Only PDGFRA, present in sub-populations of progenitors with superior regenerative capacity (Hidaka et al., 2010; J. Kim et al., 2010) was significantly altered, upregulated 1.4-fold ($p = 0.004$). In neonatal clones, a significant downregulation of Brachyury and upregulation of PDGFRA was noted (Fig. 8B). The Brachyury gene transcript is directly linked to the regulation of MESP1 (Robert David et al., 2011) a protein coding gene that promotes cardiovascular differentiation (R. David et al., 2008). Additionally, it is important to note that expression of the Oct-4 transcription factor remains unchanged in both age groups. Altogether, these findings suggest that short-term hypoxia stimulates neither differentiation nor de-differentiation in Isl-1+ c-Kit+ hCPCs.

Hypoxia Pretreatment Does Not Alter Normal Cell Cycle Progression

Hypoxia is also known to influence the cell cycle (Gardner et al., 2001; Grayson, Zhao, Bunnell, & Ma, 2007; Kook et al., 2008; Koshiji et al., 2004;

Studer et al., 2000; van Oorschot et al., 2011). A more proliferative stem cell population would undoubtedly be beneficial for the repair of damaged myocardium. To determine the effect of short-term hypoxia in regards to Isl-1+ c-Kit+ hCPC proliferation, cell cycle analysis was performed on non-treated and pretreated hCPC populations of both adult and neonatal origin. No significant difference was identified between control and experimental groups (Fig. 9A). Moreover, there was no apparent difference between normoxic and hypoxic groups, whether adult and neonatal, in their progression through the cell cycle (Fig. 9B, C). These results support the conclusion that short-term hypoxia does not alter normal cell cycle progression in Isl-1+ c-Kit+ hCPCs.

CHAPTER FOUR

DISCUSSION

In this study, Isl-1+ c-Kit+ hCPCs were expanded as clonal populations and used as a model to test the hypothesis that short-term hypoxia exposure enhances Isl-1+ c-Kit+ hCPC function *in vitro*. The results presented here show that short-term hypoxia is a feasible and practical pretreatment that benefits neonatal hCPC invasive capabilities *in vitro*.

Recent studies lend credence to the efficacy of cardiac stem cell transplants for the amelioration of cardiac dysfunction in mouse models (Hong et al., 2014; Matsuura et al., 2009). Furthermore, human trials have not only established the safety of autologous cardiac stem cell transplantation but also produced some very encouraging results (Bolli et al., 2011; Makkar et al., 2012). However, in both mice and human studies, improvements in cardiac function – typically measured using left ventricular ejection fraction – are thought to be a result of paracrine signaling stemming from the growth factors secreted by newly transplanted cells (Barile et al., 2014; Hong et al., 2014). Additionally, the functional capabilities of hCPCs are known to vary greatly between age groups with neonatal hCPCs consistently outperforming their adult counterparts (T. I. Fuentes et al., 2013). However, the incidence of cardiovascular disease is most prevalent within older adult hearts that are populated by functionally inferior

hCPC populations. Hence, administration of autologous hCPCs for treatment of the majority of cardiac injuries is limited, stimulates repair primarily by means other than direct engraftment, and leaves much to be desired. Cell-based therapies for the human heart – of all ages – may be augmented via the use of a novel cell type or pretreatment method, such as hypoxic exposure. If preconditioning indeed improves hCPC function, behavior of adult-derived clones could be selectively improved to reflect that of their functionally superior neonatal counterparts. However, CPC populations express distinct protein signatures that play an important role in overall cellular function and merit serious consideration when selecting cell populations for therapy. While the c-Kit⁺ CPC remains the most widely studied and implemented, the application of a novel Isl-1⁺ c-Kit⁺ hCPC population may aid in the effort to optimize autologous cell-based therapies for regeneration of the heart.

The idea that preconditioning cells prior to transplantation may benefit donor cell function *in vivo*, has gained a significant following in recent years (Hahn et al., 2008; Hu et al., 2014; Luo et al., 2014; Rosenblum et al., 2014; van Oorschot et al., 2011; Yan et al., 2012). However, although this research is gaining momentum, the genetic analysis and functional assays presented in this study have never been performed on Isl-1⁺ c-Kit⁺ hCPCs. Using other models, previous studies have established that hypoxia induces a persistent increase in phosphorylation of Akt for up to 24 hours with the effect peaking at six hours (Beitner-Johnson et al., 2001). Activation of Akt, a versatile kinase that regulates

several cellular functions, may result in hCPC functional improvements and enhanced cardiac repair. In the present study, enhanced Akt phosphorylation was indeed observed after only six hours of hypoxia, thus demonstrating that Isl-1+ c-Kit+ hCPCs exhibit similar behavior in response to hypoxia when compared to other cell types. However, age seems to play a significant role as the magnitude of hypoxia-mediated Akt activation was markedly reserved in adult clones. While both age groups displayed increased phosphorylation of Akt after the six-hour time point, only the neonatal group data was statistically significant. However, it is important to note that the adult clones are notoriously difficult to stimulate and, in response to SDF-1 α after starvation, levels of p-Akt significantly decrease. Short-term hypoxic preconditioning, on the other hand, rescued previous levels of phosphorylated Akt observed prior to serum deprivation in adult Isl-1+ c-Kit+ hCPCs. As hypothesized, this hypoxia-mediated activation of Akt in Isl-1+ c-Kit+ hCPCs was indeed correlated with elevated expression of PIK3CA, a protein that plays an essential role in the upstream activation of Akt (Baba et al., 2011). Experimental neonatal Isl-1+ c-Kit+ hCPCs exhibited a remarkable 43-fold significant increase of PIK3CA mRNA when compared to their respective controls. Adult Isl-1+ c-Kit+ hCPCs, on the other hand, only expressed a 1.8-fold increase in PIK3CA mRNA due to hypoxic preconditioning. The data here suggests that neonatal Isl-1+ c-Kit+ hCPCs react more strongly to the hypoxic pretreatment and transcribe more of the upstream activator of Akt.

