2011

PHOSPHODIESTERASE-5 INHIBITION: A NOVEL STRATEGY TO IMPROVE STEM CELL THERAPY IN THE HEART

Nicholas Hoke
Virginia Commonwealth University

PHOSPHODIESTERASE-5 INHIBITION: A NOVEL STRATEGY TO IMPROVE STEM CELL THERAPY IN THE HEART

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

NICHOLAS N. HOKE
B.S., Virginia Polytechnic Institute and State University, 2005

Director: DR. RAKESH C. KUKREJA, PH.D.
PROFESSOR
SCHOOL OF MEDICINE

Virginia Commonwealth University
Richmond, Virginia
May 2011

Acknowledgement

I would like to express my sincere gratitude to Dr. Rakesh Kukreja for his mentorship, guidance, and support throughout my pursuit of this advanced degree. His willingness to allow me to pursue the degree of Doctor of Philosophy in Biochemistry under his world renowned expertise in the field was vital to my completion. Futhermore, I would like to express my deepest thanks to Dr. Salloum, Dr. Das, Dr. Xi, Dr. Ockaili, Dave Durrant and all other members of the Kukreja Lab past and present that I worked with for countless hours throughout the years. Their time spent to help teach or assist with numerous experimental procedures/protocols, scientific concepts/strategies, and sharing of their knowledge in the field was pivotal to the success of this project. My appreciation of their effort will never be forgotten nor will their friendship. In particular, Dr. Salloum who imparted all his wisdom, expertise, support both personally and professionally which helped to mold me to become a better scientist. His tireless efforts with the animal occlusion models and scientific planning were essential for the development of this project. I would also like to thank the members of my committee: Dr. Holt; Dr. Ratz; Dr. Rao; and Dr. Diegelmann for their support and assistance while giving valuable intellectual critiques or suggestions to further improve and ensure completion of this project resulting in attainment of my degree. I would also like to express my appreciation for their time and kind patience in addressing any questions or concerns I faced while conducting my research. Furthermore, without the love and support of my entire family and friends this goal could never been achieved.

Table of Contents

Page

List of Tables

Page

List of Figures

Page

Chapter 3:

Abstract

PHOSPHODIESTERASE-5 INHIBITION: A NOVEL STRATEGY TO IMPROVE STEM CELL THERAPY IN THE HEART

By Nicholas N. Hoke, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2011

Major Director: Dr. Rakesh Kukreja, Ph.D
VCU School of Medicine
Division of Cardiology

Several studies have shown cellular replacement therapy as a treatment strategy of myocardial infarction but results have been limited. Therefore, enhancing the therapeutic potential of stem cells injected into ischemic microenvironments by novel preconditioning (PC) techniques is critical for improving cellular therapy. Recent studies have shown that inhibition of phosphodiesterase-5 (PDE-5) is a powerful strategy to precondition the heart and cardiomyocytes against ischemia/reperfusion injury. We therefore tested the hypothesis that inhibition of PDE-5 with sildenafil (Viagra®) or selective knockdown with a silencing vector in

adipose derived stem cells (ASCs) would improve their survival after ischemia/reoxygenation *in vitro* and enhance cardiac function following myocardial implantation *in vivo*.

ASCs were treated with sildenafil or infected with PDE-5 silencing vector shRNA ($shRNA^{PDE-5}$). The cells were subjected to simulated ischemia (SI) and reoxygenation (RO). Both sildenafil and $shRNA^{PDE-5}$ significantly reduced cell injury, as shown by improved viability, decreased lactate dehydrogenase, and apoptosis. The preconditioned ASCs also demonstrated an increase in the release of growth factors including VEGF, b-FGF, and IGF. The protective effect against SI/RO injury was abolished by inhibition of protein kinase G (PKG) using both a pharmacological inhibitor and selective knockdown with $shRNA^{PKG1\alpha}$ suggesting a PKG-mediated mechanism. To show the effect of preconditioned ASCs *in vivo*, adult male CD-1 mice underwent myocardial infarction (MI) by occlusion of the left descending coronary artery, followed by direct injection of PBS (control), non-preconditioned ASCs, or preconditioned ASCs ($4x10^5$) ASCs into the left ventricle (LV). Preconditioned ASC-treated hearts showed consistently superior cardiac function by all measures as compared with PBS and non-preconditioned ASCs after 4 weeks of treatment. Post-mortem histological analysis demonstrated that preconditioned ASC-treated mice had significantly reduced fibrosis, increased vascular density and reduced resident myocyte apoptosis as compared to mice receiving non-preconditioned ASCs or PBS. VEGF, b-FGF, and Ang-1 were also significantly elevated 4 weeks after cell therapy with preconditioned ASCs. Our data suggests that genetic or pharmacological inhibition of PDE-5 is a powerful new approach to improve stem cell therapy following myocardial infarction.

CHAPTER 1
INTRODUCTION

Cardiovascular disease remains a leading cause of morbidity and mortality in the United States, affecting approximately 5–6 million Americans, particularly those of age 65 and older. A continually aging population is expected to result in an increased number of people afflicted with heart-related conditions, requiring costly long-term medical management with an unpredictable effect on quality of life. The most common cardiovascular disease in the United States is coronary heart disease, which often appears as an acute myocardial infarction (MI) caused by the sudden occlusion of the coronary artery. In 2010, an estimated 785,000 Americans had a new coronary attack, and about 470,000 had a recurrent attack. About every 25 seconds, an American will have a coronary event, and about one every minute will die from one (1). Despite advances in treatment of MI that result in reduced mortality, congestive heart failure secondary to infarction continues to be a major complication. MI is projected to remain one of leading causes of death for years to come; therefore, there is a continuous demand for safe and efficient preventive or therapeutic strategies (2).

Etiologies of heart failure development are numerous and involve complex molecular mechanisms, not entirely understood. However, recent advances have expanded our knowledge and understanding of the cellular and molecular mechanisms involved in the development of

heart failure. The occlusion of an artery creates an ischemic microenvironment by depriving areas of the myocardium of blood, oxygen and nutrients, which if maintained for any significant amount of time will trigger a cascade changing the cellular metabolism and function within the tissue primarily from decreased amounts of energy produced in the form of ATP. The lack of sufficient amounts of ATP can eventually lead to severe cellular and tissue damage such as myocyte hypertrophy, myocyte death, and disruption of matrix metalloproteinase balance (3). The degree of injury is dependent on the period for which the myocardium is subjected to ischemia, with a longer duration leading to a decreased chance of recovery (4). Prolonged bouts of ischemia leads to a variety of pathophysiological states such as a decrease in force generation, contracture, arrhythmias, calcium overload, a decrease in pH of the tissue and eventual cell death (5). The loss of contracting myocardium and a resulting increase in the workload on the viable myocardium causes cardiac overload due to increased energy usage and supply-demand imbalance (ATP depletion), which lead to cellular necrosis and apoptosis. This subsequently promotes acute and chronic transformation of both the necrotic infarct zone and the nonnecrotic, peri-infarct tissue, leading to global alterations that have collectively been termed "ventricular remodeling" (6, 7). Progressive cardiac hypertrophy that occurs in response to MI is known to increase the risk of heart failure, although it is believed to be compensatory at the initial stages of remodeling (8). Because of the loss of cardiomyocytes during an MI, the heart, as a result, is unable to maintain a cardiac output appropriate for the requirements of the body.

The main factor leading to the progression of heart failure is the irreversible loss of cardiomyocytes due to necrosis and apoptosis. To overcome myocyte loss and the heart's limited self-regeneration capacity, mesenchymal stem cell-based therapies are becoming increasingly

recognized for their potential to repair the damaged myocardium post injury. Mesenchymal stem cells are characterized for their self-renewing capacity and ability to undergo multi-lineage differentiation. However, the use of mesenchymal stem cells for the purpose of regenerative medicine should adhere to the following set of criteria: (i) available in abundant quantities; (ii) collected and harvested by a minimally invasive procedure; (iii) differentiated along multiple cell lineage pathways in a reproducible manner; (iv) can be safely and effectively transplanted to either an autologous or allogeneic host (9).

Typically, mesenchymal stem cells are isolated from either bone marrow or adipose tissue. Adipose tissue is an attractive source of mesenchymal stem cells for researchers and clinicians due to the simple surgical procedure, the abundance of subcutaneous adipose tissue, and the easy enzyme-based isolation procedures (10, 11). Adipose tissue-derived stem cells (ASCs) have the ability to differentiate into multiple lineages of tissues, such as skeletal muscle, bone, and fat, using specific culturing conditions containing hormones or growth factors (12-14). Interestingly, ASCs have been shown to differentiate into spontaneous contractile myocytes (15). Transplanted stem cells function through paracrine mechanisms to promote endogenous repair of cardiac tissue (16-18). Moreover, differentiation of ASCs into endothelial cells has been shown to have a strong regenerative angiogenic potential because of their ability to secrete angiogenic and pro-survival paracrine factors (19, 20). Due to their multi-lineage differentiation potential, ASCs are becoming a widely studied alternative to bone marrow-derived stem cells (BMSCs) for therapeutic treatment of cardiac diseases.

Characterization of Adipose Tissue-Derived Stem Cells

Numerous experiments have documented the benefits of BMSCs for treating myocardial infarction (21, 22). Yet, only in the past few years have groups started to study the benefits of cells obtained from adipose tissue for treating heart disease. Interestingly, there are several advantages of using adipose tissue versus bone marrow-derived cells. It is known that ASCs, along with BMSCs, can be maintained *in vitro* for extended periods of time with a stable population doubling time and low senescence levels, thus implying that there is no deterioration in their proliferation rate (23). Multipotent stem cells from adipose tissue can be harvested from patients by a simple surgical procedure that is less expensive and minimally invasive when compared to obtaining cells from bone marrow. In fact, a greater frequency and yield of multipotent stem cells from adipose tissue was reported when compared to bone marrow isolations, approximately 5×10^5 cells versus 1×10^5, respectively (24). Neither the type of surgical procedure nor the anatomical site of the adipose tissue affects the total number of viable cells that can be obtained in the stroma vascular fraction (25). Furthermore, it has been shown that ASCs possess a higher stem cell proliferation rate than BMSCs (24). Moreover, ASCs can be held in culture for up to 1 month without passage while maintaining their proliferation and differentiation potential, thus minimizing time and expenses of tissue culture maintenance. The high yield of isolation, proliferation rate, and maintenance of differentiation potential suggests that it is possible to obtain an autologous line of ASCs from patients undergoing elective liposuction. Cells can also be cryogenically frozen for storage or transplanted either immediately or after expansion in culture if needed for treatment of cardiac injury.

The frequency and yield of ASCs and BMSCs cellular preparations are different, but they share similar biology. It is imperative to determine the gene and protein expression profile of ASCs to improve culturing conditions, lineage-specific differentiation, and enrichment of the stem cell population prior to transplantation. Using fluorescence-activated cell sorting analysis, the surface immunophenotype of ASCs was determined to resemble the phenotypes of BMSCs, stromal cells, or skeletal muscle-derived cells (25-27). Previously reported immunophenotypes of human ASCs and human BMSCs show them to be approximately 90% identical (28). Both cell populations display similar mitogen-activated protein kinase (MAPK) phosphorylation in response to tumor necrosis factor, adiponectin and leptin secretion, and lipolytic response to adrenergic agents (13). Unfortunately, studies comparing the direct gene and protein expression profile of ASCs and BMSCs are extremely rare. However, an important comparative micro-array analysis of mesenchymal stem cells obtained from bone marrow, adipose tissue, and umbilical cord clearly showed that no significant differences in morphology, immune phenotype, and differentiation capacity were apparent between the groups (29). When compared to fibroblasts, 25 genes overlapped and were up-regulated in all cellular preparations (29). No phenotypic differences could be determined comparing 22 surface antigens. Interestingly, hundreds of expressed sequence tags that were differentially expressed between groups. There is some controversy in the literature regarding the expression of the widely used marker of hematopoietic stem cells, CD34, in ASCs (30-32). Data suggest that CD34 is absent in cultured ASCs but, by contrast, higher levels of expression especially early in passage or lower subculture number have been documented (32). The Stro-1 antigen, a typical BMSC surface antigen, has also been reported to have controversial expression levels as it has been described to be absent and present

in human ASCs (13, 33). The conflicting data might be due to the differences in either the epitope recognized, the duration in culture, hematopoietic cell contamination, or the detection methods, typically flow cytometry or immunohistochemistry. While both cell types share numerous surface markers, there are some differences in protein expression between the groups as seen when comparing ASCs and BMSCs taken from the same individual. BMSCs express the marker CD106 while ASCs do not, but conversely ASCs express CD49d unlike bone marrow cells (33).

The expression of important transcription factors in cultured ASCs is still present even after 30 passages. The mRNA expression of Nanog, Oct-4, and Sox-2 was determined at passage 30 with the expression levels of all genes being significantly lower than passage 4, yet still detectable (24). These factors have been shown to play an important role in differentiation of ASCs (24). Furthermore, gene expression is also affected by the time spent in culture between passages. Culturing of cells every 14 days instead of the typical 5 days resulted in increased expression of the stem cell-related genes thus suggesting that these cells might have a stronger differentiation potential. Therefore, the ideal cells for therapeutic transplantation would be of a low passage with a longer time spent in between passages.

It is important for transplanted cells not to induce an immune response from the host. Like cells isolated from bone marrow, ASCs do not provoke *in vitro* alloreactivity of incompatible lymphocytes, and they suppress mixed lymphocyte reaction and lymphocyte proliferative response to mitogens in a dose and time dependent manner (34). This is most likely due to the fact that less than 1% of ASCs express the HLA-DR protein, which is known to mediate the rejection of transplanted tissue in the graft-versus-host immune response (35).

Moreover, Rodiguez et al (35) demonstrated that adipose derived cells are immunopriviledged *in vitro* and *in vivo* being relatively resistant to rejection after transplantation. These findings support the idea that ASCs share immunosuppressive properties with BMSCs and therefore might represent an alternative cellular source suitable for allogenic transplantation procedures lacking the risk of tissue rejection.

Differentiation Capacity

In order for ASC transplantation to function properly and repair the damaged myocardium, ASCs must have the capability to differentiate into cardiac cells thus being able to repopulate cellular loss after infarction due to ischemia. Fortunately, differentiation of ASCs into cells that phenotypically resemble myocytes has been detailed in several *in vitro* experiments (36-38). Lineage specific differentiation of stem cells can be controlled by chemical treatment, co-incubation with other cells, or adding growth factors to the culture medium. It has been demonstrated that treatment with the commonly used DNA demethylating agent, 5-azacytadine (9 μmol/L for 24 hours), results in differentiation of ASCs isolated from New Zealand White rabbits into myocytes after 3 weeks in culture. The differentiated cells were multinucleated, began to beat spontaneously in culture and positively expressed myosin heavy chain, α-actinin, and Troponin-I (39).

Besides chemical treatment, addition of growth factors to the culture medium will also influence the differentiation pathway. It has been documented that medium supplemented with interlueukin-3,-6, and stem cell factor induces differentiation of ASCs taken from male C57Bl/6N mice into myocytes that also have pacemaker activity (15). After 24 days in culture,

cells were beating in unison independently of 5-azacytidine treatment and clones expressed several cardiac-specific mRNA such as GATA-4, Nkx2.5, ventricular and atrial myosin light chains. Clones were also positive for the cardiac markers: myosin-enhancing factor 2C, α-actinin, myosin heavy chain and connexin 43, while being negative for skeletal muscle markers. Structural analysis by electron microscopy revealed multinuclear cells with morphology consistent with cardiac myocytes. Cellular electrical activity was recorded on cells in a current clamp and revealed an action potential characteristic to cardiac pacemaker cells. The differentiated cells were also capable of responding to both adrenergic and cholinergic agonists. As expected for myocyte-like cells, isoproterenol, a β-agonist, induced a dose-dependent increase of the spontaneous contraction rate while propranolol, a nonselective β-adrenergic antagonist, reversed the effects. In contrast, the nonselective acetylcholine agonist, carbamylcholine stopped the spontaneous contractions. Furthermore, reversibly permeabilized human ASCs co-incubated with nuclear and cytoplasmic extracts from neonatal rat myocytes resulted in differentiation into binucleated striated spontaneously beating myocytes (39). Finally, it has been reported by Song et al (41), using standard culture conditions without any addition of growth factors or cytokines, that ASCs isolated from humans can spontaneously differentiate into myocytes after 12 days in culture. Vascular endothelial growth factor (VEGF) was identified as being critical for cardiomyogenesis (41). In fact, significant amounts of VEGF were found in the conditioned medium which is known to significantly enhance MHC-α, cTN-I, and Nkx2.5 expression in differentiated embryonic stem cells suggesting that VEGF is partly responsible for the differentiation into cardiac cells via a paracrine mechanism. It has been clearly demonstrated that ASCs will differentiate into cardiac myocytes *in vitro,* but a limited

number of studies have detailed differentiation in an *in vivo* animal model of myocardial infarction. Injection of ASCs taken from β-galactosidase transgene expressing Rosa26 mice into B61295 mice immediately after permanent occlusion resulted in β-galactosidase positive cells expressing the cardiac specific genes, myosin heavy chain, Nkx2.5, and Tropinin I (26). Similar results were obtained when using green fluorescent protein (GFP) labeled ASCs in a rat model of heart failure in which cells were immunohistochemically stained positive for cardiac markers 30 days post surgery (40). Moreover, it has been reported that ASCs grown in temperature responsive culture dishes formed a monolayer sheet due to cell-to-cell adhesions that, when transplanted onto the ligated myocardium 4 weeks post surgery, resulted in a thickened layer of newly generated vessels and mycoytes over the damaged area that were positive for Tropinin I and desmin (41). These data strongly suggest that there is a great potential for ASC cellular therapy as a treatment to repopulate the injured myocardium with differentiated cells that have a functional pacemaker activity while phenotypically and structurally resemble myocytes.