Altogether, these findings confirm that six hours of hypoxia exposure significantly increases Akt activation in neonatal Isl-1+ c-Kit+ hCPCs.

Nonetheless, hypoxia is a stressor that, as demonstrated here, triggers a physiological response in hCPCs. As oxygen levels decline, the mitochondria within a cell increase the production of reactive oxygen species (ROS) (Chandel et al., 2000; Guzy et al., 2005). The accumulation of ROS after short periods of hypoxia has been shown to confer resistance against future oxygen shortages (Hoek, Becker, Shao, Li, & Schumacker, 1998). However, longer periods of hypoxia coincide with excessive accumulation of ROS that are known to promote cell death via caspase activation and DNA damage (Filomeni, De Zio, & Cecconi, 2015; Kamata et al., 2005; J.-Y. Kim & Park, 2003; Mounjaroen et al., 2006). Altogether, chronic oxidative stress has the potential to impair the functional capacity and overall health of a population of cells (van Oorschot et al., 2011). The extent of this stress response in Isl-1+ c-Kit+ hCPCs was evaluated by measuring the impact of short-term hypoxia on the expression of several HSP mRNAs. The induction of the heat-shock pathway during hypoxia has been well documented in other cell types (Baird, Turnbull, & Johnson, 2006). Genetic analysis of adult and neonatal Isl-1+ c-Kit+ hCPCs revealed a trend towards hypoxia-mediated activation of transcripts for several heat shock proteins. However, only the upregulation of HSP70 mRNA was statistically significant and was found only in the neonatal group. HSP70 is known to play a role in stabilizing Akt (Koren et al., 2010) and in the inhibition of apoptosis (Jiang et al., 2009;

Powers, Clarke, & Workman, 2008). Under hypoxia, Akt and other proteins are at risk of degradation, thus, the survival response likely includes upregulation of HSP mRNAs as an attempt to promote stabilization of proteins that are needed for cell survival.

Accordingly, pro-survival gene expression was also found to be upregulated in hypoxic Isl-1+ c-Kit+ hCPCs. Preconditioned hCPCs displayed a dramatic increase in transcripts encoding HMOX1, a pro-survival gene that becomes upregulated in response to hypoxia and affords protection against future oxidative damage (Poss & Tonegawa, 1997). Furthermore, the transcript encoding the NF- κ B p65 subunit (RELA), a known Akt downstream effector that inhibits programmed cell death (Beg & Baltimore, 1996; Madrid, Mayo, Reuther, & Baldwin, 2001), was significantly upregulated, but only in the pretreated neonatal group. Although a significant difference in apoptosis was not observed, hypoxia-preconditioned hCPCs indeed exhibited significantly enhanced invasion capabilities when compared to their normoxic counterparts. Moreover, when additional time points were tested, the six-hour time point yielded the greatest number of cells that successfully invaded through the transwell membrane. Applied to multiple biological replicates, the six-hour pretreatment of hCPCs resulted in significantly improved chemotaxis in response to SDF-1 α , which is in parallel to what has been observed in other models (Filippi et al., 2014; van Oorschot et al., 2011; Yan et al., 2012). Not surprisingly, however, these results were significant only in neonatal hCPCs. According to the PCR data, the

pretreated adult hCPCs did not upregulate PIK3CA mRNA transcripts to the same extent as the pretreated neonatal hCPCs. This suggests that the Akt pathway is not sufficiently activated by short-term hypoxia in adult hCPCs and may explain why only the neonatal clones displayed significantly elevated p-Akt and enhanced invasion capacity.

Furthermore, depending on the cell type, hypoxic exposure may either enhance proliferation (Grayson et al., 2007; Kook et al., 2008; Studer et al., 2000; van Oorschot et al., 2011), lead to G1 arrest (Gardner et al., 2001; Koshiji et al., 2004; Utting et al., 2006), or influence the differentiation process (Lin, Lee, & Yun, 2006; Studer et al., 2000; Utting et al., 2006). After treatment with hypoxia, human mesenchymal stem cells acquire enhanced proliferative abilities (Grayson et al., 2007) while, on the other hand, murine embryonic fibroblasts encounter G1 arrest (Gardner et al., 2001). Ideally, in the early stages after transplantation, donor cells must survive, continue to divide, migrate to the damaged myocardium, and remain multipotent as they engraft. Isl-1+ c-Kit+ hCPCs indeed migrate more readily, progress normally through the cell cycle, and retain expression of pluripotency markers after short-term hypoxic treatment. The results reported here using Isl-1+ c-Kit+ hCPC clones are in line with those of other cardiovascular progenitors (Hu et al., 2014; van Oorschot et al., 2011), suggesting that pretreatment with short-term hypoxia will enhance functional efficacy. Short-term hypoxia yielded mild improvements in adult CPCs, procured significant benefit to neonatal CPCs, and therefore, is a promising method for

improving cellular function. However, it is important to acknowledge that the most significant results were obtained in the neonatal group – enhanced Akt activation, upregulated pro-survival transcripts (RELA, HSP70), and improved invasion capabilities. Additional work is required to maximize the stimulation of adult hCPCs to mirror the function of neonatal CPCs and, in the process, optimize autologous adult CPCs for superior transplantation. Nevertheless, the results presented here demonstrate that outcomes of surgical procedures involving neonatal CPCs – for the treatment of CHDs or for transplant in HLA-matched adult patients – may be improved by preconditioning donor cells using short-term hypoxia.

Conclusions

Short-term hypoxia, as a pretreatment, is a viable approach for supporting cell survival and enhancing migratory capabilities in neonatal Isl-1+ c-Kit+ hCPCs. While the benefits accrued by adult Isl-1+ c-Kit+ hCPCs via preconditioning were reserved in comparison to neonatal clones, the applicability of neonatal CPCs in the clinical setting is significant and, therefore, hypoxia-preconditioned Isl-1+ c-Kit+ hCPCs warrant further investigation in animal models. The positive effects of short-term hypoxia include: 1) enhanced chemotaxis, which would render the cells more likely to reach damaged tissues and successfully engraft, and 2) elevated levels of PIK3CA, HSP70, RELA, and HMOX1 mRNA transcripts, which are important for cellular signaling and survival.

These findings, if implemented *in vivo*, may improve cardiac repair after infarction. Thus, in order to validate the efficacy of short-term hypoxia as an effective pretreatment strategy to optimize cell-based repair, future *in vivo* experiments comparing the performance of hypoxia-preconditioned CPCs to non-treated CPCs are currently in the design phase.

APPENDIX A
FIGURES

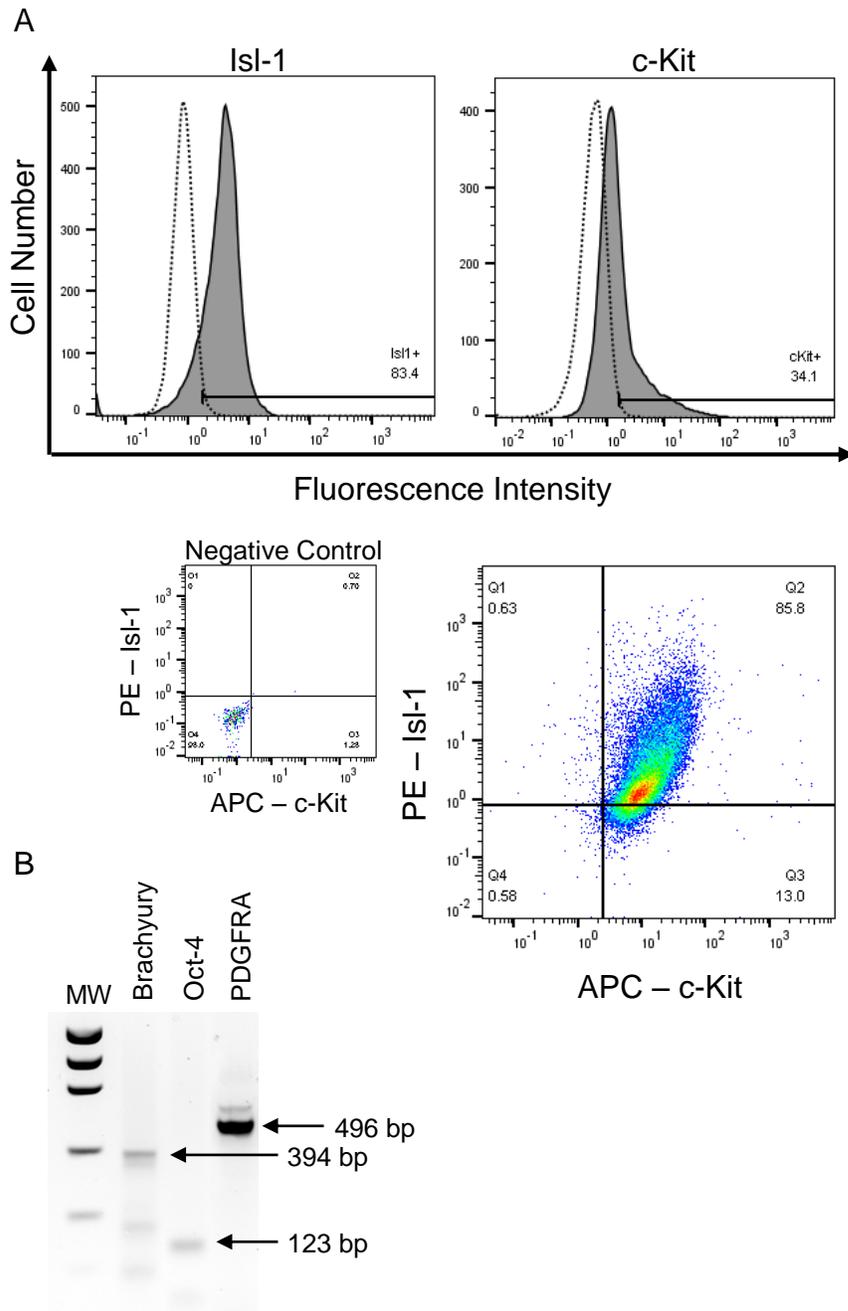


Figure 1. Expression of early progenitor markers within a representative clonal hCPC population. (A) Isl-1 and c-Kit expression within a representative clonal hCPC population as measured by flow cytometry. Dotted lines indicate isotype controls and solid lines indicate Isl-1 or c-Kit antibody-labeled hCPC. Double-labeled cells are shown in dot-plot. (B) Expression of stem cell markers in a representative Isl-1+ c-Kit+ hCPC clone is shown here by electrophoresis of PCR products. MW = molecular weight marker.