Therapeutic enhancement of neovascularization is an important strategy needed to limit the complications of post ischemic injury. Stem cell therapy has been shown to be promising in neoangiogenesis in models of hind-leg ischemia (19, 42). The ability to differentiate into mature endothelial cells, which is critical for formation of new blood vessels, gives ASCs great angiogenic potential. Planat-Benard et al (43) observed vascular-like structure formation in Matrigel plug using ASCs taken from mice. Cells were positive for the endothelial markers: CD31, VE-cadherin, and von Willebrand factor. Moreover, the cells formed branching networks, consistent with the formation of vascular structures that lead to enhancement of the neovascularization reaction in ischemic tissue. It has also been shown that ASCs can form

numerous tube-like structures, and, while in the presence of erythrocytes, demonstrated the existence of a functional vascular structure (44). Similarly, CD31 expression and differentiation into vascular structures is enhanced by VEGF. If VEGF were added to the growth medium, human ASCs display an endothelial phenotype (45). All these results clearly demonstrate that there is a relationship between VEGF and lineage specific differentiation. *In vivo* studies also support the potential of endothelial differentiation. Using a model of hind-limb ischemia in rats, transplantation of ASCs improves angiogenesis and recovery of vascular blood supply (19). Several groups have reported similar results that transplanted ASCs can integrate as fully functional and differentiated endothelial cells (26, 46-48). Furthermore, Zhang et al (48) demonstrated that BrdU-labeled ASCs differentiate into myocytes and endothelial cells that participate in vessel-like structure formation. It is clear that cellular transplantation of stem cells derived from adipose tissue is a viable option for repairing damaged myocardium through differentiation into myocytes and endothelial cells that become an integral part of new vascular structures.

Potential for Myocardial Regeneration

Over the past several years, experimental findings suggest there is a therapeutic potential for cellular replacement therapy as treatment of MI and other progressive chronic cardiac diseases such as left ventricular (LV) remodeling and heart failure. Since cardiovascular disease remains a worldwide problem, the development of novel effective cell-based therapies is crucial to improve patient outcome post MI. Current treatment of MI still leaves a significant number of patients with impaired cardiac function that leads to more severe LV dysfunction and adverse

remodeling. Remodeling of the ventricle is a result of increased apoptosis in the ischemic zones after infarction. While apoptosis influences remodeling, the other form of cellular death that occurs in the heart, necrosis, provokes inflammatory reactions, neoangiogenesis, fibroblast activation, and scar formation. To date, most of the cellular based therapies have involved the use of myoblasts or BMSCs that often result in an improvement in LV function but have little effect on preventing LV remodeling (22, 49, 50). Recently, studies have shown that ASCs have become a viable alternative option to further limit remodeling and the progression to LV dysfunction post MI (29, 47, 48, 51, 52). In a model of acute MI, rats underwent ligation of the left anterior descending coronary artery for 45 minutes, then were allowed to reperfuse for 15 minutes before receiving an intramyocardial injection of GFP-labeled ASCs. Interestingly, 12 weeks later, there was very poor engraftment, but cell-treated animals had more capillaries and arterioles per mm^2 in the infarct border zone with a similar trend in the infarct area. The remodeling seen in the control animals was not detected in the ASC-treated group (51). Furthermore, a significant increase in LV ejection fraction and fractional shortening compared to control mice at 2 weeks following permanent occlusion of the coronary artery has been reported. The improvement in cardiac function also correlated with a significant decrease in LV end-systolic diameter (53). Interestingly, reversed wall thinning in the scar area has been seen at 30 days after permanent ligation (40). The reconstruction of thick myocardial tissue reduces the stress on the LV wall subsequently improving cardiac function. Similar results from a direct comparison of intracoronary injection of ASCs and BMSCs in a porcine model of MI revealed that ASC treatment substantially improved LV perfusion, function and attenuated adverse remodeling. The capillary vessel density was found to be greater in the ASC treatment group

versus the group receiving BMSCs (40). This data suggest that angiogenesis may contribute to the maintenance of cardiac function by preservation of the remaining viable myocytes and through neovascularization, thus protecting the myocardium in the border zone that would normally undergo apoptosis.

Mechanisms of Action

There are three proposed mechanisms through which ASCs can be used to repair and regenerate damaged myocardium: myocyte regeneration, vasculogenesis, and paracrine actions. Cell therapy as treatment of cardiovascular diseases was originally thought to repopulate the myocardium, but growing evidence supports the hypothesis that paracrine mechanisms play an essential role in repairing the damaged myocardium. Paracrine factors are released from endogenous cells of the heart in response to injury. These pro-survival growth factors or cytokines mediate multiple mechanisms such as increased blood flow to ischemic tissue, reduction in myocyte apoptosis, regulation of inflammatory response, and recruiting endogenous stem cells to regenerate injured tissue. Additionally, administration of conditioned media from hypoxic ASCs significantly increased endothelial cell growth and reduced endothelial cell apoptosis, while transplantation of these cells into ischemic hind limbs led to improved perfusion, suggesting that paracrine factors from these cells promote neovascularization (54). Similar effects have been seen in the heart as injection of conditioned media decreased apoptosis, fibrosis, and LV dilatation and increased myocardial thickness after infarction (48). The absence of cells in the treatment proves that a paracrine mechanism from growth factors released in the conditioned media plays an important beneficial role in repairing the damaged myocardium.

Induction of neovascularization is crucial for limiting the damage in ischemic tissue; however, studies suggest that only a small number of blood vessels contain transplanted donor cells. It is known that angiogenesis and arteriogenesis typically involve mediators such as nitric oxide, VEGF, basic fibroblast growth factor (b-FGF), hepatocyte growth factor (HGF), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and the angiopoietins. Interestingly, hypoxic preconditioning of cells induces expression of activated Akt and endothelial nitric oxide synthase (eNOS), while also secreting higher levels of VEGF, b-FGF, HGF, insulin-like growth factor (IGF)-1 when compared to cells cultured under normal culture conditions. The transplantation of the cells led to a significant increase in blood vessel density (55). The release of the angiogenic factors augmented the vessel number without incorporation into mature vessels. Tissue levels of VEGF and b-FGF are significantly increased in infarcted hearts that are treated with ASCs (56). Moreover, the expression of these growth factors correlated with increased angiogenesis and reduction in infarct size.

Besides the increase in new blood vessel formation, it is important that the paracrine factors function to protect resident cells, particularly myocytes, from apoptosis. Transplantation of ASCs along with their secretion of VEGF, b-FGF, IGF, and SDF-1 has been shown to upregulate the anti-apoptotic protein, Bcl-2, which results in the decrease in myocyte apoptosis *in vitro* and *in vivo* (57). Furthermore, intramyocardial injection of adenoviruses over-expressing VEGF or b-FGF decreases infarct size and increases expression of Bcl-2 (21). It has been shown that stem cells over-expressing Akt led to a decline in myocyte apoptosis *in vitro* and that transplantation led to a decrease in infarct size (57, 59). The Akt over-expressing cells secreted several paracrine factors such as VEGF, b-FGF, thymosin β4 (TB4) and HGF. HGF has been

shown to have anti-apoptotic effects in acute MI (60). Additionally, transplantation of ASCs over-expressing HGF into MI rat models induced myocardial angiogenesis, suppressed fibrosis and improved cardiac function better than transplantation of ASCs alone (61). Also the G-actin sequestering peptide, TB4, promotes survival of embryonic and postnatal cardiac myocytes, and treatment results in enhanced myocardial survival suggesting that ASCs protect the myocardium from apoptosis through paracrine effects (62). Myocardial injury provokes an inflammatory response resulting in an increased expression of a variety of both pro-inflammatory and anti-inflammatory cytokines. Initially, inflammatory cytokine expression is necessary for maintaining homeostasis in the heart after stress or injury, however, sustained upregulation of certain cytokines leads to adverse remodeling and heart failure (63). There is increasing evidence that ASCs secrete cytokines that may directly act to limit deleterious, sustained endogenous inflammation. In fact, cellular administration led to a downregulation of TNF-α, IL-1β, and IL-6, which are involved in adverse LV remodeling (64). Furthermore, cell transplantation attenuated myocardial dysfunction in a rat model of acute myocarditis (65). Also, conditioned media protected isolated adult rat cardiac myocytes from MCP-1 induced injury, suggesting that the anti-inflammatory effects were due to paracrine factors. Data also suggests that ASCs may directly modulate T lymphocyte function in the heart, possibly leading to protection against their cytotoxicity or alternatively modulate their role in cardiac remodeling. T lymphocytes co-cultured with cardiac fibroblasts led to an increase in fibroblast pro-collagen expression, suggesting that suppression of T lymphocyte accumulation may inhibit fibrosis (66). Therefore, alterations in the immune response by cellular therapy may serve to improve LV function and attenuate adverse LV remodeling.

The direct effect on fibrosis by stem cell therapy has been demonstrated. Conditioned media significantly attenuated proliferation of cardiac fibroblasts and up-regulated elastin, myocardin, and DNA-damage inducible transcript 3 (67). Furthermore, type I and III collagen expression and type III collagen promoter activity were significantly down-regulated. Gene expression analysis revealed that stem cells had several matrix-modulating factors up-regulated such as matrix metalloproteinase-2 (MMP-2), tissue inhibitors of matrix metalloproteinases (TIMP)-1 and TIMP-2, thrombospondin-1, and tenacin C, suggesting a direct effect on extracellular matrix remodeling (68). A reduction in fibrosis, along with a reduction in levels of MMP-2 and MMP-9, has been documented after injection of ASCs in models of MI (68). IL-1β, which is secreted by ASCs, has a direct anti-proliferative effect on cardiac fibroblasts (20). The paracrine factors secreted by ASCs may play a crucial role in extracellular matrix remodeling that contributes to improvements in LV function. Figure 1 depicts the proposed mechanism of how ASC transplantation mediates cardioprotection through differentiation, neovascularization, and paracrine effects on the host cells.

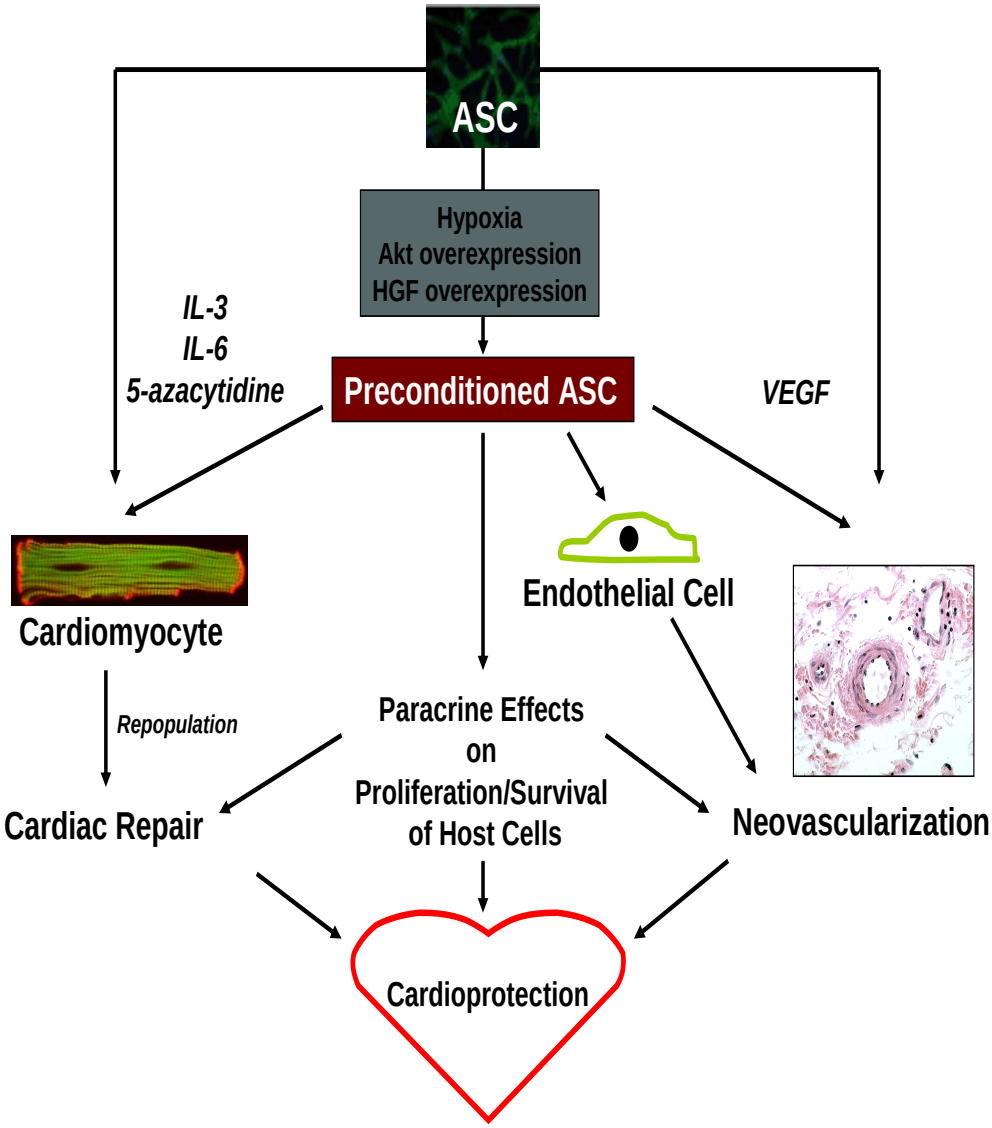

Figure 1. Proposed mechanism(s) of ASC transplantation in mediating cardioprotection. Addition of growth factors to the medium, preconditioning with hypoxia or adenoviral over-expression of Akt or HGF enhances differentiation into cardiomyocytes or endothelial cells while also inducing a greater survival paracrine effect in host cells.

Preconditioning of Stem Cells

Various animal studies show the potential to regenerate myocardium, improve perfusion to the infarct area, and improve cardiac function (69-72). Phase II and III clinical studies indicate that stem cell transplantation is feasible and may have beneficial effects on ventricular remodeling after myocardial infarction. However, the majority of transplanted cells are readily lost after transplantation because of the poor blood supply, ischemia/reperfusion injury, and inflammatory factors. Disconcerting reports have shown that up to 99% of transplanted cells are lost within the first 24 hours (73). Therefore, enhancing cell viability and reduction of apoptosis of ASCs in an ischemic microenvironment of the infarcted heart is critical for improving the efficiency of cell therapy. To improve the effectiveness of stem cell transplantation various methods have been employed to increase cell survival. Recently, Zhang et al. demonstrated that ischemic preconditioning of stem cells attenuated apoptosis induced by simulated ischemia (SI) and re-oxygenation (RO) (74). Subjecting cells to sublethal bouts of hypoxia prior to SI/RO resulted in preconditioning that correlated with stabilized membrane potential, upregulation of Bcl-2 and VEGF. Furthermore, there was an increased phosphorylation of ERK and Akt (74). Other models have shown that the effect of ischemic preconditioning can be mimicked pharmacologically by using phopshodiesterase-5 (PDE-5) inhibitor, sildenafil (75). Using mitoK_{ATP} channel opener, diazoxide, Ashraf et al. preconditioned skeletal myoblasts to promote their survival in the infarcted heart (76). Diazoxide preconditioning of the cells significantly induced expression of p-Akt, b-FGF, HGF, and COX-2. Treatment of cells with wortmannin prior to preconditioning abolished the effects and significantly reduced their survival thus

demonstrating the importance of the PI3K-Akt signaling cascade. Positive results have been demonstrated using genetic modulation of stem cells with transgenes that overexpress angiogenic growth factors such as Ang-1, VEGF, or Akt to improve cell survival, neovascularization, and cardiac function by limiting the remodeling process in the scar while decreasing apoptosis of myocytes in the peri-infarct region (77, 78). Moreover, genetically modified cells seem to function in autocrine and paracrine manner to confer therapeutic effects. Transplantation of cells over-expressing VEGF not only were protected from apoptosis but also reduced the apoptotic index of host myocytes. There was also improvement in regional blood flow in the myocardium leading to preservation of cells and myocardial structure. Jian et al. (77) showed the cytoprotective effects of co-overexpression of Ang-1 and Akt. Transduction with Ang-1 and Akt genes resulted in marked survival of the transplanted cells *in vivo*, their differentiation into myocytes and participation in neovascularization, which caused a reduced infarct size and optimally preserved cardiac function after MI (77).

PDE-5 Inhibitors

Phosphodiesterase type-5 (PDE-5) inhibitors are a class of vasoactive drugs that have been extensively used for treatment of heart failure, pulmonary hypertension, and coronary artery disease besides their use for the treatment of erectile dysfunction (79-82). The mechanism of action involves active inhibition of the PDE-5 enzyme resulting increase in cyclic guanosine monophosphate (cGMP) and smooth muscle relaxation in the penis. There are 11 families of PDEs that have been identified in mammalian tissues (83, 84). The PDEs vary in their substrate specificity for cyclic adenosine monophosphate (cAMP) and cGMP: PDE-5, PDE-6 and PDE-9

are specific for cGMP; PDE-4, PDE-7 and PDE-8 are specific for cAMP; and PDE-1, PDE-2, PDE-3, PDE-10 and PDE-11 have mixed specificity for cAMP/cGMP (85). PDE-5 inhibitors compete with the substrate cGMP for binding to the protein at the catalytic site. Although cGMP binding to the catalytic site stimulates cyclic-nucleotide binding to the allosteric sites, inhibitors do not elicit the same property, and Ser92 phosphorylation has no effect on inhibitor binding. PDE-5 is the primary enzyme with cGMP-hydrolyzing activity in human corpus cavernosal tissue (85). PDE-5 inhibitors have been studied extensively for their role in regulation of vascular tone and blood-flow balance during erection. During erection, nitric oxide (NO) is released from non-cholinergic, non-adrenergic neurons and from endothelial cells. NO diffuses into cells, where it activates soluble guanylyl cyclase, the enzyme that converts GTP to cGMP, which then stimulates protein kinase G (PKG) and initiates a protein phosphorylation cascade. This results in a decrease in intracellular levels of Ca2+ ions, leading to dilation of the arteries that bring blood to the penis and compression of the corpus cavernosum. A PDE-5 inhibitor inhibits enzymatic hydrolysis of cGMP by binding to the cGMP-catalytic sites thereby allowing the accumulation of cGMP in the erectile tissue (85).