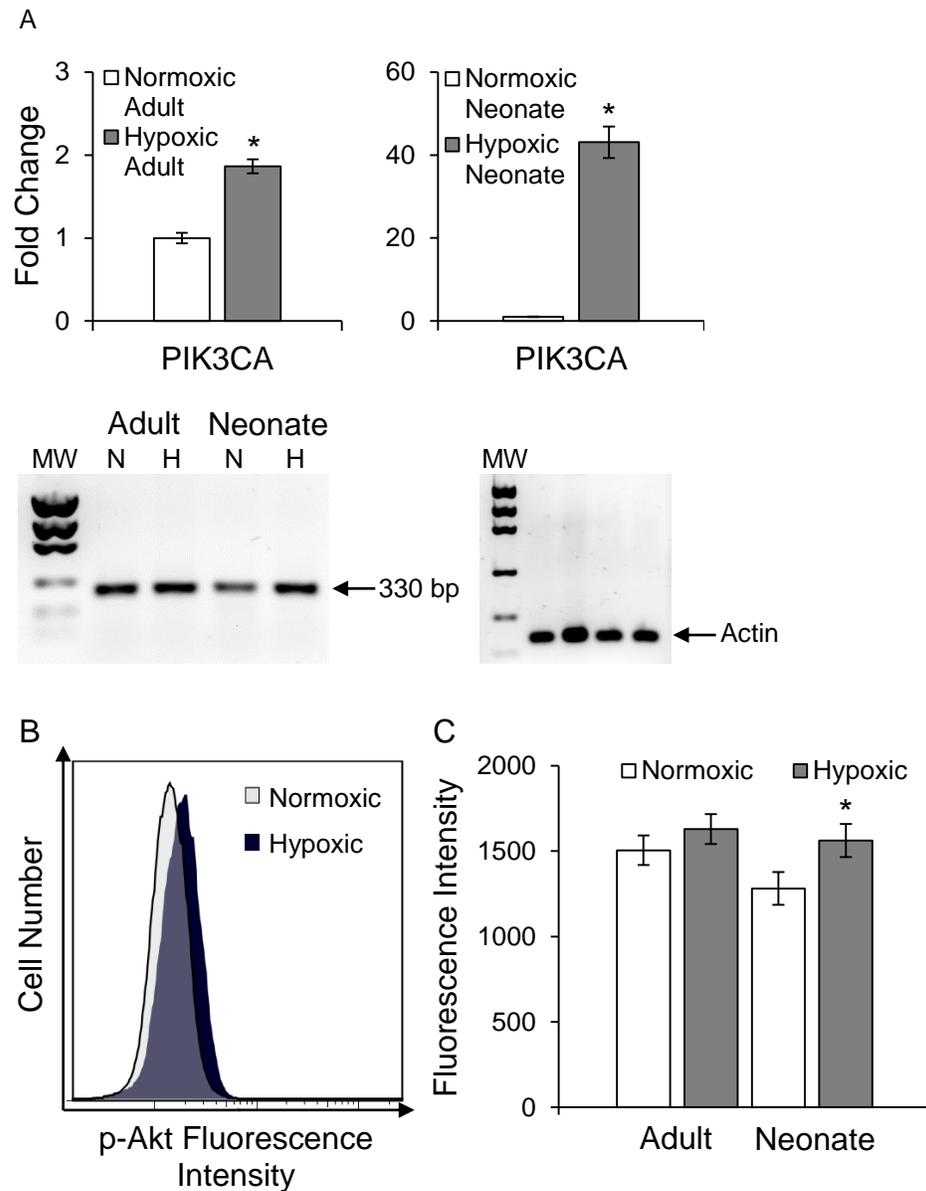


Figure 2. Short-term hypoxia upregulates phosphorylation of Akt in Isl-1+ c-Kit+ hCPCs. (A) Adult and neonatal Isl-1+ c-Kit+ hCPCs exposed to short-term hypoxia were compared to their respective normoxic control via qRT-PCR (n = 8). Quantification of results revealed that hypoxia preconditioning yields significant upregulation of PIK3CA transcripts within both age groups. Additionally, electrophoresis of PIK3CA primer products confirms PCR amplification of the target gene segment. Subsequently, phosphorylation of Akt was then measured in preconditioned Isl-1+ c-Kit+ hCPCs and their respective controls by flow cytometry. (B) Representative histogram of increased p-Akt monoclonal antibody binding after exposure to six hours of hypoxia. (C) Quantification of seven independent hCPCs revealed significantly increased (15.4%, p = 0.019) Akt phosphorylation in hypoxia-pretreated neonatal groups. Adult clones displayed modest Akt activation, but was deemed non-significant after further investigation.