PDEs are found in all tissues besides the human corpus cavernosal tissue, but the distribution of the PDEs varies among different tissues and cell types (86). PDE-5A has a wide distribution in the body tissues and cells where it exists in three isoforms; PDE-5A1, PDE-5A2, PDE-5A3 that only differ in their N-terminal sequence (87). Immuohistochemical studies have demonstrated the presence of PDE-5A isoforms in vascular and bronchial smooth muscle and in platelets. It is not clear whether PDE-5A is present in the human myocytes. However, a recent study by Senzaki et al. (88) provided evidence for PDE-5A expression in canine cardiomyocytes

and our laboratory reported expression in mouse heart (75). Despite these interesting observations, little is known about the distribution of PDE-5A in stem and progenitor cells. However, recently it has been reported that cultured bone marrow derived mesenchymal stem cells predominantly express PDE-5A (89).

The cytoprotective effects of PDE-5 inhibitors have been observed in heart cells, neurons and glia, and epithelial cells. The precise mechanisms for these protective effects are quite complex. We have demonstrated that sildenafil (Viagra®) and other PDE-5 inhibitors induce powerful protective effect against ischemia/reperfusion injury in the rabbit and mouse heart (90-94), DOX-induced cardiomyopathy (95, 96) and myocardial infarction-induced heart failure in mice (97). Furthermore, we showed that PDE-5 inhibition protects isolated adult cardiomyocytes from SI/RO. The cardioprotective effect is attributed to limiting apoptosis and necrosis through enhanced expression of nitric oxide synthases (NOS), particularly, eNOS/iNOS, activation of protein kinase C and PKG, phosphorylation of ERK1/2, PKG-dependent phosphorylation of GSK-3β, NO-dependent upregulation of Bcl-2/Bax and opening of the mitochondrial K_{ATP} channels (75, 91-93, 97, 98).

Nitric Oxide/cGMP/Protein Kinase G Signaling

Nitric Oxide (NO) is well recognized as a key mediator in cell signaling processes. It is produced from L-arginine through chemical reaction catalyzed by at least three major isoforms of nitric oxide synthase, *i.e.* neuronal (nNOS), inducible (iNOS), endothelial (eNOS). NO/cGMP/PKG signaling is a widely studied pathway in many tissues and cells, and reduced production and function of NO has been shown to participate in a number of disorders such as

cardiovascular, pulmonary, endothelial, renal, and hepatic diseases and erectile dysfunction. NO is produced and released from many cell types in the body where it acts as a paracrine signal in a number of systems, including the vasculature. NO at nanomolar levels binds tightly to a prosthetic heme on the β-subunit of NO-GC, also known as the soluble guanylyl cyclase, and causes activation of the enzyme (99, 100). Activation of NO-GC increases conversion of GTP to cGMP, resulting in elevation of cGMP, which initiates the cGMP-signaling pathway and subsequent physiological changes (101). NO-induced elevation of cGMP regulates numerous physiological processes including: relaxation of vascular smooth muscle, inhibition of platelet aggregation, inhibition of cytokine production, blunting of cardiac hypertrophy, and protection against myocardial ischemia/reperfusion injury (102-104). At the cellular level NO and cGMP regulate important processes such as growth, survival, differentiation, proliferation, and migration. These effects are largely mediated through activation of cGMP dependent protein kinase I, PKG. (105-108). cGMP-gated channels, PDEs and PKGII are also important targets for cGMP actions.

PKG is a serine/threonine protein kinase and is one of the major intracellular receptors for cGMP. PKG is present in high concentrations in smooth muscle, platelets, cerebellum, hippocampus, dorsal root ganglia, neuromuscular end plate, and the kidney vasculature (109). PKGI isozymes (PKGIα and PKGIβ) are products of alternative splicing and differ only in the N-terminal amino acids (110, 111). PKG Iα is more sensitive to activation by cGMP. The activation of PKG phosphorylates many intracellular proteins and regulates important physiological functions such as relaxation of vascular smooth muscle, inhibition of cell differentiation and proliferation, and inhibition of platelet aggregation and apoptosis (109, 112). PKG also plays an

important role in the cardiovascular system. Recently, we reported that sildenafil-induced protection is dependent upon activation of PKG in adult mouse heart and cardiomyocytes (113). This notion is based on the fact that PKG inhibitors and selective knockdown of PKG by adenoviral vector containing short-hairpin RNA of PKG abolished the antinecrotic and antiapoptotic effect of sildenafil in cardiomyocytes, and PKG inhibition also abrogated the infarct size reduction by sildenafil in isolated mouse hearts. Furthermore, our labroratory also has shown the effect of PKG Iα overexpression in protecting cardiomyocytes from SI/RO induced necrosis and apoptosis (114).

Although research on stem cells has increased dramatically in recent years, there are very few studies on the role of the NO/cGMP/PKG pathway in stem cells (115- 121). It has been reported that the NO/cGMP/PKG signaling pathway has been proposed to promote stem cell-like characteristics in glioma cells in the tumor perivascular niche of medulloglioma (118). Interestingly, enhanced endogenous NO generation has been shown to positively regulate proliferation of neural progenitor cells through activation of cGMP/PKG signaling pathway (119). Recent studies have suggested the role cGMP-mediated NO signaling plays in the differentiation of embroyonic stem cells into myocardial cells (120, 121). Furthermore, expressions of mRNA and protein levels of the three NOSs and sGC, along with expression of PKG have been shown to increase during differentiation (116). However, the role of NO/cGMP/PKG signaling in protection of ASCs against ischemia has not been investigated.

Rationale for Study:

The present study, a series of novel investigations designed to examine the feasibility of effect of PDE-5 inhibition as a strategy to precondition human ASCs for improving their efficacy *in vivo* after cardiac transplantation. The rationale for this approach was the established powerful preconditioning-like effect of PDE-5 inhibitors in cardiomyocytes (75, 98, 114) and against ischemia/reperfusion injury in heart (92-94) previously established by our laboratory. The purpose of the following study is to investigate the effect of PDE-5 inhibition of ASCs for transplantation to attenuate adverse cardiac remodeling and preserve of cardiac function in a chronic model of MI. Furthermore, we wanted to attain a better understanding of the signaling pathways involved, which ultimately lead to enhancement of stem cell therapy.

Accordingly, the main aims of the present study were:

Aim #1) to investigate whether PDE-5 inhibition could confer cytoprotection of ASCs against SI/RO injury *in vitro*;

Aim #2) to demonstrate the role of cGMP-dependent PKG signaling in protection of ASCs;

Aim #3) to show that *in vivo* transplantation of ASCs after *ex vivo* PDE-5 inhibition improve LV function following myocardial infarction;

Aim #4) to examine the possible role of paracrine mechanism in enhancing the cytoprotective effects of PDE-5 inhibition.

CHAPTER 2
PDE-5 Inhibition Protects Adipose-Tissue Derived Stems Cells Against Ischemia Through a PKG-dependent Mechanism

ABSTRACT

Several studies have implicated cellular replacement therapy as a treatment strategy for myocardial infarction, although the results have been limited. Therefore, enhancing the therapeutic potential of stem cells injected into ischemic microenvironments by novel preconditioning strategies is critical for improving cellular therapy. We tested the hypothesis that inhibition of PDE-5 with sildenafil (Viagra®) or selective knockdown with a silencing vector in adipose derived stem cells (ASCs) would improve their survival after simulated ischemia/reoxygenation (SI/RO). ASCs were treated with sildenafil or infected with a PDE-5 silencing vector shRNA ($shRNA^{PDE-5}$) and subjected to SI/RO. Both sildenafil and $shRNA^{PDE-5}$ significantly reduced cell injury with improved viability, decreased lactate dehydrogenase release and reduced apoptosis. The preconditioned ASCs demonstrated an increase in the release of nitric oxide metabolites and growth factors including VEGF, b-FGF, and IGF into conditioned medium in which treatment protected adult cardiomyocytes from SI/RO. The cytoprotective effect seen in ASCs against SI/RO injury was abolished by inhibition of protein kinase G (PKG) with a pharmacological inhibitor and selective knockdown with $shRNA^{PKG}$. Our data shows that *in vitro* inhibition of PDE-5 using either genetic of pharmacological approaches can improve stem cell therapy following myocardial infarction.

Introduction

The main factor leading to the progression of heart failure is the irreversible loss of cardiomyocytes due to necrosis and apoptosis following ischemic injury. To overcome myocyte loss and the heart's limited self-regeneration capacity, recent research has focused on transplantation of stem cells to differentiate and replenish the loss of myocytes. Various animal studies have shown the potential to regenerate myocardium, improve perfusion to the infarct area, and improve cardiac function (69-72). Although cardiac performance by cell-based therapy has improved, unsatisfactory cell retention and transplant survival still plague this technique. The available transplantation strategies achieve modest engraftment of donor stem cells in the infarcted myocardium, primarily due to the rapid and massive loss of donor stem cells (122, 123). Several factors influence the accelerated cell death in the infarcted myocardium, including the ischemic and cytokine-rich microenvironment, mechanical injury, maladaptation, and the origin and quality of the donor cell preparation (124). Therefore, strategies targeted toward enhancing stem cell survival in the ischemic microenvironment are of paramount importance for improving cardiac regeneration. Previous studies have shown that treatment of bone marrow stem cells (BMSCs) with hypoxia improved survival post engraftment in the infarcted heart (125), increased proliferation rates and differentiation, and modulated their paracrine activity (126). In addition, various pharmacological preconditioning agents including diazoxide, an opener of mitochondrial K_{ATP} channel (77), vascular endothelial growth factor 2 (127), and IGF-1 (128) have been shown to promote myogenic response of stem cells following transplantation in the myocardium. Nevertheless, progressive strategies to improve the regenerative potential of stem cells are critical for their utility.

In the present study, we tested the hypothesis whether PDE-5 inhibition could improve the survival of adipose derived stem cells (ASCs,) leading to enhanced cardiac function following myocardial infarction in mice. Specifically we addressed the following questions: 1) Does PDE-5 inhibition by sildenafil or genetic knock-down with a silencing vector improve survival following simulated ischemia/reoxygenation (SI/RO) injury *in vitro*?; 2) What is the role of cGMP-dependent PKG signaling pathway in protection of ASCs?; 3) What is the role of paracrine mechanisms for enhancing cytoprotective effects of PDE-5 inhibition? Our results show that preconditioning of ASCs enhances release of cytokines such as VEGF, b-FGF, and IGF-1, stimulates NO metabolites, activates PKG, and increases cell viability after SI/RO, all of which were was abolished with PKG inhibition.

Materials and Methods

Isolation of Adipose Derived Stem Cells

Epicardial adipose tissue was harvested from voluntary patients undergoing transplant. Adipose tissue was mechanically disrupted with a scalpel and washed twice with phosphate buffered saline. Minced fat tissue was digested for an incubation period of 90 min at 37°C on a shaker in 20 mL of sterile filtered PBS containing 25 mg of Collagenase type VIII (Sigma-Aldrich) and 5 mM calcium chloride. The digested tissue was filtered through a 100 μm nylon mesh filter (Millipore). Filtrate was centrifuged at 800 x g for 10 min. The supernatant containing adipocytes and debris was discarded and the pelleted cells were washed twice with 40 mL Hank's Balanced Salt Solution (Cellgro). Freshly isolated ASCs were plated with α-MEM containing 20% FBS, 1% L-glutamine (0.2 M, Cellgro) and 1% Penicillin (10,000 U/mL) with Streptomycin (10 mg/mL, Cellgro). Plastic adherent cells were named human adipose tissue derived stem cells (ASCs) and were grown in culture at 37° C in a humidified incubator at 5% CO_2 followed by daily washing for three days to remove red blood cells and non-adherent hematopoietic cells. ASCs were then plated after 3 days for subsequent experiments at a density of 1000 cells/cm^2. Subsequent passages were performed with a 0.25% trypsin solution containing 0.01% EDTA for 6 minutes at 37°C.

Flow Cytometry

Cell surface antigen phenotyping was performed on ASCs that were harvested upon reaching 90% confluency. Cells were pelleted at 500 x g for 5 minutes at 4°C. Approximately,

1 x 10^6 ASCs were incubated for 1 h at 37°C with primary FITC conjugated antibodies at a dilution of 1:1000 for CD 14, CD29, CD44, CD45, CD105 and HLA-DR2 (Invitrogen, Molecular Probes.) Mild agitation was used every 10 min to further mix the solution and prevent cell clumping. No antibody controls were tested for each individual antibody. Cells were counted on a Beckman CoulterElite XL-MCL single-laser flow cytometer at a minimum of 10,000 counts. Positive results were defined as over 97% of cells expressing the surface protein of interest.

Differentiation of ASCs

Adipogenesis was induced in ASCs using a mesenchymal stem cell adipogenesis kit (Millipore) according to manufacturer's instructions. In brief, ASCs were plated at a density of 60,000 cells per well in a 24- well culture dish in 1 mL of medium and incubated at 37°C in a 5% CO_2 humidified incubator overnight. Once reaching 100% confluence, medium was aspirated and 1 mL of adipogenesis induction medium (low glucose DMEM containing 10% FBS, 1 μM dexamethasone, 0.5 mM IBMX, insulin (10 μg/mL), 100 μM Indomethacin, and 1 % Pen/Strep) was added. This medium change corresponds to differentiation day 1. Adipogenesis induction medium was changed every 2-3 days for 21 days. Positive staining of Oil Red-O indicated adipogenic phenotype.

Osteogenesis was induced in ASCs using HyClone AdvanceSTEM Osteogenic Differentiation Kit (Thermo Scientific) according to manufacturer's instructions. In brief, ASCs were plated at a density of 60,000 cells per well in a 24 well culture dish with 1 mL of medium and incubated at 37°C in a 5% CO_2 humidified incubator overnight. Once reaching 100%

confluence, medium was aspirated and 1 mL of osteogenic induction medium supplemented with 1 nM dexamethasone, 2 mM β-glycerophosphate, and 50 μM ascorbate-2-phosphate. ASCs were induced for 14 days and the the osteogenic medium was replaced every 2-3 days. Osteogenic mineralization was assessed after 21 days by staining with 40 mM Alizarin red (Sigma).

Immunocytochemistry

ASCs were cultured on sterile glass cover slips and fixed by incubation in 4% paraformaldehyde/ PBS for 20 min and permeabilized with 1.0% Triton X-100 in PBS for 10 min. Intracellular staining patterns and distribution of Oct-4, Sox-2, Nanog, and PDE-5 proteins were analyzed by immunostaining with incubation of respective antibodies at 4°C overnight (1:500 dilution) followed by incubation of FITC conjugated secondary antibodies at 37°C for 1 h (1:1000 dilution). Staining of 4',6-diamino-2-phenylindole (DAPI; Sigma) was used to visualize all nuclei.

Western blot analysis

Total soluble protein was extracted from ASCs with extraction buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 50 μg/ml PMSF, protease inhibitor cocktail (10μl/ml, Sigma), 0.3% β-mercaptoethanol as described by Qiu et al. (129). Homogenate was centrifuged at 14,000 x g for 10 min at 4 °C, and the supernatant was recovered as the total cellular protein. Total protein (50 μg) from each sample was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and then blocked with 5% nonfat dry milk in Tris-

buffered saline. The membrane was subsequently incubated with a primary antibody at a dilution of 1:500 for each of the respective proteins, *i.e.* PDE-5A, Oct-4 (rabbit polyconal), PKG, β-actin (goat polyclonal, Santa Cruz), SOX-2, and Nanog (mouse monoclonal, BD Biosciences). The membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution, 1 h at room temperature). The membranes were developed using enhanced chemiluminescence system (ECL Plus; Amersham Biosciences) and exposed to X-ray film.

Preparation of shRNAs

PDE-5 gene silencing shRNA (inserted into miRNA-155 cassette) and gfp-PDE-5 fusion protein both coupled to a CMV promoter and incorporated into adenoviral vectors were generated by Zhang et al (130). In brief**,** short-hairpin RNAs were designed based on mouse PDE5A sequence

(shRNA$^{PDE5\text{-}1899}$: FORWARD 5′-TGCTGTTTCAGAGCAGCAAACATGCAGTTTTGGCCACTGACTGACTGCATGTTCTGCTCTGAAA-3′ and REVERSE 5′-CCTGTTTCAGAGCAGAACATGCAGTCAGTCAGTGGCCAAAACTGCATGTTTGCTGCTCTGAAAC-3′; shRNA$^{PDE5\text{-}2066}$ FORWARD 5′-TGCTGAAATGATGGTGTTCCATGATGGTTTTGGCCACTGACTGACCATCATGGCACCATCATTT-3′ and REVERSE 5′-CCTGAAATGATGGTGCCATGATGGTCAGTCAGTGGCCAAAACCATCATGGAA-3′)

and inserted into pcDNA 6.2-GW/EmGFP-miR-155 vector (Invitrogen) retaining miRNA regulatory sequences required for efficient shRNA processing. This was transferred by recombinase cloning into pAd/CMV/V5-DEST™ vector (Invitrogen) to generate AdV-*gfp*-shRNA^{PDE5A}. AdV was CsCl purified and titered at 1.5–3.0 x 10^{10} pfu/ml. Green fluorescent protein (*gfp*) enabled infection to be confirmed (AdV-*gfp* virus was the control), and the miRNA construct allowed use of the CMV promotor enhancing gene knock-down.