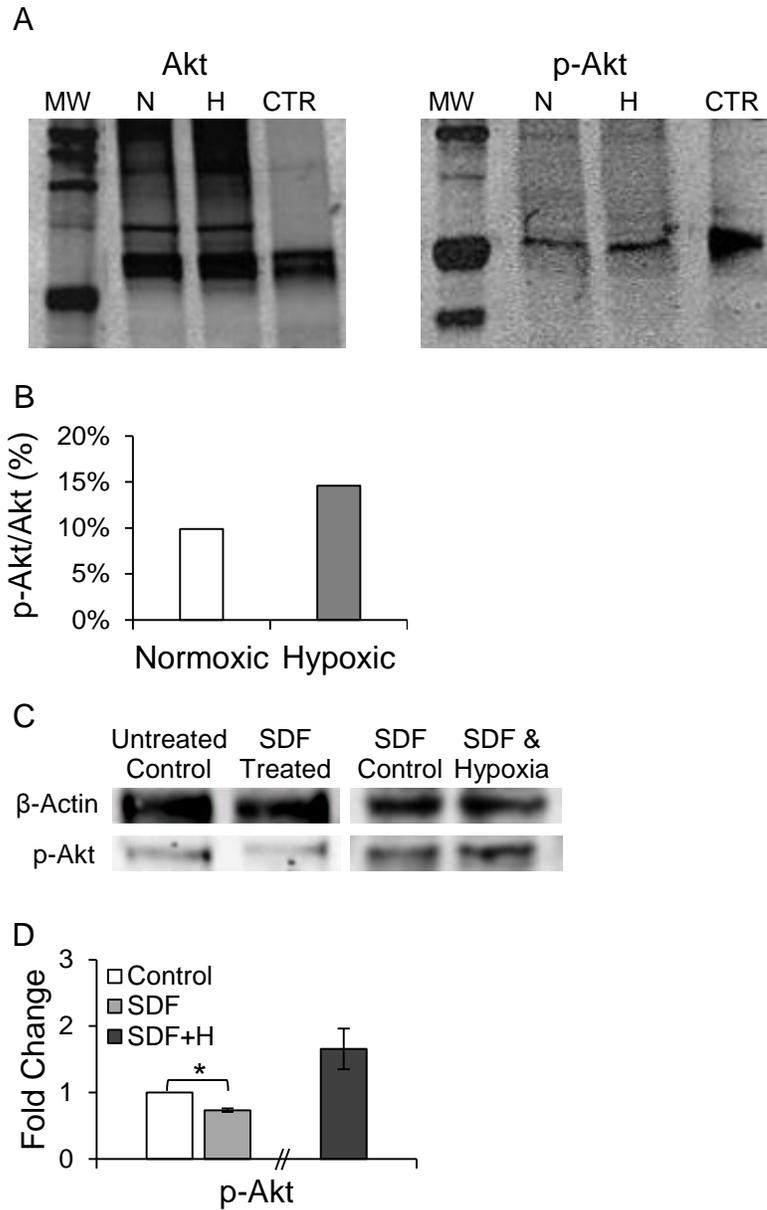


Figure 3. Hypoxia-induced Akt activation in adult Isl-1+ c-Kit+ hCPCs. (A) Protein immunoblots depicting Akt and p-Akt protein bands from normoxic and hypoxic representative adult hCPC samples. (B) Percentage of phosphorylated Akt in total Akt of non-treated and pretreated adult hCPC protein – quantified using ImageJ. (C) Western blots illustrating β -Actin and p-Akt protein from a representative normoxic adult clone, a SDF-1 α -treated normoxic adult clone, and an adult clone treated with both SDF-1 α and hypoxia. (D) Fold change of phosphorylated Akt, relative to β -Actin, was quantified using ImageJ.

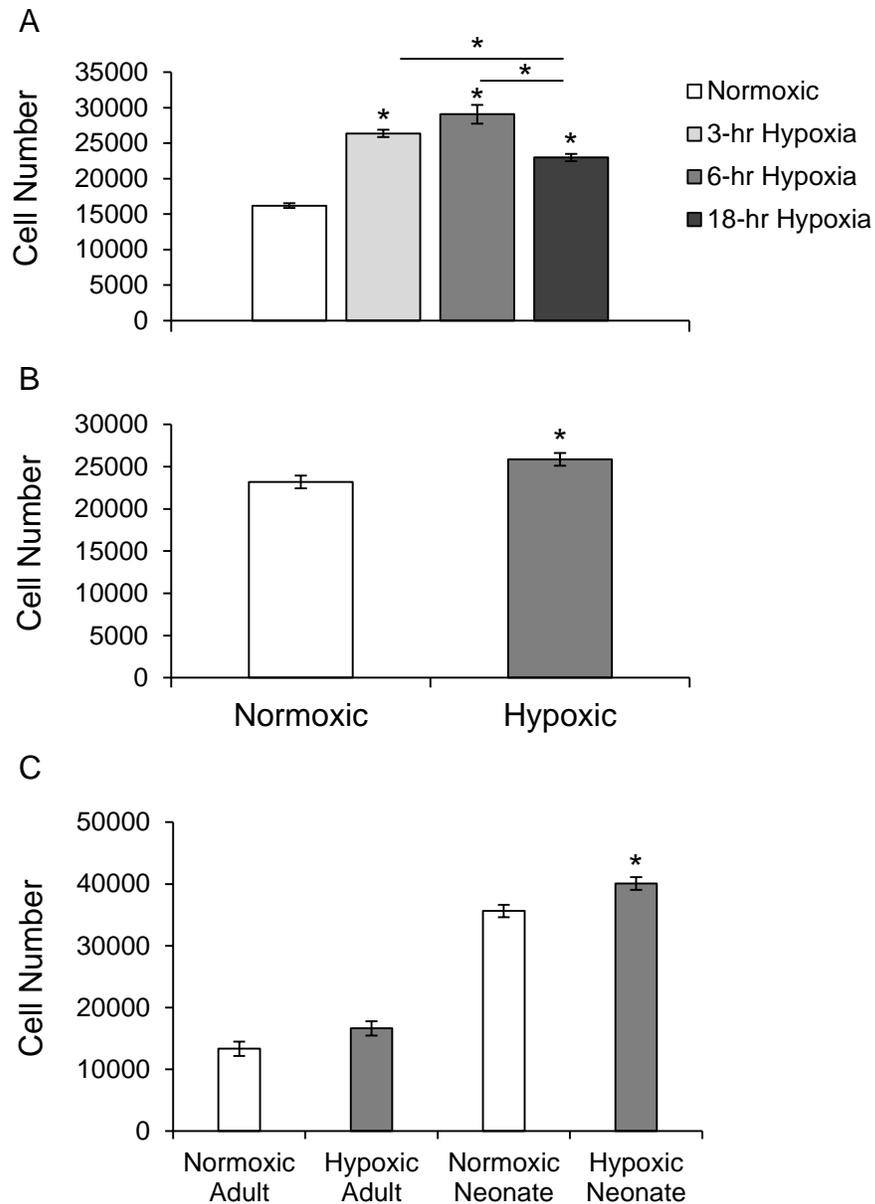


Figure 4. Invasion capabilities of Isl-1+ c-Kit+ hCPCs in response to SDF-1 α . (A) Quantification of hCPC performance in Transwell® invasion assay as influenced by duration of hypoxic exposure. Cell numbers were measured by calcein AM in quadruplicate using a representative Isl-1+ c-Kit+ hCPC. A significant increase in cell number was noted for each time point, with six hours of hypoxia yielding the greatest improvement. (B) Pooled data from both adult and neonate Isl-1+ c-Kit+ hCPCs, after six hours of hypoxia, show that hypoxia-pretreated hCPCs exhibit significantly improved invasion ($n = 10$, $p = 0.017$). (C) Invasion assay results organized by age group reveals that benefits afforded by hypoxia are age dependent. Adult hCPCs exhibited slight enhancements of their invasion capabilities, but only neonatal hCPCs procured statistically significant improvements.

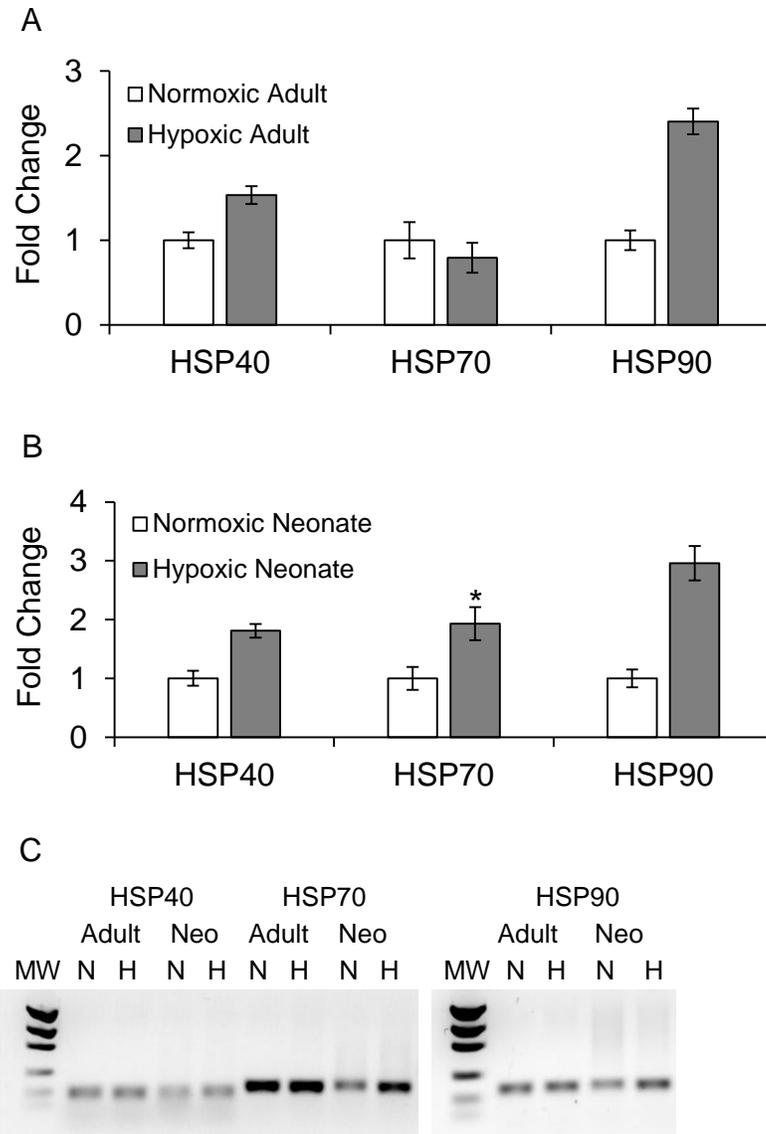


Figure 5. Transcription of heat shock proteins (HSPs) in response to short-term hypoxia. (A) Fold change of HSP mRNAs in adult *Isl-1+* *c-Kit+* hCPCs resulting from pretreatment with short-term hypoxia (n = 4). (B) Neonatal *Isl-1+* *c-Kit+* hCPC HSP mRNA expression reveals a significant two-fold elevation of HSP70 in response to short-term hypoxia (p = 0.022, n = 5). (C) PCR products of HSP mRNAs upregulated in adult and neonatal hCPCs, as visualized by agarose gel electrophoresis, validate the significant increase of HSP70 in neonatal hCPCs after short-term hypoxic preconditioning. MW = molecular weight marker, N = normoxic, H = hypoxic.

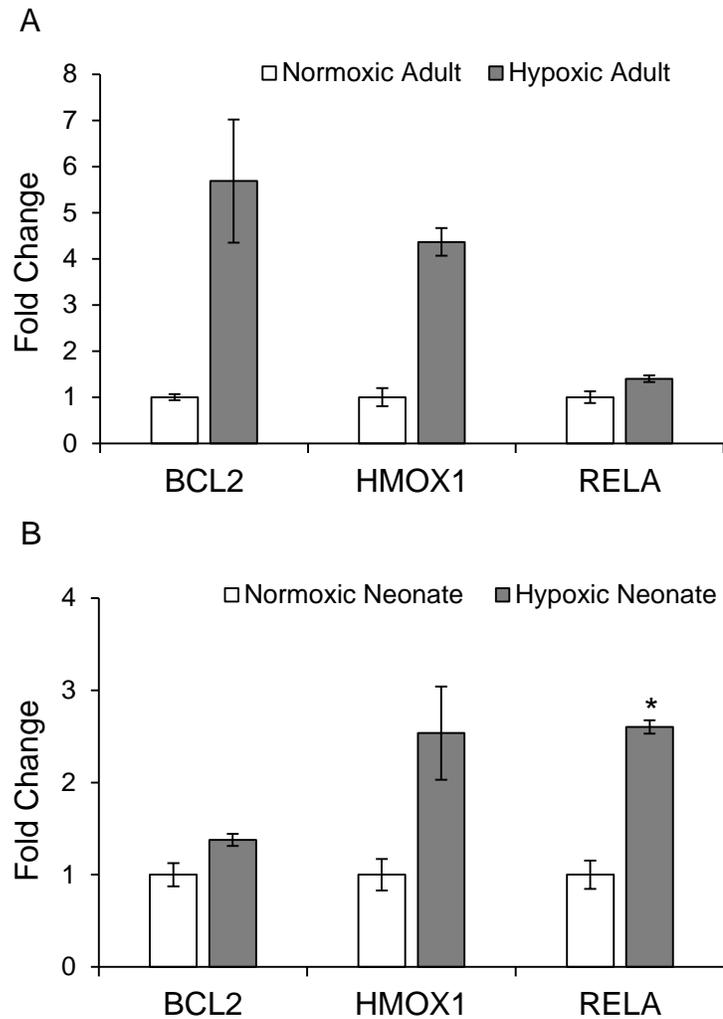


Figure 6. Select genes associated with cell survival are elevated in response to short-term hypoxia. (A) Expression of mRNA transcripts associated with cell survival in adult Isl-1+ c-Kit+ hCPCs and relative fold changes after hypoxic exposure. BCL2 and HMOX1 are strongly upregulated in response to hypoxia, but the changes are not statistically significant (n = 3). (B) Neonatal Isl-1+ c-Kit+ hCPC pro-survival gene expression in response to short-term hypoxia. RELA and HMOX1 are elevated in response to short-term hypoxia, but only RELA is significant (p = 0.02, n = 3).