The mouse PKGI, shRNA expression vector for PKG was constructed as described previously (98). In brief, to knockdown the expression of PKG, we used shRNA, targeting the mouse cDNA of PKG type I (GenBank™ accession number NM_001013833): corresponding to bases 1593 to 1611, targeting the sequence 5′-GAACAAAGGCCATGACATT-3′, synthesized by Dharmacon Research Inc. (Lafayette, CO). A non-targeting scrambled RNA duplex siRNA control (NTSC, Dharmacon) containing 21-nucleotide sequences demonstrating no homology to murine genes was also used as a control for transfection. Transient transfections of duplex siRNAs (100 nM) were performed in H9C2 cells using siPORT™ amine (Ambion). After 48 h, RNA was isolated by Tri-Reagent (Molecular Research Center), and RT-PCR and quantitative real-time PCR were performed. After confirming the significant reduction of PKG expression in H9C2 cells using this duplex siRNA targeting the mouse PKGI, shRNA expression vector for PKG was constructed using the pSilencer™ adeno1.0-CMV system from Ambion (Adenoviral siRNA expression Vector System). The hairpin siRNA oligonucleotide (55-mer) sequence 5′-TCGAGGAACAAAGGCCATGACATT*ttcaagaga*AATGTCATGGCCTTTGTTCAGA-3′ (mouse PKGI with sequence in capital letters and loop in lowercase italics) and its antisense with

XhoI and SpeI were synthesized, annealed, and subcloned into the pSilencer adeno 1.0-CMV shuttle vector. HEK293 cells were transfected with linearized shRNA vector together with adenoviral LacZ backbone to generate a recombinant adenovirus.

Simulated Ischemia/Reoxygenation Protocol

ASCs were incubated at 37 °C and 5% CO_2 for 2 h, with or without 10 μM sildenafil. This dose was selected based on its protective effect against SI/RO injury in adult cardiomyocytes (75). A subset of ASCs were treated with PKG inhibitor KT 5823 (2 μM) with or without sildenafil for 2 h. Another subset of ASCs were transduced with an adenoviral vector containing scrambled control shRNA ($shRNA^{Con}$ ASC), PDE-5 shRNA ($shRNA^{PDE-5}$ ASC), or PKG shRNA ($shRNA^{PKG}$ ASC) in serum-free growth medium for 24 h. (Fig. 2). The cells were infected with the viruses at a concentration of $1x10^3$ particles/cell. ASCs were then subjected to SI for 15 h by replacing the cell medium with an "ischemia buffer" that contained 118 mM NaCl, 24 mM $NaHCO_3$, 1.0 mM NaH_2PO_4, 2.5 mM $CaCl_2$-$2H_2O$, 1.2 mM $MgCl_2$, 20 mM sodium lactate, 16 mM KCl, 10 mM 2-deoxyglucose (pH adjusted to 6.2) as reported previously (75). Cells were incubated in an anoxic chamber at 37°C during the entire SI period. RO was accomplished by replacing ischemic buffer with normal cell medium under normoxic conditions. Cell necrosis and apoptosis were assessed after 1 or 18 h of reoxygenation, respectively.

Evaluation of Cell Viability and Apoptosis

Cell viability, trypan blue exclusion assay and lactate dehydrogenase release into the medium were used to assess cell necrosis. Cell viability assessment was performed with

CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (Promega), per the manufacturer's instructions. In brief, approximately 5,000 ASCs/well were plated into a 96-well dish and allowed to attach overnight prior to SI/RO protocol. Following completion of SI/RO, 20µl of CellTiter 96® AQueous One Solution Reagent was added into each well and incubated for 2 h at 37°C in a 5% CO_2 before recording absorbance at 490 nm using a VersaMax microplate reader with SoftMaxPro software (Molecular Devices). CellTiter 96® AQueous One Solution uses a MTS tetrazolium compound which is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. The quantity of formazan product as measured by the absorbance at 490 nm is directly proportional to the number of living cells in culture.

ASCs were plated at a density of $2x10^5$ cells/mL in 2-well chamber slides prior to SI/RO protocol. Following SI/RO, 100 µL of cellular medium was collected for LDH measurements, and enzyme activity was monitored spectrophotometrically using an LDH assay kit (Sigma). NAD+ is reduced to NADH/H+ by LDH-catalyzed conversion of lactate to pyruvate. The catalyst (diaphorase) transfers H/H+ from NADH/H+ to tetrazolium salt which is reduced to formazan. An increase in the number of dead cells leads to an increase in LDH activity in the culture medium which correlates to amount of formazan dye formed which is measured at 490 nm. Trypan blue exclusion assay was performed as follows. Following SI/RO, floating and attached cells were collected by centrifugation and cell pellets were resuspended and mixed with 20 µl of 0.4% trypan blue (Sigma). After ~ 5 min of equilibration, dead cells, stained by trypan

blue, were counted using a hemocytometer. The number of dead cells was counted from five randomly chosen fields and expressed as a percentage of the total number of cells.

Apoptosis was analyzed by the terminal dUTP nick-end labeling method (TUNEL) staining using ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore) that detects nuclear DNA fragmentation as previously reported (98). After SI and 18 h of reoxygenation, cells were fixed by 4% formaldehyde/PBS at 4 °C for 25 min and subjected to TUNEL assay according to the manufacturer's protocol. In brief, cells were washed twice in PBS for 5 min before being permeabilized with precooled ethanol:acetic acid 2:1 for 7 min at -20°C. Cells were washed twice in PBS for 5 min before incubation 3.0% hydrogen peroxide in PBS for 5 minutes at room temperature to quench endogenous peroxidase. Cells were washed twice and incubated with terminal deoxynucleotidyl Transferase (TdT) enzyme for 1 h at 37°C in a humidified chamber. After the 1 h incubation period, cells were washed 3 times in PBS prior to incubation with anti-digoxignenin conjugate for 30 min at room temperature. Slides were developed with Nova Red peroxidase substrate (Vector Lab), counterstained with hemotoxylin and mounted using Permount solution (Fisher Scientific). Stained cells were examined under a Nikon Eclipse TE 800 microscope. TdT catalyzes a template-independent addition of nucleotide triphosphates to the 3'- OH ends of double-stranded or single-stranded DNA. The incorporated nucleotides form an oligomer composed of digoxigenin-conjugated nucleotide and unlabeled nucleotide in a random sequence. DNA fragments which have been labeled with the digoxigenin-nucleotide are then allowed to bind an anti-digoxigenin antibody that is conjugated to a peroxidase reporter molecule. The bound peroxidase antibody conjugate enzymatically generates a permanent,

intense, localized stain from chromogenic substrates, providing sensitive detection of apoptotic bodies.

cGMP, cAMP and Protein Kinase G Activity

cGMP activity assay was performed using cGMP Direct Immunoassay Kit (Biovision) which provided a direct competitive immunoassay for sensitive and quantitative determination of cGMP as per manufacturer's instructions. Briefly, the cell lysate is incubated with a cGMP polyclonal antibody at room temperature for 1 h and the excess reagents are washed away. The substrate is added and after a short incubation period, the enzyme reaction was stopped and the yellow color intensity was measured using VersaMax microplate reader at 405nm. The intensity of the bound yellow color is inversely proportional to the concentration of cGMP in either standards or samples. The measured optical density is used to calculate the concentration of cGMP. Protein concentration of lysate was measured spectrophotometrically at 595 nm. The results are expressed as pmol/mg of protein.

Cellular levels of cAMP were measured using bioluminescent assay, cAMP-Glo (Promega) as per manufacturer's instructions. In brief, approximately 5,000 ASCs/well were plated into a 96-well dish and allowed to attach overnight prior to respective treatment. Cells were lysed with incubation of cAMP-Glo Lysis buffer for 15 minutes at room temperature prior to addition of cAMP-Glo Detection Solution. After 20 min at room temperature, Kinase-Glo Reagent was added, mixed for 60 s and incubated at room temperature for 10 min. Luminescence was measured using VersaMax microplate reader. Luminescence is inversely proportional to cAMP levels. The results are expressed as pmol/mg of protein.

Cardiac protein kinase G activity was examined using a commercially available PKG activity kit (Cyclex) in whole cell lysates (n = 4/group). Activity was measured according to the manufacturer's instructions. In brief, total soluble protein was extracted from ASCs with extraction buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 50μg/ml PMSF, protease inhibitor cocktail (10μl/mL), and 0.3% β-mercaptoethanol. Cell extracts (10 μL) were plated in duplicate in a 96-well plate along with 90 μL of cGMP plus Kinase Reaction Buffer per well. The plate was covered with plate sealer, and incubated at 30°C for 30 min. Wells were washed 5 times with Wash buffer prior to addition of 100 μL of HRP conjugated Detection Antibody 10H11. The plate was recovered and incubated at room temperature for 60 min. The enzyme reaction was stopped and the spectrophotometric absorbance was measured at 450 nm. Results were normalized as per mg of protein.

Measurement of VEGF, b-FGF, Ang-1and IGF by ELISA

The levels of VEGF, b-FGF and IGF released from ASCs into culture medium were directly measured by ELISA kit according to manufacturer's instructions (R & D Systems). In brief, conditioned medium was added to a microplate pre-coated with the respective growth factors for a 2 h incubation period at room temperature. Basal medium was used as a control. The plate was washed 3 times to remove any unbound substances prior to incubation with the respective enzyme-linked polyclonal antibody conjugate. Following washing to remove any unbound antibody-enzyme reagent, substrate solution is added to the wells and color develops in proportion to amount bound of respective growth factor. The absorbance was measure at 450 and 570 nm.

Measurement of Nitrate, Nitrite and NOx

ASCs were plated at a density of $2x10^5$ cells/mL in 10 mm^2 culture dishes. ASCs were incubated at 37 °C and 5% CO_2 for 2 h, with or without 10 μM sildenafil A subset of ASCs were transduced with an adenoviral vector containing scrambled control shRNA ($shRNA^{Con}$ ASC), PDE-5 shRNA ($shRNA^{PDE-5}$ ASC), in serum-free growth medium for 24 h. Conditioned medium was collected 24 h after treatment and was subsequently centrifuged using Amicon Ultra-4 centrifugal filter devices at 7500 *g* in 4^oC to eliminate large molecules (molecular weight >30 kDa) from the medium. The levels of nitrate and nitrite in the conditioned medium were measured with a SIEVERS nitric oxide analyzer (model 280NOA). The reducing agents used were either vanadium (III) chloride (VCl_3) in 1 M HCl (for nitrate) or 1% sodium iodide (NaI) in glacial acetic acid (for nitrite). Five to six mL of a reagent plus 100 μL of 1:30 diluted anti-foaming agent were loaded into the purge vessel for analysis. These reducing agents converted nitrite and nitrate respectively to gaseous NO at 90°C, which was quantified by the analyzer. The results are expressed in μM of nitrite, nitrate, and NOx (total levels of nitrate and nitrate).

Isolation of Adult Mouse Ventricular Cardiomyocytes

Adult male CD1 (Harlan Sprague Dawley) were used in this study. The animal experimental protocols were approved by the Institutional Animal Care and Use committee of Virginia Commonwealth University. The ventricular cardiomyocytes were isolated using an enzymatic technique modified from the previously reported method (131, 132). In brief, the animal was anesthetized with pentobarbital sodium (100 mg/kg, i.p.) and heart was quickly

removed. Within 3 min, the aortic opening was cannulated onto a Langendorff perfusion system and heart was retrogradely perfused (37 ^{o}C) at a constant pressure of 55 mmHg for ~5 min with a Ca^{2+} free bicarbonate-based buffer containing: 120 mM NaCl, 5.4 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 5.6 mM glucose, 20 mM $NaHCO_3$, 10 mM 2,3-butanedione monoxime, and 5 mM taurine, which was continuously bubbled with 95% O_2 + 5% CO_2. The enzymatic digestion was commenced by adding collagenase type II (Worthington, 0.5 mg/mL each) and protease type XIV (0.02 mg/mL) to the perfusion buffer and continued for ~15 min. 50 μM Ca^{2+} was then added in to the enzyme solution for perfusing the heart for another 10-15 min. The digested ventricular tissue was cut into chunks and gently aspirated with a transfer pipette for facilitating the cell dissociation. The cell pellet was resuspended for a 3-step Ca restoration procedure (i.e. 125, 250, 500 μM Ca). The freshly isolated cardiomyocytes were then suspended in minimal essential medium (Sigma) containing 1.2 mM Ca, 12 mM $NaHCO_3$, 2.5% fetal bovine serum and 1% penicillin-streptomycin. The cells were then plated onto the 35 mm^2 cell culture dishes, which were pre-coated with 20 μg/mL mouse laminin in PBS + 1% penicillin-streptomycin for 1 hour. The cardiomyocytes were cultured in the presence of 5% CO_2 for 1 hour in a humidified incubator at 37°C, which allowed cardiomyocytes to attach to the dish surface prior to the experimental protocol.

In Vitro Experiments with Conditioned Medium

Conditioned medium was generated as follows: 90% confluent ASCs were treated with or without 10 μM sildenafil for 2 h prior to being subjected to 15 h SI and 1 h RO. The medium

was then collected and used for *in vitro* experiments. Adult mice cardiomyocytes were obtained from Langendorff-perfused hearts of adult CD-1 mice, as described previously. Cells were seeded in 2-well chamber slides precoated with laminin (30 µg/ml in PBS) and left to attach for 1 h. After attachment, the α-MEM medium was replaced with serum- free α-MEM (normal medium) or conditioned medium from either control or sildenafil-treated ASCs. The cardiomyocytes were then subjected to 40 min SI and 1 h RO for necrosis studies and 18 h RO for apoptosis studies.

Cardiomyocyte Viability and Apoptosis

Cell viability was assessed by trypan blue exclusion assay as reported previously (75, 98). Cardiomyocyte apoptosis was evaluated via TUNEL that detects nuclear DNA fragmentation via a fluorescence assay as previously reported (75, 98) using the ApoAlert™ DNA Fragmentation Assay Kit (BD Biosciences) according to manufacturer's instructions. In brief, after SI and 18 h of reoxygenation, cardiomyocytes in two chamber slides were fixed by 4% formaldehyde/phosphate-buffered saline at 4°C for 25 min and subjected to TUNEL assay according to the manufacturer's protocol. The slides were then counterstained with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (a DNA intercalating dye for visualizing nuclei in fixed cells, Vector Laboratories). The stained cells were examined under an Olympus IX70 fluorescence microscope. Apoptotic index (AI) was determined from counting TUNEL-positive myocyte nuclei from ten separate fields per treatment and expressed as a percentage.

Data Analysis and Statistics

Data are presented as mean ± S.E. The differences between groups were analyzed with one way analysis of variance followed by Student-Newman-Keuls post hoc test for pair-wise comparison. $P<0.05$ was considered to be statistically significant.

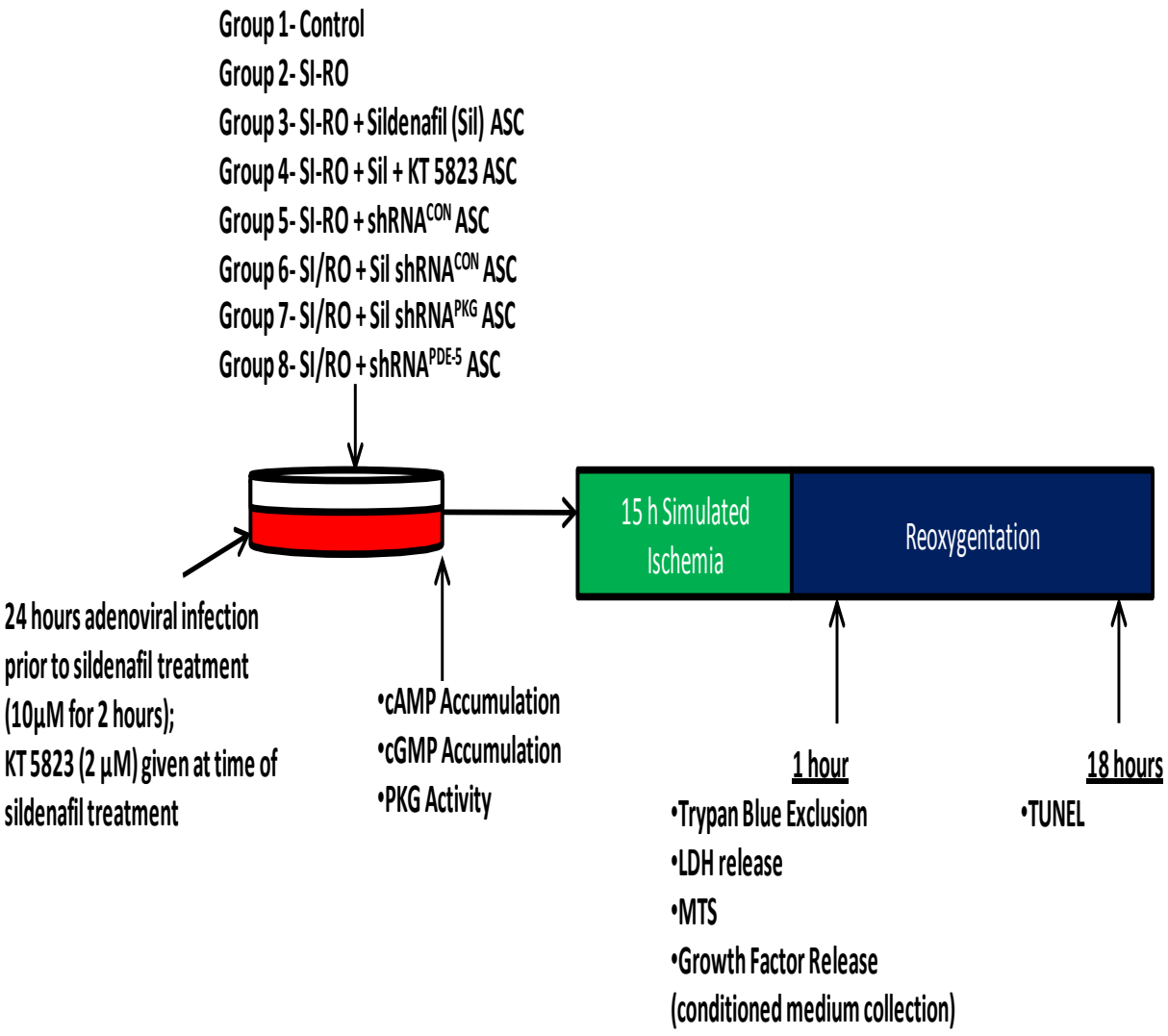

Figure 2. Experimental protocol. *In vitro* protocol, arrows indicate time points for treatment, performance of simulated ischemia/reoxygenation, and measurement of various parameters for each experimental group.