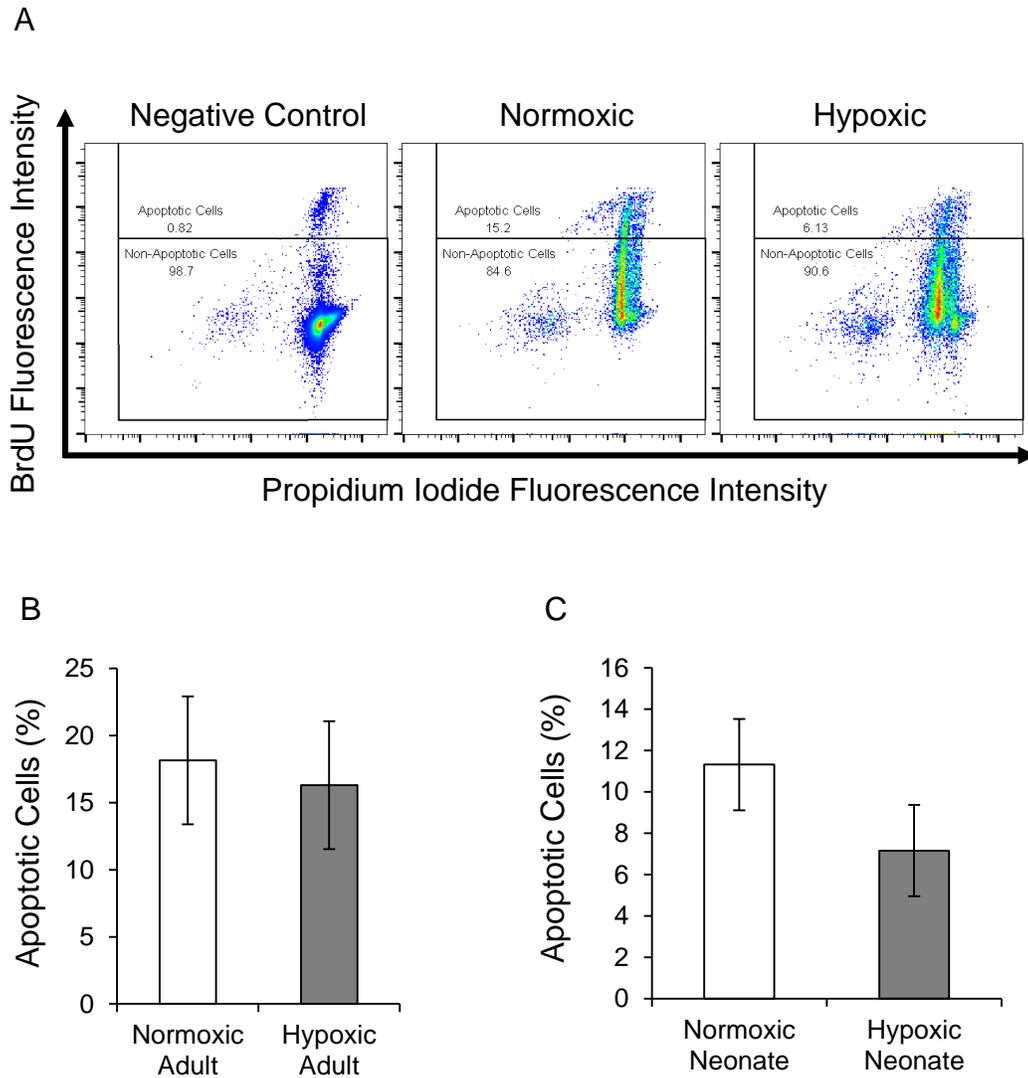


Figure 7. Programmed cell death in response to oxidative damage is not significantly reduced. Using 0.5 mM H₂O₂, apoptosis was induced in non-treated and preconditioned Isl-1+ c-Kit+ hCPCs of both adult and neonatal origin. Relative DNA fragmentation was measured using Brd-U and anti-BrdU antibody. (A) Cytometric analysis of BrdU-DNA binding within a representative clone is shown here, with and without hypoxic pretreatment. The group treated with short-term hypoxia exhibited an approximate 50% decrease in apoptotic cells after induction of cellular death by 0.5 mM H₂O₂. (B) Quantification of TUNEL assay results obtained using three independent adult clones are pictured here, showing that apoptosis is not significantly reduced ($p = 0.89$). (C) Pooled results of TUNEL assay for three neonatal clones with and without hypoxic pretreatment. Apoptosis in response to oxidative stress trends downward in the hypoxia preconditioned group but not to a significant degree ($p = 0.34$).

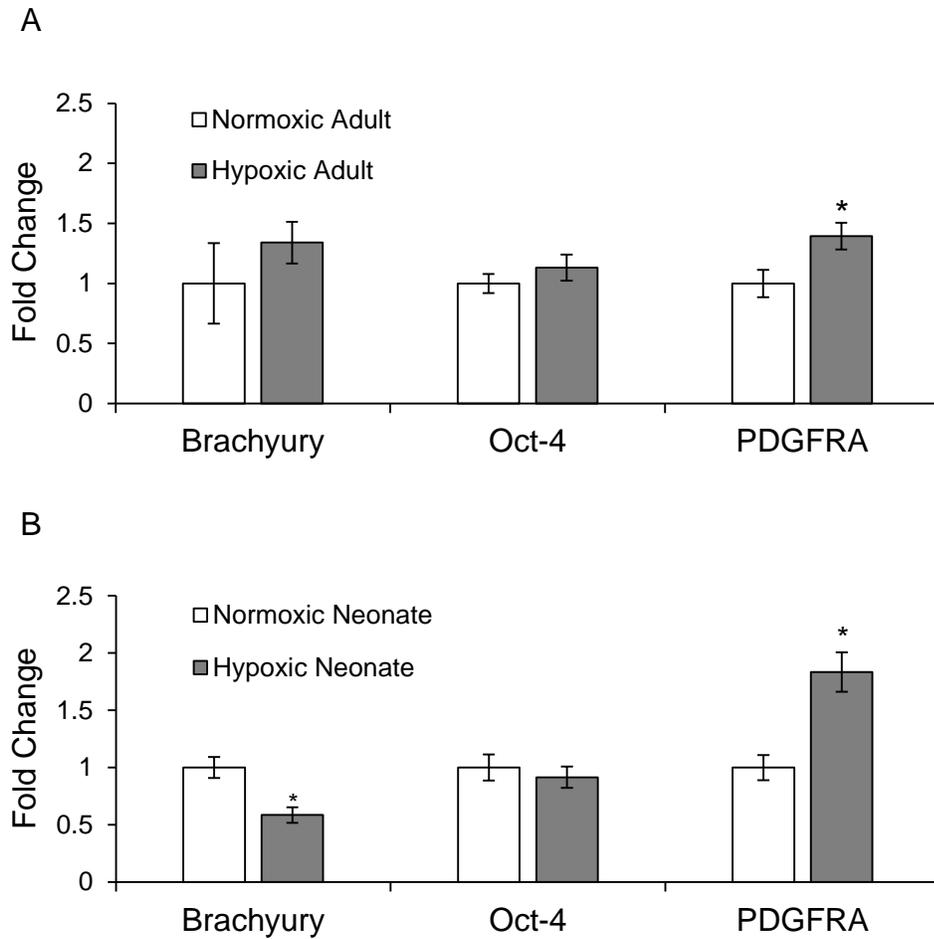


Figure 8. *Isl-1+* *c-Kit+* hCPCs maintain expression of early differentiation markers after hypoxic exposure. Quantitative RT-PCR was used to examine the effects of short-term hypoxia on the expression of differentiation markers present in cardiovascular progenitors. (A) Pooled PCR results from three individual adult hCPC clones reveals no significant change in differentiation markers with the exception of PDGFRA (1.4-fold increase, $p = 0.004$). (B) Quantification of PCR data from four independent neonatal clones reveals a similar upregulation of PDGFRA (1.8-fold, $p = 0.0003$), as well as a decrease in Brachyury transcripts (0.58-fold, $p = 0.004$).

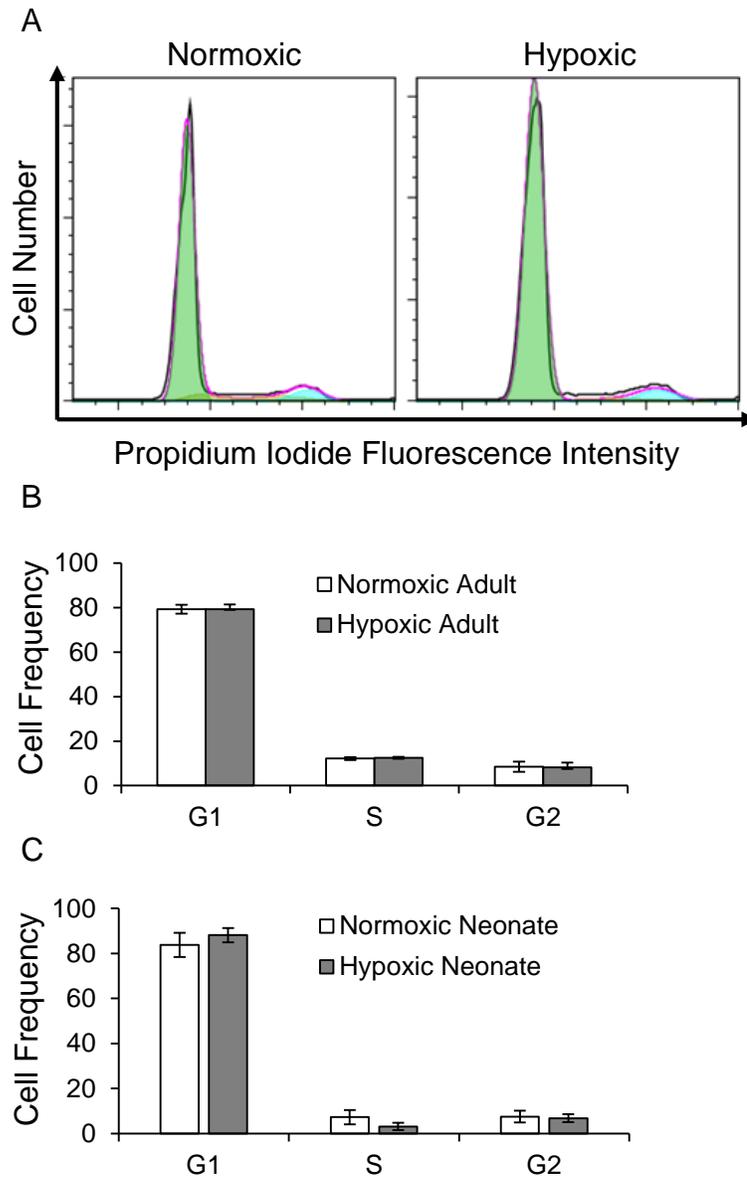


Figure 9. Short-term hypoxia does not alter normal cell cycle progression. Preconditioned Isl-1+ c-Kit+ hCPCs, of both neonate and adult origin, were stained using propidium iodide and compared to their respective normoxic controls via flow cytometry. (A) Flow cytometry histogram of cell cycle analysis in a representative clonal population, both with and without hypoxic pretreatment. (B) Quantification of nine individual results from three independent adult clones reveals that short-term hypoxia does not significantly affect normal cell cycle progression in adult Isl-1+ c-Kit+ hCPCs. (C) Quantification of data from six technical replicates using three independent neonatal clones confirms that normal cell cycle progression is also unaffected by short-term hypoxia in neonatal Isl-1+ c-Kit+ hCPCs.

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