Results

Characterization of ASCs

Fluorescence activated cell sorting analysis showed that isolated ASCs expressed common surface expression markers CD29 (immune response), CD44 (cell-cell interactions, cell adhesion and migration), and CD105 (angiogenesis). The cells were devoid of markers such as CD14 (monocytes), CD45 (hematopoietic cells), and Human Leukocyte Antigen receptor DR2 (HLA-DR2) (Table 1). Immunostaining showed intense nuclear and cytosolic expression of the pluripotent stem cell transcription factors including Oct-4, Sox-2 and Nanog in the ASCs (Fig. 3A). The expression of these proteins was also confirmed by Western blot analysis. Treatment with sildenafil did not alter their expression (Fig. 3B). To demonstrate the differentiation capacity of ASCs *in vitro*, adipogenic and osteogenic lineage specific induction factors were used in the normal culture conditions. Adipogenic and osteogenic phenotype was determined by staining monolayers of ASCs with Oil Red-O and Alizarin Red respectively. The ASCs showed multiple intracellular lipid filled droplets in ~30% of cells confirming adipogenic differentiation (Fig. 3C) and calcium deposition with Alizarin Red confirming osteogenic differentiation (Fig. 3D).

Table 1. Surface Characterization of Isolated ASCs. Surface Marker	**Positive or Negative**	**Involvement**
CD29	+	immune response
C105	+	angiogenesis
CD44	+	cell-cell interactions, cell adhesion and migration
CD14	-	monocytes
CD45	-	hematopoietic cells
Human Leukocyte Antigen receptor DR2 (HLA-DR2)	-	graft vs. host immune response

Positive results were defined as over 97% of cells expressing the surface protein of interest.

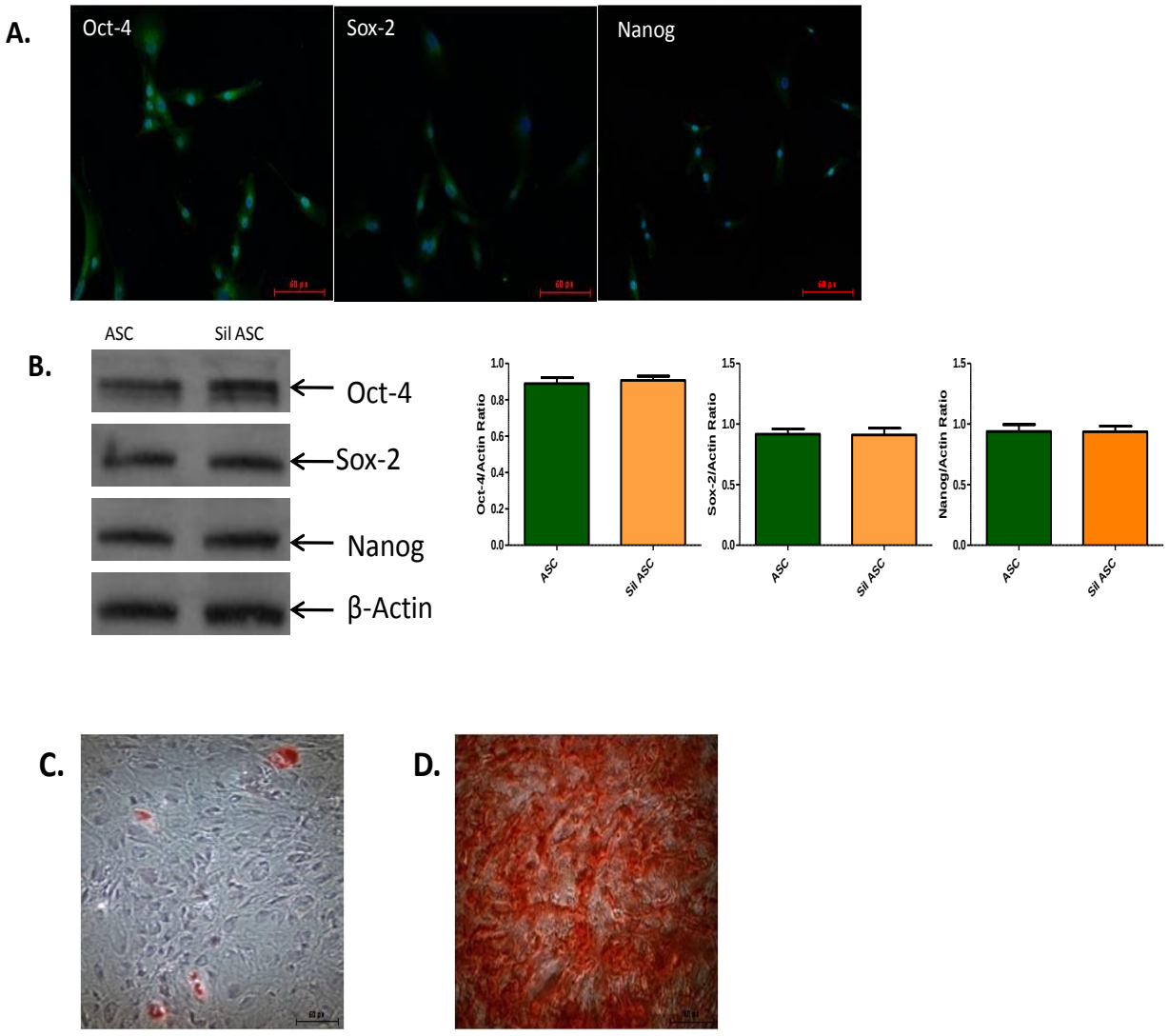

Figure 3. Expression of transcription factors and differentiation of ASCs. (A) Immunohistochemical staining of Alexa 488 labeled Oct-4 (left panel- green), Sox-2 (middle panel- green), and Nanog (right panel- green) overlayed with DAPI staining of nuclei (blue). (B) Western Blot analysis from ASC lysate show expression of stem cell transcription factors Oct-4, Sox-2 and Nanog. Treatment with sildenafil (SIL) did not alter their expression. (C) Adipogenesis and lipid vesicle formation was determined by Oil Red-O staining. (D) Mineralization was detected in osteogenic-differentiated ASCs using Alizarin red staining.

Cytoprotection by PDE-5 Inhibition

Immunostaining showed that ASCs express PDE-5, which was localized within the cytoplasm (Fig. 4). Moreover, adenoviral infection was confirmed by the presence of GFP expression in $shRNA^{CON}$ and $shRNA^{PDE-5}$. PDE-5 expression was also confirmed by Western blot and real time-PCR. $shRNA^{PDE-5}$ efficiently silenced PDE-5 in ASCs at gene and protein expression levels (Figs. 4 and 5). Adenovirally transduced or normal cultured ASCs were treated with or without 10 μM sildenafil for 2 h. The percentage of trypan blue-positive (necrotic) cells increased to 24.2±3.7 as compared to non-SI/RO controls (0.6±0.1) following SI (15 h)/RO (1 h) ($n=8$; $p<0.01$). Sildenafil treatment reduced cell death as measured by MTS-based cell incorporation (Fig. 6A) and necrosis as shown by decrease in trypan blue-positive cells to 5.7 ±1.6% ($n=8$; $p<0.01$, Fig. 5B). PDE-5 knockdown by $shRNA^{PDE-5}$ conferred a similar protective effect when compared to the scrambled $shRNA^{CON}$ ASCs (7.1 ±0.9 vs. 29.3 ±1.3%, $p < 0.01$, $n = 8$; Fig. 6). Also, combination of $shRNA^{CON}$ with sildenafil increased cell viability and reduced necrosis (Fig. 6). Similar results were obtained when lactate dehydrogenase (LDH) release in the medium was used as a marker of necrosis in ASCs (Fig. 6C).

After 15 h of SI and 18 h of RO, apoptotic nuclei (TUNEL-positive cells) increased from 2.0±0.5 % (in non-ischemic control group) to 18.3±2.5 % of total cells ($p < 0.01$, $n = 8$;). PDE-5 inhibition resulted in reduction of TUNEL-positive cells to 5.7± 2.1% in sildenafil-treated ASCs and 6.4± 0.9% in $shRNA^{PDE-5}$ ASCs ($p < 0.01$ vs. SI-RO, $n = 8$; Fig. 7). These results suggest that PDE-5 inhibition in ASCs exerts a cytoprotective against ischemic injury.

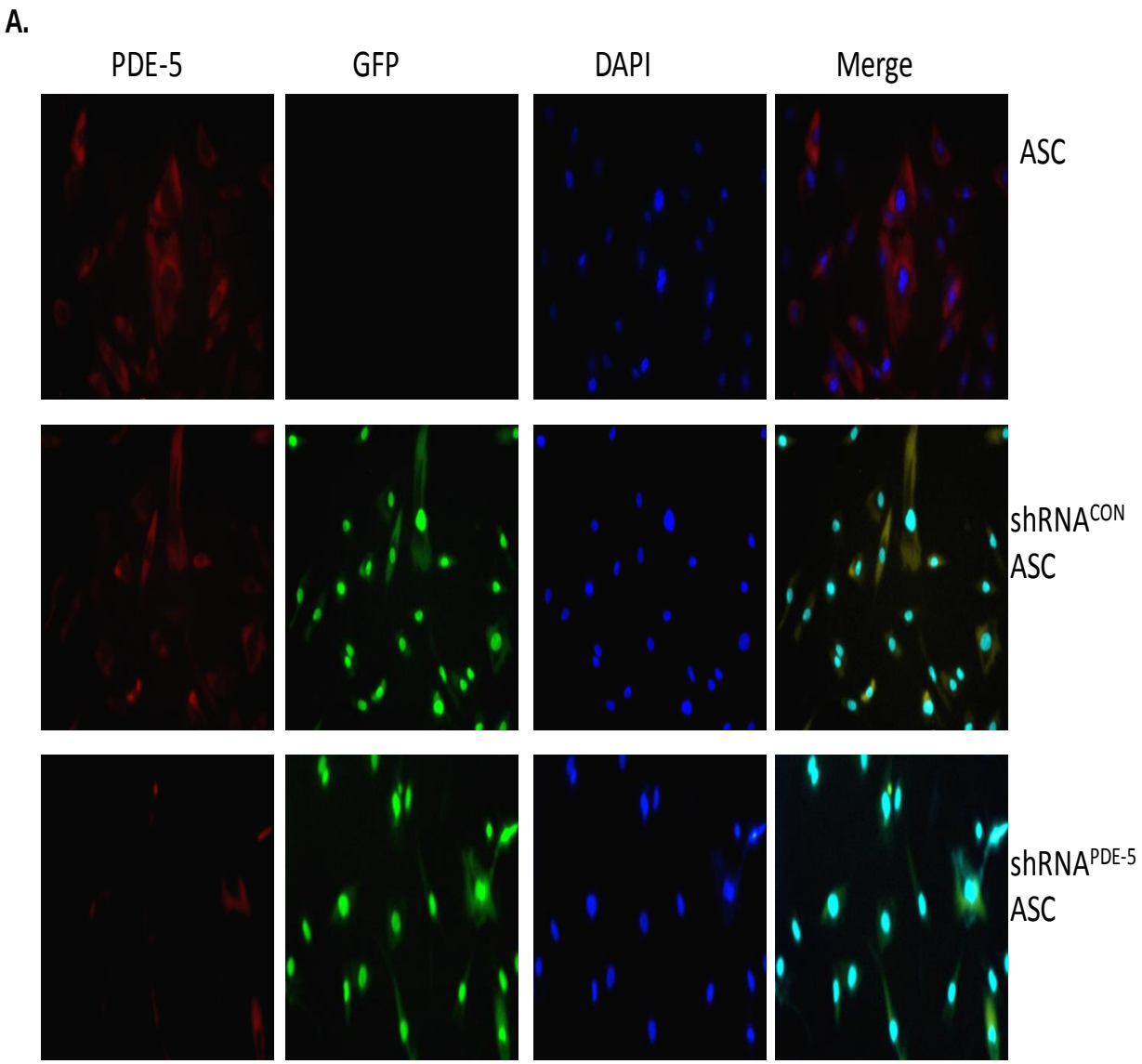

Figure 4. PDE-5 immunofluoresence in ASCs. Immunohistochemical staining of PDE-5 (red), GFP (green), DAPI staining of nuclei (blue) and overlay in ASCs with $shRNA^{PDE-5}$ as compared to $shRNA^{CON}$ ASCs and control ASCs.

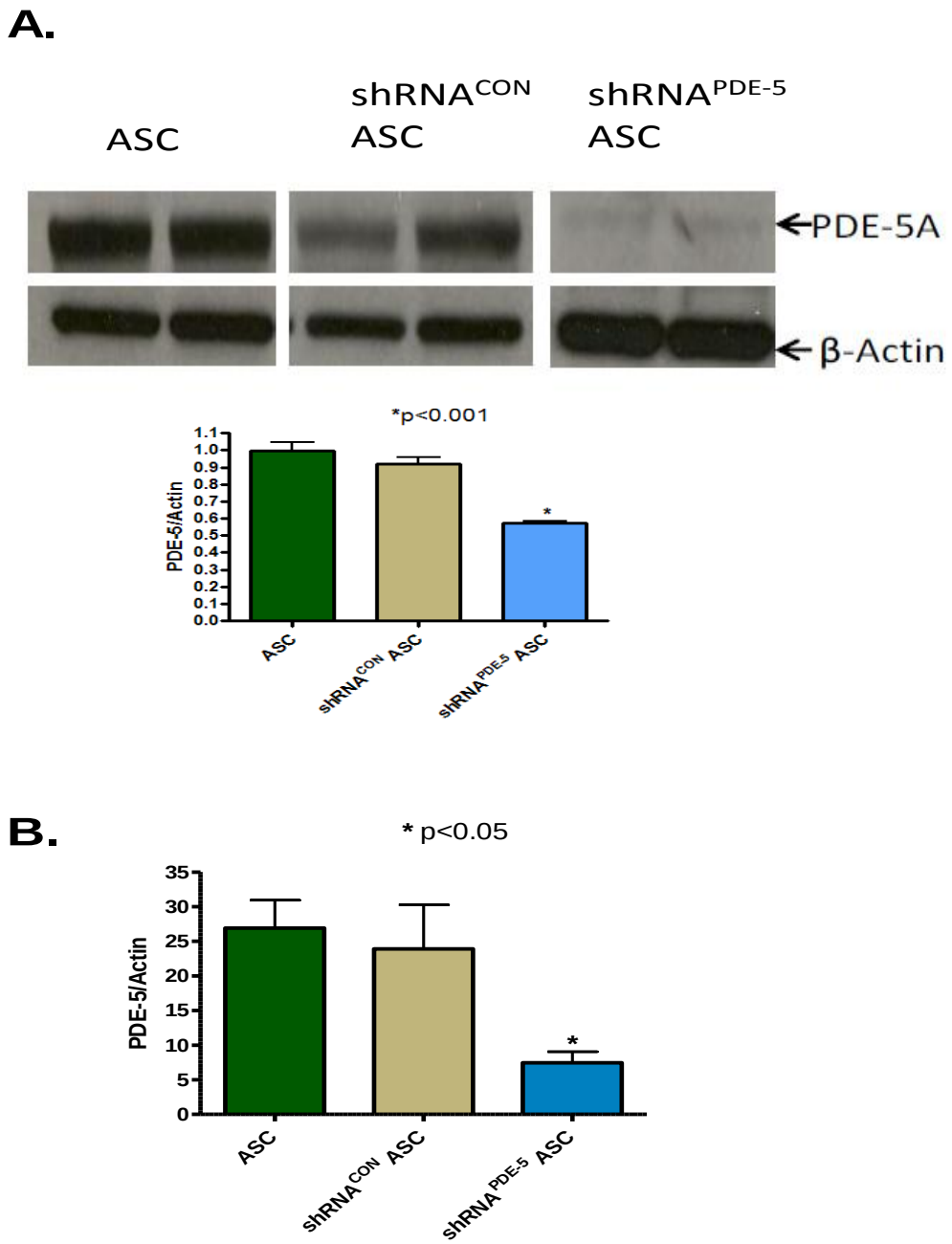

Figure 5. Expression of PDE-5 in ASCs (A) Western blot analysis showing knock-down of PDE-5 in ASCs with shRNA^{PDE-5} as compared to shRNA^{CON} ASCs and control ASCs (representative lanes from contiguous blot; n=3). (B) Real-time PCR showing reduced PDE-5 expression; (n=3).

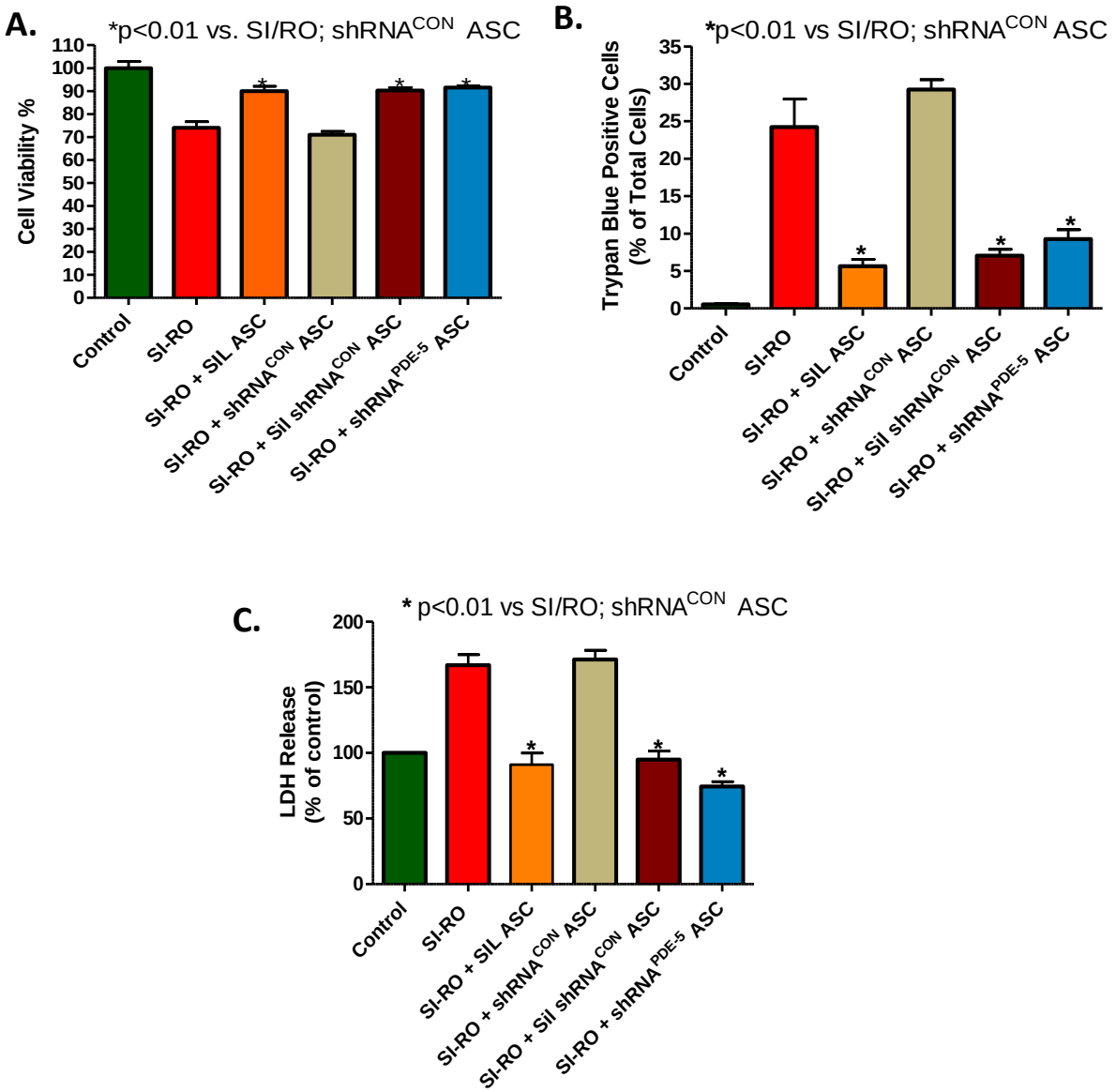

Figure 6. Effect of PDE-5 iinhibition on protection of ASCs from necrosis. Quantitative data showing the effect of sildenafil (SIL) or shRNA^{PDE-5} on necrosis following SI-RO as determined by MTS cell viability assay (A), trypan blue staining (B), and LDH release (C);(n=8).

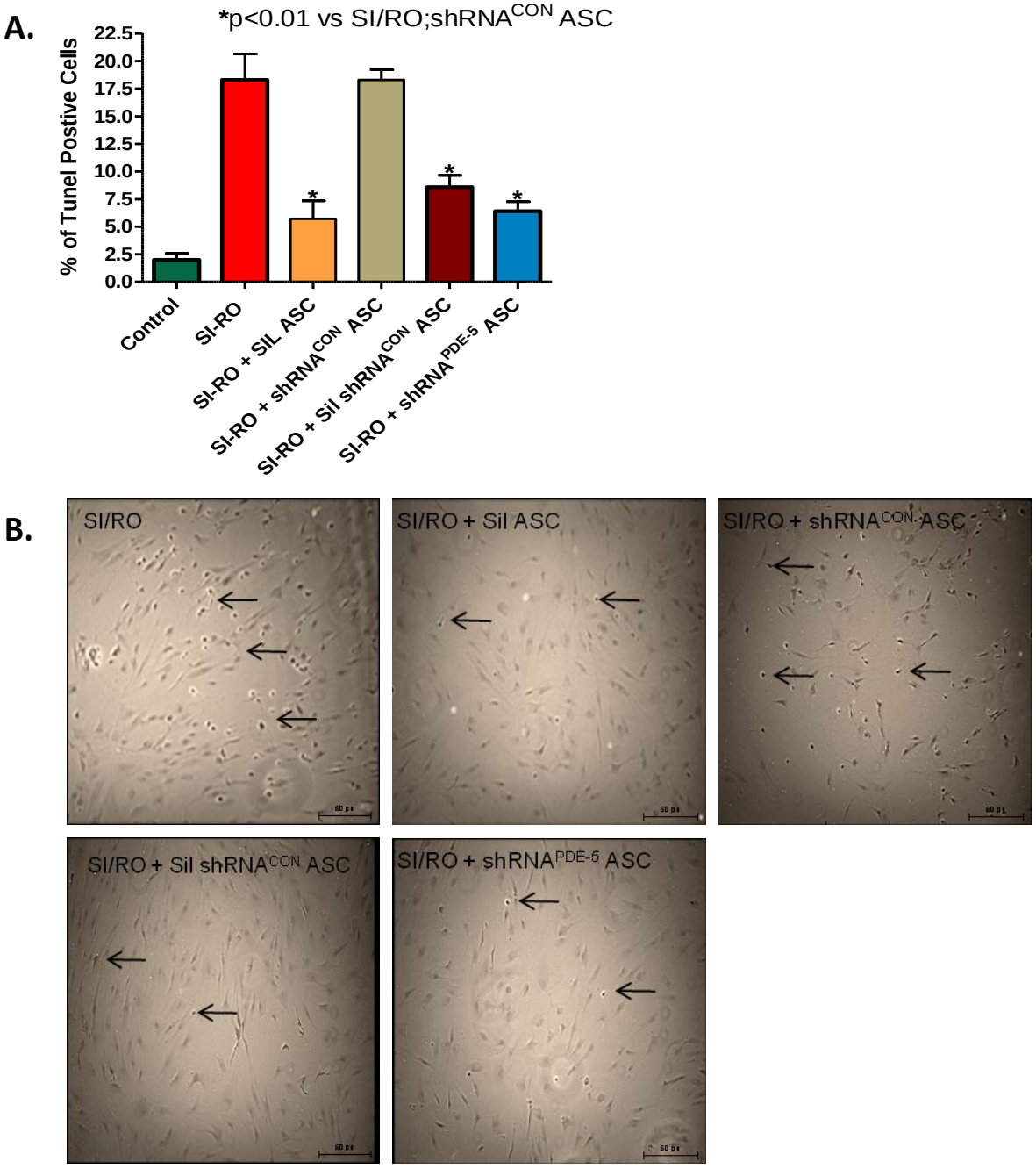

Figure 7. Effect of PDE-5 inhibition on protection of ASCs from apoptosis. Quantitative data showing the effect of sildenafil (SIL) or shRNA^{PDE-5} on apoptosis following SI-RO as determined by TUNEL assay (A) and representative images (B); n=8).

Effect of PDE-5 Inhibition on cGMP and PKG Activity

PDE-5 inhibition by sildenafil or shRNA^{PDE-5} resulted in nearly identical increase in cGMP levels (0.9±0.01 and 0.9±0.02 pmol/mg of protein, respectively) as compared to non-treated ASCs (0.7±0.03) and shRNA^{CON} ASCs (0.7±0.03) ($p<0.05$, $n=4$). shRNA^{CON} had no effect on cGMP formation with sildenafil treatment (Fig. 8A). Both sildenafil and shRNA^{PDE-5} had no effect on cAMP levels (Fig 8B). Also, sildenafil and shRNA^{PDE-5} increased PKG enzymatic activity (A450/mg protein) as compared to control ASCs (Fig. 8D).

To determine the cause and effect relationship of PKG in sildenafil-induced survival of ASCs following SI/RO, we used shRNA knockdown of PKG and pharmacological inhibition approach as reported previously (99). ASCs infected with shRNA^{PKG} caused at least 60% knock-down of PKG (Figs. 8C). Moreover, sildenafil induced PKG activity was inhibited in shRNA^{PKG} ASCs or by co-treatment with PKG inhibitor, KT 5823 (Fig. 8D). The protective effect of sildenafil against necrosis and apoptosis (Figs. 6, 7) were attenuated by KT 5823 and in shRNA^{PKG} ASCs (Figs. 9, 10) suggesting that inhibition of PDE-5 induced protection involves a PKG dependent pathway.

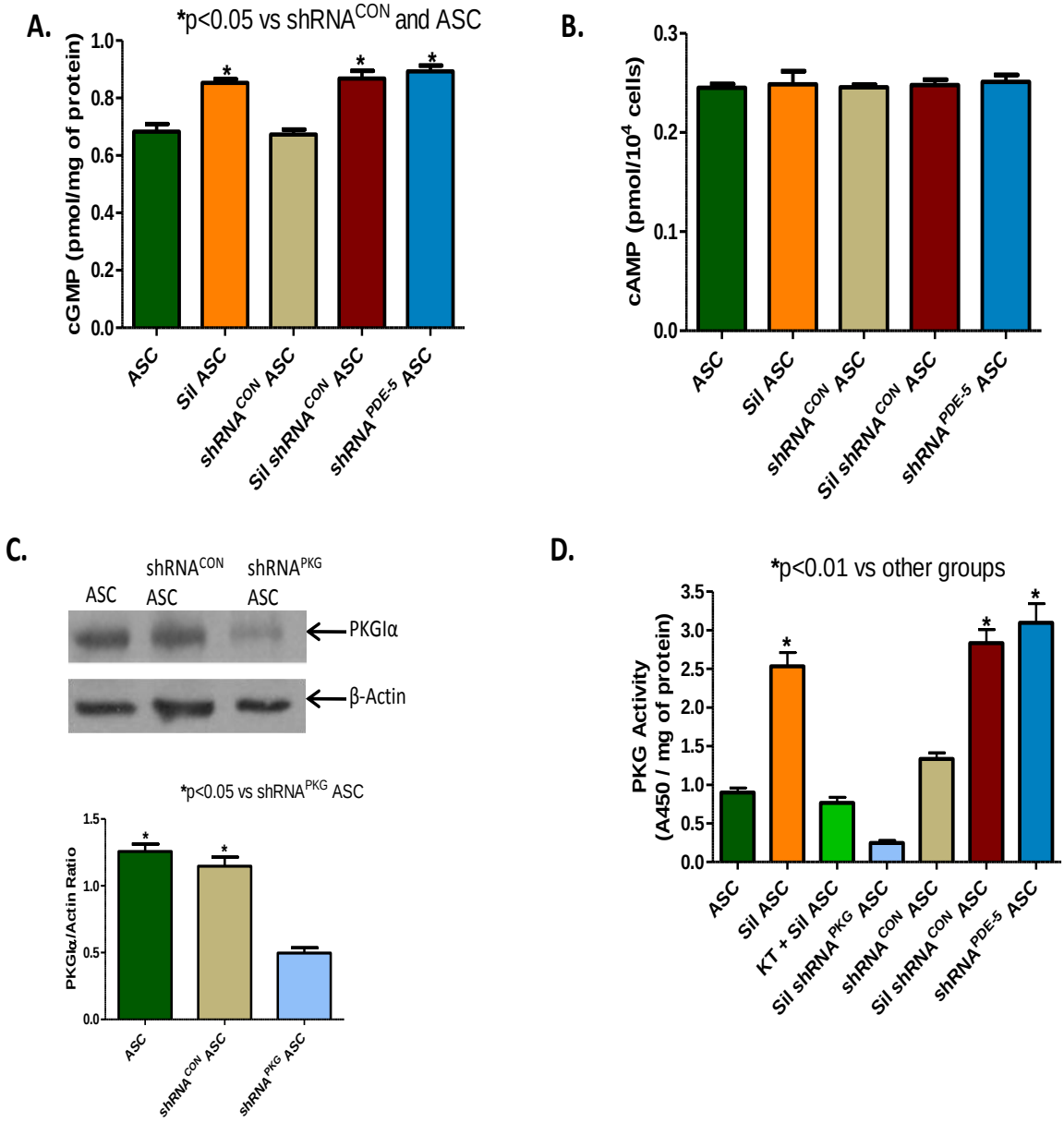

Figure 8. PDE-5 inhibition increases cGMP and PKG activity. (A) cGMP levels in ASCs following preconditioning with sildenafil and shRNA^{PDE-5} as compared to shRNA^{CON} and non-treated ASCs. (B) cAMP levels in the same groups as in A. (C) Western blot analysis showing knockdown of PKG expression as compared to controls (D) PKG activity in ASCs following treatment with sildenafil or shRNA^{PDE-5}. Treatment with 2 µM KT 5823 (KT) or shRNA^{PKG} inhibited PKG activity; (n=4).

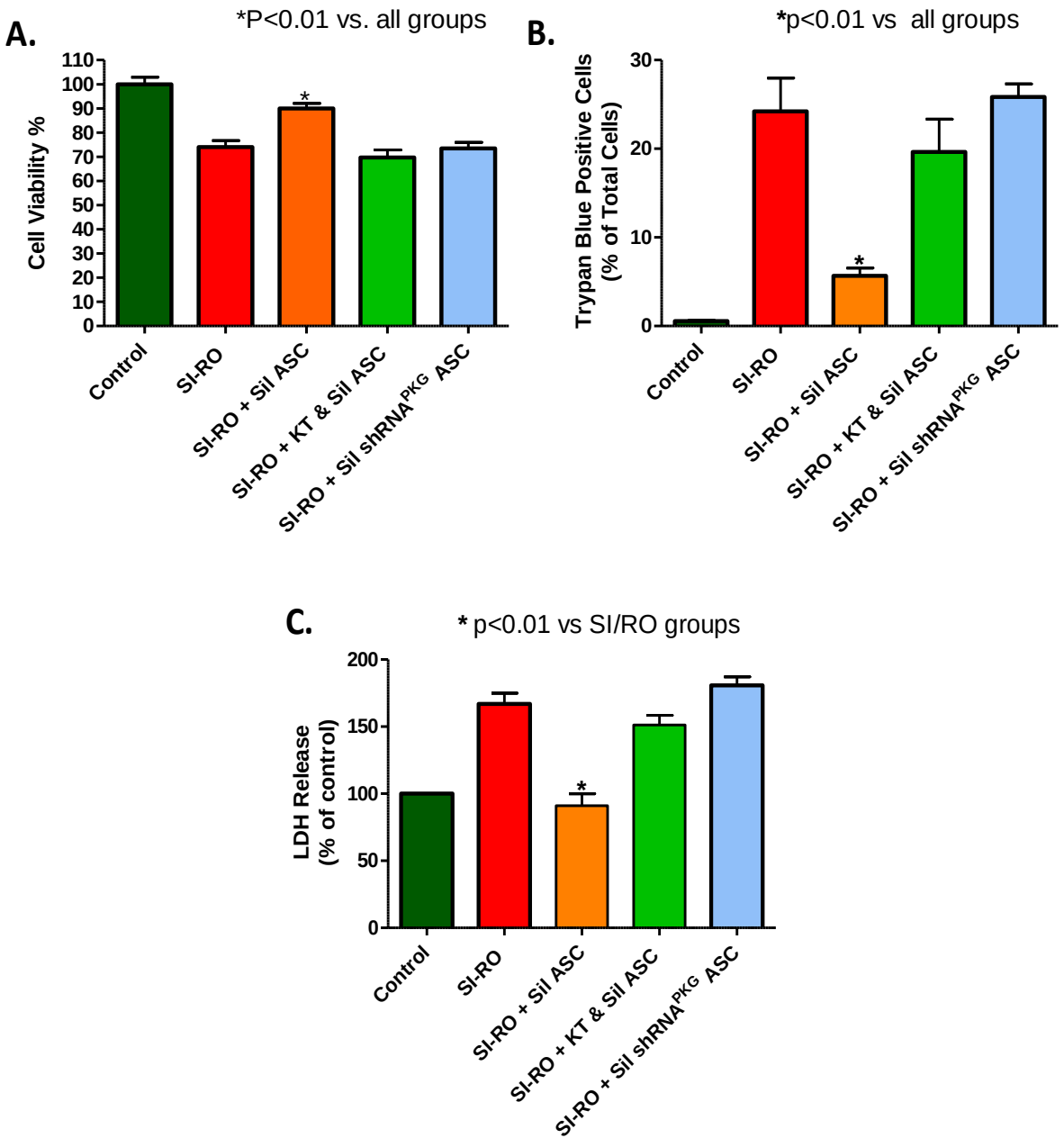

Figure 9. Sildenafil protects ASCs against necrosis through PKG dependent mechanism. Quantitative data showing cell viability as determined by MTS assay (A), cell necrosis assessed by trypan blue exclusion assay (B), and LDH release into the medium (C); (n=8).

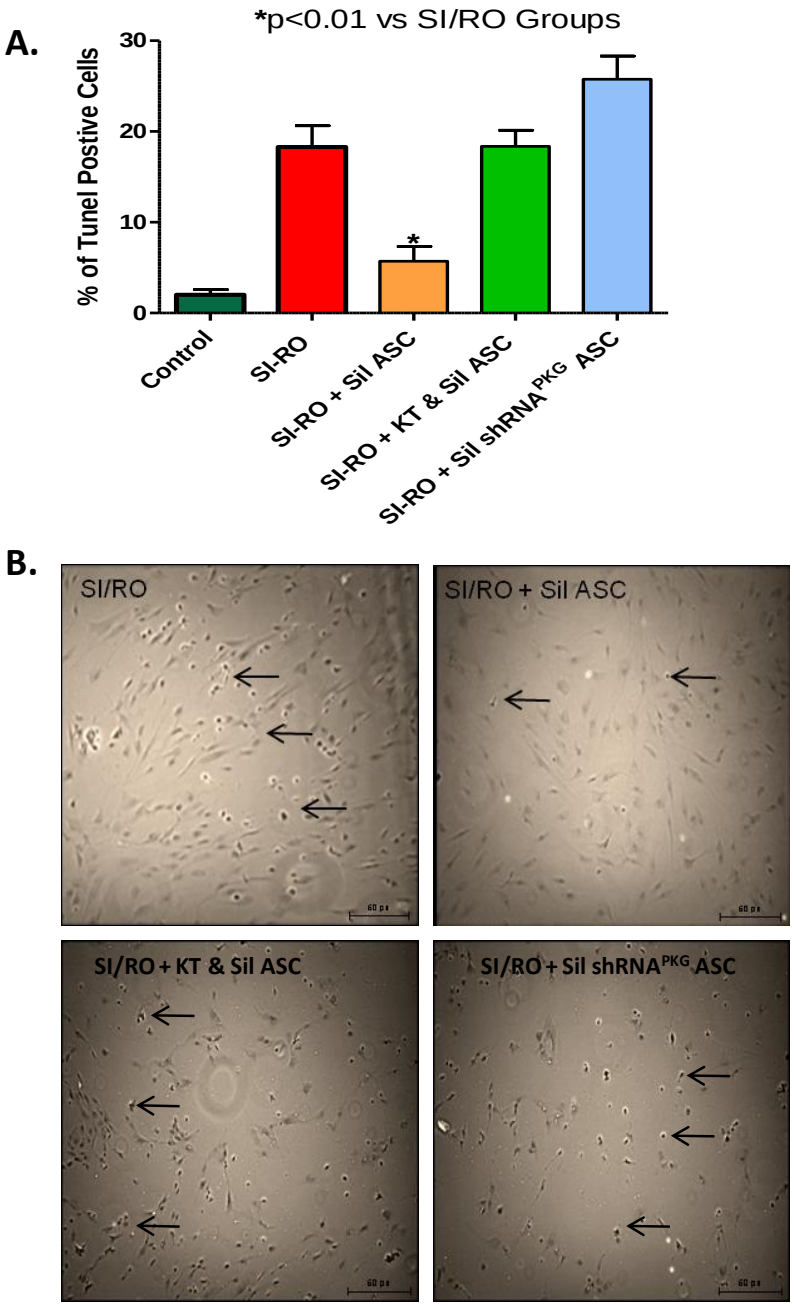

Figure 10. Sildenafil protects ASCs against apoptosis through PKG. Quantitative data showing the effect of PKG inhibitor KT 5823 (KT) and shRNA^{PKG} on apoptosis following SI-RO as determined by TUNEL assay (A) and representative images (B). The PKG inhibitor KT 5823 (KT) and shRNA^{PKG} abolished the protective effect of sildenafil against necrosis and apoptosis; (n=8).

PDE-5 Inhibition Enhances Release of Growth Factors

To identify potential paracrine mechanisms responsible for the therapeutic effect of preconditioning, we examined the effect of PDE-5 inhibition in ASCs on release of growth factors, VEGF, b-FGF, and IGF-1 *in vitro.* No differences in their secretion was observed between sildenafil-treated, $shRNA^{PDE-5}$ and non-treated ASCs under normal conditions. Following SI/RO, both sildenafil and knockdown with $shRNA^{PDE-5}$ increased the release of basic-fibrolast growth factor (b-FGF) (1.7 fold), IGF-1 (1.5 fold) and VEGF (1.4 fold) as compared to SI/RO control. Inhibition of PKG blocked the enhanced secretion of the growth factors (Fig. 11). These data suggests that the high-level secretion of growth factors from preconditioned ASCs may provide cardioprotective and proangiogenic effects as a result of PKG activation.

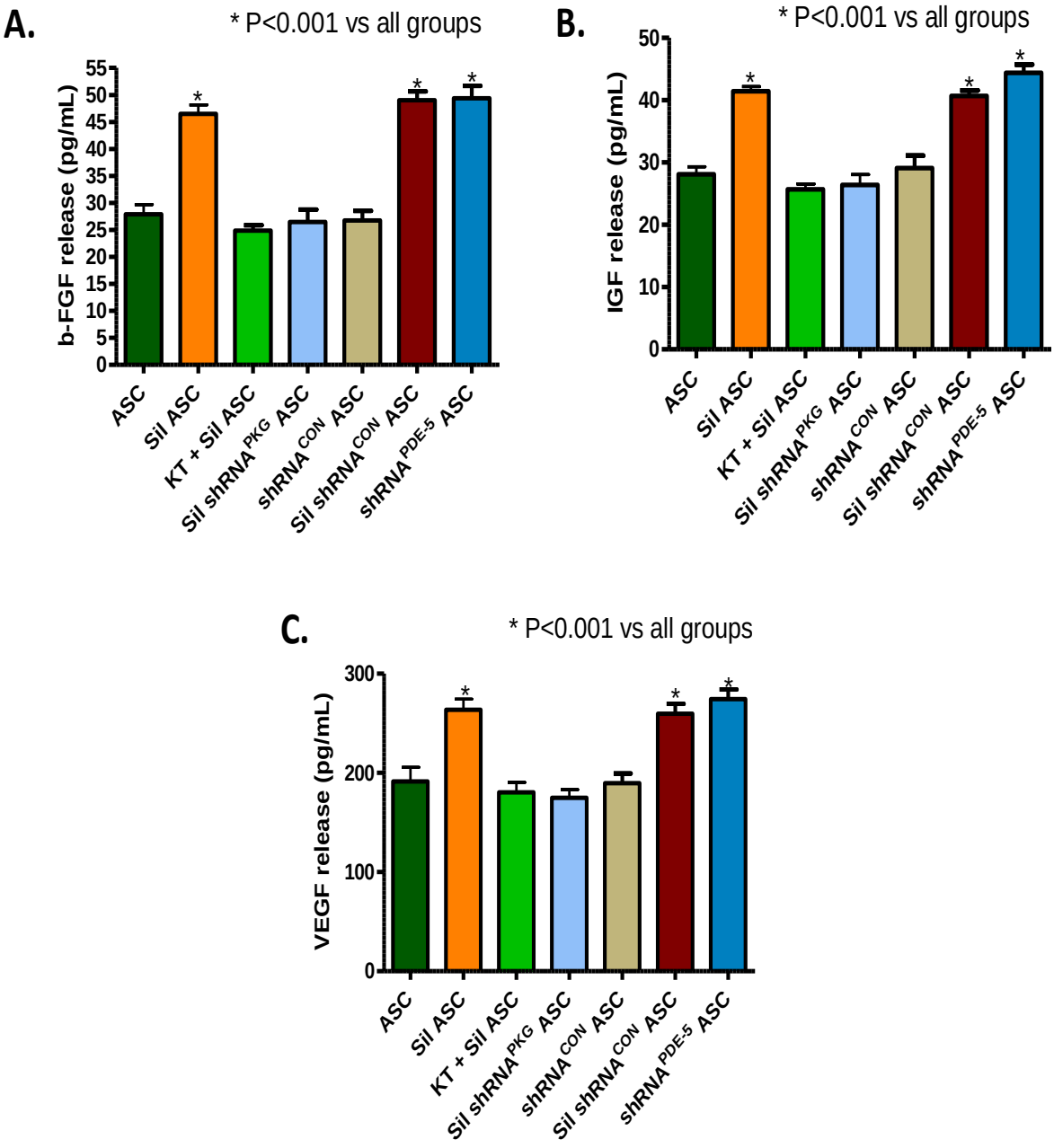

Figure 11. PDE-5 inhibition increases the release of growth factors following ischemia/reoxygenation in ASCs. PDE-5 inhibition by sildenafil or its knockdown with silencing vector shRNA^{PDE-5} augmented the release of (A) b-FGF, (B) IGF, and (C) VEGF after SI/RO which is blocked by PKG inhibitor KT 5823 (KT) and shRNA^{PKG}. shRNA^{CON} had no effect; (n=4).

Effect of Sildenafil Preconditioning on Nitrate, Nitrite and NOx

The concentrations of nitrate, nitrite and NOx (nitrate + nitrite) secreted into culture medium was examined following PDE-5 inhibition in ASCs. Sildenafil treatment of ASCs resulted in significant increases of nitrate (26±1.7 μM) and NOx (30.5±0.8 μM) as compared to non-treated controls (17.9±1.7 μM and 22.3±1.7 μM, respectively, $p<0.05$, $n=3$). Interestingly, PDE-5 inhibition through a silencing vector exhibited a trend towards enhanced secretion of nitrate and NOx as compared to non-treated ASCs; however, did not confer a similar increase as compared to ASCs infected with scrambled control vector (Fig. 12). Furthermore, the levels of nitrite secreted into the medium were similar in each treatment group.

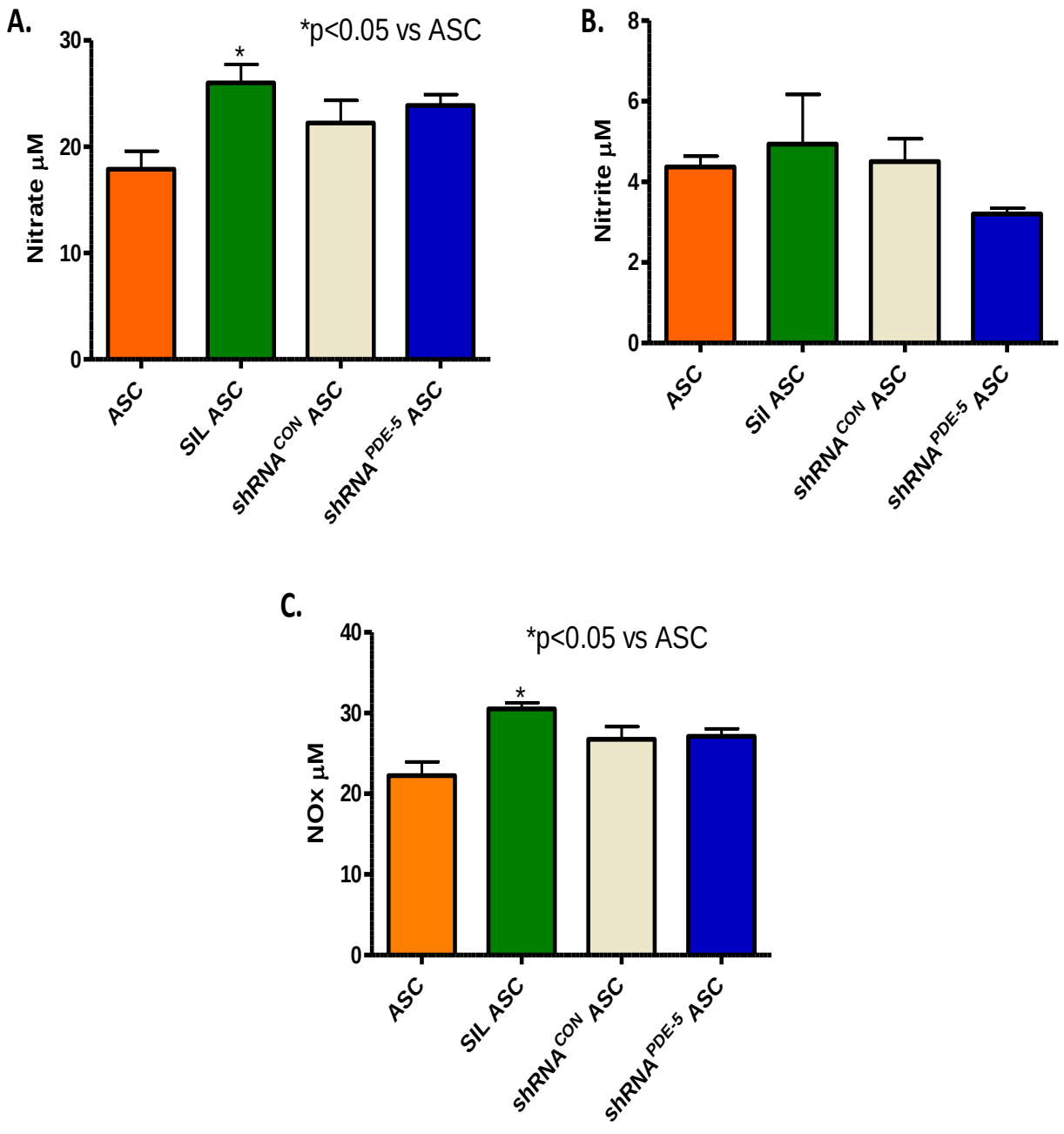

Figure 12. Increased NOx levels following sildenafil treatment. Quantitative data showing the effect of sildenafil (SIL) or shRNA^{PDE-5} on levels of nitrate (A); nitrite (B); and NOx (C) in conditioned medium; (n=3).

Cytoprotection of Adult Mouse Cardiomyocytes with Conditioned Medium:

We further evaluated the possible paracrine effects of conditioned medium from cultured ASCs on isolated primary adult cardiomyocytes. Our method for isolation and cell preparation yielded at least 85% of the cardiomyocytes with rod-shaped morphology (Fig. 13 B). Initially, the mouse cardiomyocyte standard growth medium was replaced with normal α-MEM or conditioned medium from control or sildenafil-treated ASCs; cardiomyocytes were subsequently subjected to SI-RO (Fig. 13 A). Necrosis was measured by trypan blue exclusion and LDH release. The percentage of trypan blue-positive (necrotic) cardiomyocytes increased to 41.3±5.8% as compared to non-SI/RO controls (2.0±0.8%) following SI (40 min)/RO (1 h) ($p<0.01$, $n=4$). Treatment with conditioned medium (CM) reduced necrosis as shown by decrease in trypan blue-positive cells. Although the cytoprotective effect was observed in the presence of both control and sildenafil-treated ASC-CM, the greater degree of protection was conferred by the SIL ASC-CM (24.5±2.6% vs 32.3 ±3.9%, $p<0.01$, $n=4$, Fig. 13 C). Similar results were obtained by measurement of LDH release in the medium (Fig. 13 D). Furthermore, the relative number of apoptotic cardiomyocytes following 18 h of RO was measured by TUNEL analysis. In the presence of SIL ASC-CM, the number of apoptotic nuclei was reduced to 11.5±2.9% as compared with that of normal medium (26.3±4.2%) or the control ASC-CM (18.5±2.1%), respectively (Fig. 14). Thus, these results indicate that a paracrine cytoprotective mechanism is involved with growth factors secreted by the ASCs under ischemic conditions.

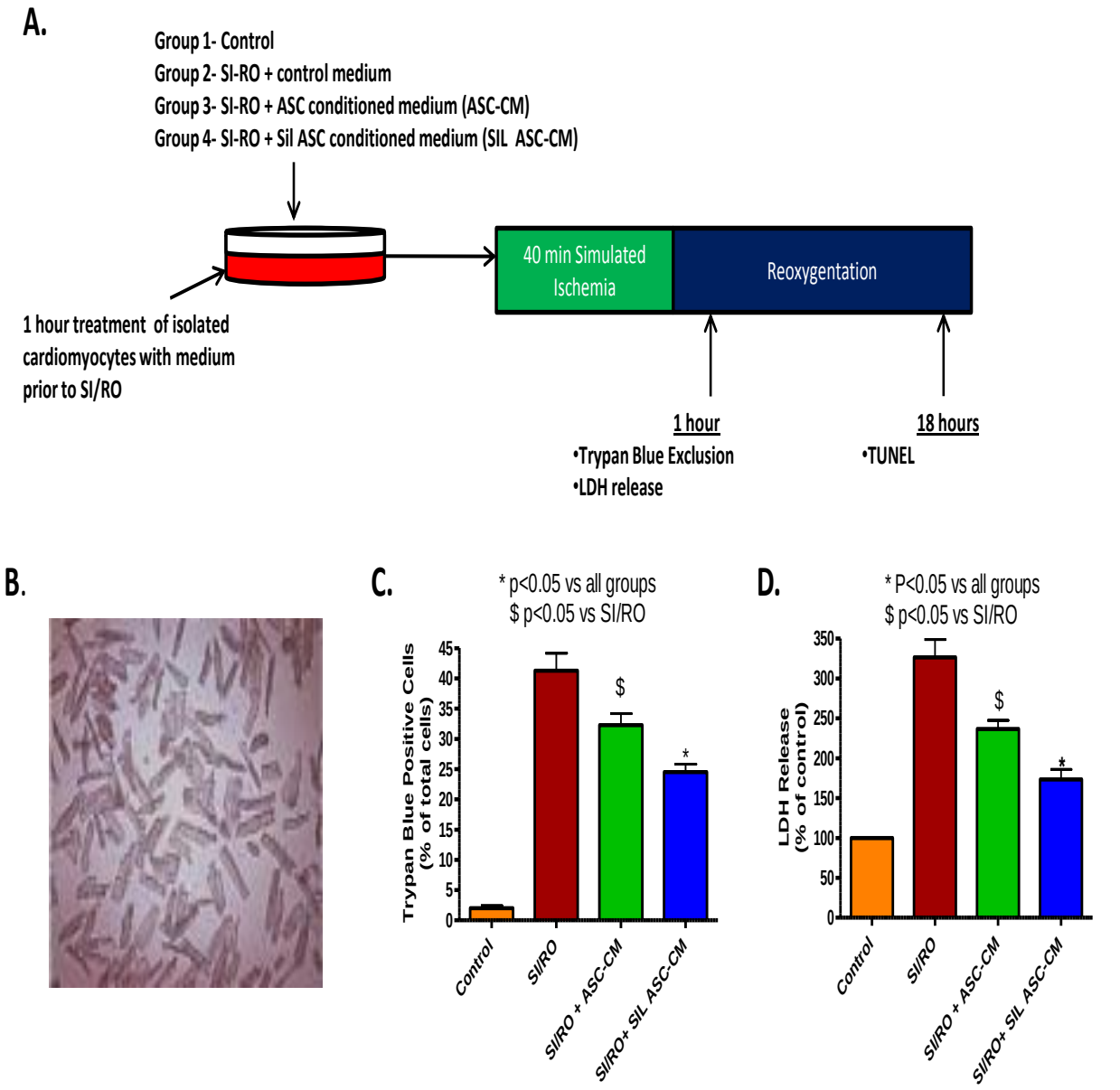

Figure 13. Conditioned media attenuates necrosis of cardiomyocytes following ischemia/reoxygenation. (A) *In vitro* protocol, arrows indicate time points for treatment, performance of simulated ischemia/reoxygenation, and measurement of various parameters for each experimental group. (B) Representative image of isolated mouse cardiomyocytes. Quantitative data showing cell necrosis assessed by trypan blue exclusion assay (C), and LDH release into the medium (D); (n=4).

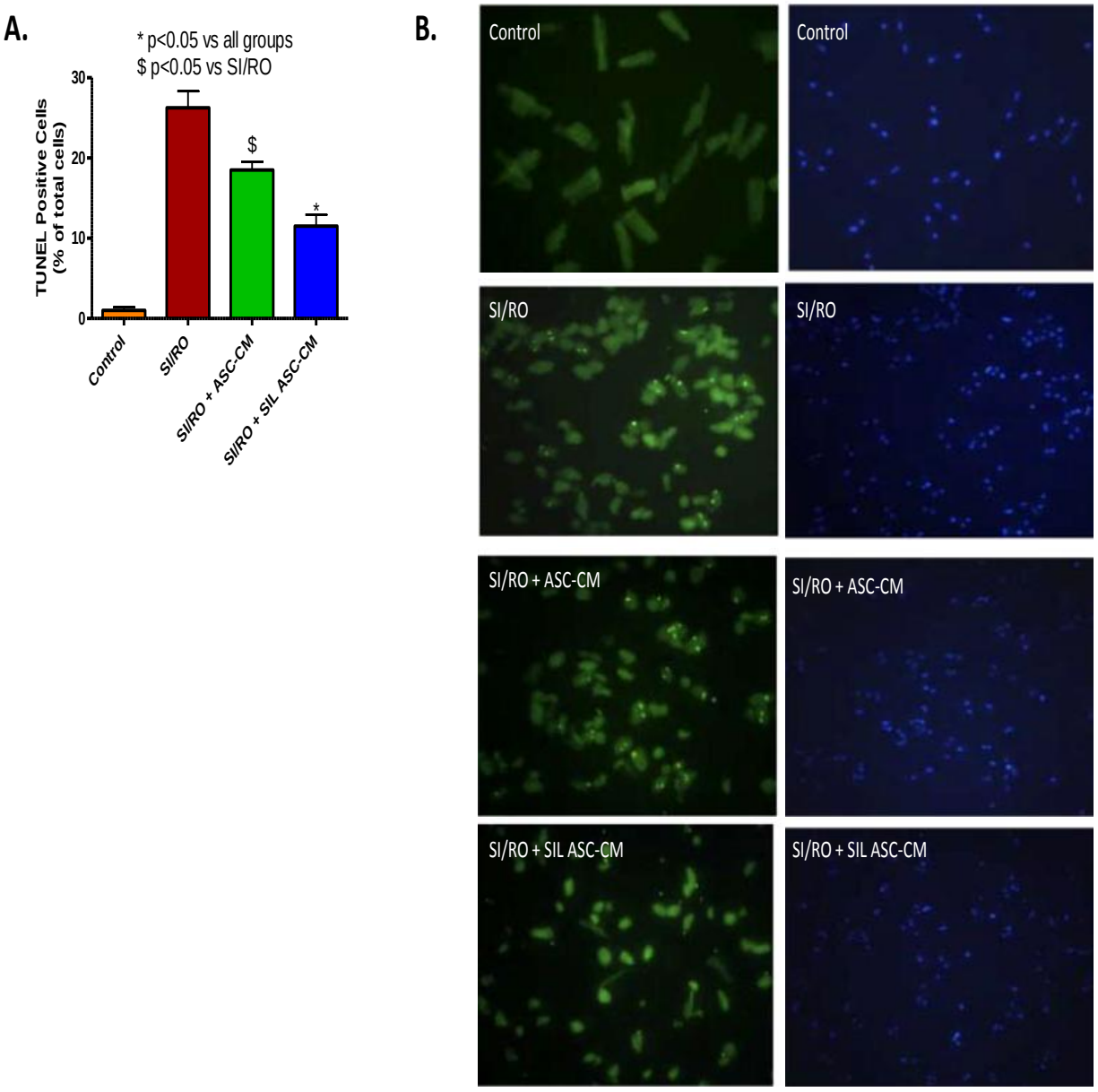

Figure 14. Conditioned media attenuates apoptosis of cardiomyocytes following ischemia/reoxygenation. Quantitative data showing the effect of conditioned medium from sildenafil-treated ASCs (SIL ASC-CM) or control ASCs (ASC-CM) on apoptosis following SI-RO as determined by TUNEL assay (A) and representative images (B); (n=4).

Discussion

Cardiac repair via cellular transplantation has generated considerable enthusiasm in recent years although the optimal cells for cardiac repair remain to be identified. We chose adipose-derived stromal/stem cells which have the ability to differentiate into multiple mesenchymal cell types including endothelial cells (45, 46) and cardiomyocytes (15, 52). Human ASCs have been shown to preserve heart function following myocardial infarction (133). In the present study, we investigated the feasibility of PDE-5 inhibition as a strategy to precondition human ASCs. The rationale for this approach was the established powerful preconditioning-like effect of PDE-5 inhibitors in cardiomyocytes (75, 98) and against ischemia/reperfusion injury in heart (92-94) established by us. Our results show preconditioning of ASCs by PDE-5 inhibition significantly improved their ability to survive SI/RO injury *in vitro*. Moreover, we observed significant release of pro-angiogenic/pro-survival growth factors including VEGF, b-FGF, and IGF-1. More importantly, we provide the first evidence for robust expression of PDE-5 in the isolated ASCs. PDE-5 knockdown with a silencing vector significantly reproduced the effect of sildenafil in survival against cell death as well as release of growth factors. These data not only rule out the potential off target effects of sildenafil in ASCs but also provide us a genetic approach to precondition the stem cells possibly for improving survival after transplantation. To our knowledge, this is the first study showing PDE-5 as a target gene/enzyme to improve survival of ASCs under ischemic conditions *in vitro*. These are clinically significant observations because improving stem cell survival by exploiting novel therapeutic targets with clinically approved drugs would directly impact the prognostic outcome of stem cell therapy in the heart.

We have shown that sildenafil reduced myocardial infarction in the intact heart and apoptosis and necrosis in cardiomyocytes subjected to simulated ischemia and reoxygenation through nitric oxide (NO)-dependent pathway (75). Sildenafil induced a delayed cardioprotective effect in the mouse heart that involved the upregulation of iNOS and eNOS (91). ASCs have been shown to endogenously generate NO which can be measured in biological systems as metabolites of NO such as nitrite and nitrate (134). Our results indicate a significant increase in concentrations of nitrate and NOx (nitrate + nitrite) in culture medium following sildenafil treatment of ASCs as compared to non-treated cells. Interestingly, $shRNA^{PDE\text{-}5}$ ASCs exhibited a trend towards increased NO generation as compared to control ASCs; however, there was not a significant difference in NO metabolites as compared the $shRNA^{CON}$ ASCs. The downstream targets and cellular actions of NO are known to be dependent on its local concentrations with normal *in vivo* concentrations of NO ranging from low nanomolar to low micromolar level. Therefore, even modest increases in NO concentration can result in activation of the major NO receptor, soluble guanylyl cyclase which in turn elevates intracellular cGMP (101). Enhanced accumulation of cGMP leads to activation of PKG which has been shown to be involved in cardioprotective effects against I/R injury (102-104). In the present study, there is no clear evidence that PKG activation seen in preconditioning of ASCs is a result of increased NO production, although recently, we demonstrated that direct adenoviral overexpression of PKG Iα in cardiomyocytes induced both iNOS and eNOS and protected these cells against SI/RO injury. Similarly in isolated cardiomyocytes (75), sildenafil caused a significant increase in mRNA and protein expression of iNOS and eNOS. Also, sildenafil-induced protection against necrosis and apoptosis was abolished in cardiomyocytes derived from iNOS but not from eNOS gene

knockout mice (91). ASCs have been shown to express all three nitric oxide synthases, iNOS (135), eNOS (45, 136), and nNOS (137), and endogenous generation of NO has been attenuated with the iNOS/eNOS inhibitor, L-NAME (134). Therefore, it is plausible that the increase in NO concentrations following sildenafil treatment might be a result of a similar mechanism involving iNOS/eNOS up-regulation. However, further studies are needed to delineate the exact source of NO in our model of ASC preconditioning with sildenafil.

Inhibition of PDE-5 by siRNA has been documented to increase levels of cGMP and increase the angiogenic phenotype of endothelial cells (98, 130). PDE-5 inhibition and activation of cGMP has also been shown to increase mobilization and exert pro-survival effects on stem cells (138, 139). In ours and other previous studies, PDE-5 inhibition lead to accumulation of cGMP to levels that lead to activation of PKG. Phosphorylation of downstream proteins by PKG regulates such important physiological functions as relaxation of vascular smooth muscle, inhibition of platelet aggregation and apoptosis, and induction of VEGF (15, 97). We have demonstrated previously that sildenafil induced activation of PKG and adenoviral overexpression of PKG in isolated cardiomyocytes resulted in attenuation from necrosis and apoptosis following ischemia/reoxygenation (75, 114). The present results demonstrate that preconditioning of ASCs with PDE-5 inhibition exerted a similar cytoprotective effect through a PKG-dependent pathway as PKG inhibitor, KT 5823, and short-hairpin RNA knock-down of PKG abolished protection.

There are at least two mechanisms contributing to the resistance of ASCs in which PDE-5 is inhibited against hypoxic/ischemic stress. We have previously shown that sildenafil induces a cytoprotective effect in cardiomyocytes through NO and cGMP dependent activation of PKG which results in opening of mitochondrial K_{ATP} channels (75, 98, 140). While the

comprehensive cytoprotective signaling pathways following PDE-5 inhibition in ASCs remains to be investigated, it is clear that cGMP (but not cAMP) was elevated and PKG was involved in protecting ASCs against ischemic injury (Figs. 8, 9, 10). PKG is a serine/threonine protein kinase that has two isozymes (type I and type II; *i.e.* PKGI and PKGII). PKGIα is mainly found in lung, heart, platelets, and cerebellum, whereas PKGIβ is highly expressed in smooth muscles of uterus, vessels, intestine, and trachea (141). As discussed elsewhere, sildenafil activated PKG-dependent signaling cascade that involved phosphorylation of ERK and inhibition of GSK-3β thus leading to cytoprotection (98). Moreover, gene transfer of PKGIα in cardiomyocytes in the absence of sildenafil or other pathophysiological stimuli (such as ischemic preconditioning) resulted in a cytoprotective phenotype that was associated with the phosphorylation of Akt, ERK, and JNK and increased Bcl-2 expression (114). It is quite likely that a similar cascade of signaling events leads to survival of ASCs after SI.

Another cytoprotective mechanism of PDE-5 inhibition in ASCs may involve the paracrine effects by secretion of growth factors with angiogenic potential. The present study clearly demonstrated the increased secretion of VEGF, b-FGF, and IGF-1 from sildenafil- and $shRNA^{PDE-5}$-treated ASCs in response to the ischemic conditions. Moreover, the release of growth factors was blunted under conditions where PKG signaling was disrupted (Fig. 6) suggesting a critical role of cGMP-PKG pathway in their secretion. These results are supported by other studies on downregulation of VEGF expression with the inhibition of the downstream kinase of PKG, GSK3β (142), which are also activated by sildenafil treatment in cardiomyocytes and exert protective effect against SI/RO injury (98). ASCs have been shown to secrete VEGF and HGF which possess both angiogenic and anti-apoptotic effects on both myocardial and

endothelial cells (47). Furthermore, these paracrine factors from stem cells have been shown to protect co-cultured adult cardiomyocytes against hypoxic/ischemic stress (144-146). Accordingly, we observed that adult mouse cardiomyocytes cultured with conditioned medium from sildenafil treated ASCs survived better than those cultured with conditioned medium from non-treated cells. Therefore, transplantation of ASCs preconditioned through PDE-5 inhibition could provide adequate magnitude and duration of VEGF, b-FGF, and IGF-1 release in the ischemic myocardium, which would provide cardioprotective effects leading to the salvaging of ischemic myocardium and decrease the infarcted area.

In summary, this study demonstrates that preconditioning of ASCs through PDE-5 inhibition, improved survival under conditions of ischemia/reoxygenation via PKG activation and enhanced paracrine action. We propose that *in vitro* preconditioning of ASCs by inhibition of PDE-5 with small molecule drugs or gene silencing vectors can be a powerful new approach to improve stem cell therapy following myocardial infarction in patients. Particularly the easy availability of ASCs from humans combined with the preconditioning by inhibition of PDE-5 may hold great promise for initiation of clinical trials in heart failure patients.

Funding Sources: This study was supported by grants from National Institutes of Health (HL51045, HL79424 and HL93685) to Rakesh C. Kukreja, National Scientist Development Grant from the American Heart Association (10SDG3770011) to Fadi N. Salloum, and Predoctoral Fellowship from the American Heart Association (09PRE2250905) to Nicholas N. Hoke.

CHAPTER 3

Preconditioning by Phosphodiesterase-5 Inhibition Improves Therapeutic Efficacy of Adipose Derived Stem Cells Following Myocardial Infarction

ABSTRACT

Cell-based therapies for the prevention and treatment of cardiac dysfunction offer the potential to significantly alter cardiac function and improve the outcome of patients with cardiovascular disease. To date several clinical studies have suggested the potential efficacy of stem cell therapy; however, the benefits have been limited and inconsistent. We have demonstrated that preconditioning of adipose-derived stem cells (ASCs) through PDE-5 inhibition improves their viability following simulated ischemia and re-oxygenation. We therefore tested the hypothesis that *ex vivo* preconditioning of ASCs with sildenafil (Viagra) or selective knockdown with a silencing vector would enhance cardiac function following myocardial implantation *in vivo.* ASCs were preconditioned by treatment with sildenafil or PDE-5 silencing vector shRNA ($shRNA^{PDE-5}$). Adult male CD-1 mice underwent myocardial infarction (MI) by occlusion of left descending coronary artery. Animals received PBS and preconditioned ASCs ($4x10^5$) by direct intramyocardial injection into left ventricular wall. Cardiac function and structure were evaluated by serial echocardiography and histology. Preconditioned ASCs-treated hearts showed consistently superior cardiac function by all measures as compared with PBS and non-

preconditioned ASCs 4 weeks after treatment. Post-mortem histological analysis demonstrated that preconditioned ASCs-treated mice had significantly reduced fibrosis, increased vascular density and decreased resident myocyte apoptosis as compared to mice that received non-preconditioned ASCs or PBS. Plasma levels of VEGF, b-FGF, and Ang-1 were also significantly elevated 4 weeks after cell therapy with preconditioned ASCs. We conclude that *ex vivo* inhibition of PDE-5 prior to transplantation can be a potent new approach to improve stem cell therapy following myocardial infarction.

Introduction

Over the past several years, experimental findings suggest there is a therapeutic potential for cellular replacement therapy as treatment of acute myocardial infarction (MI) and other progressive chronic cardiac diseases such as left ventricular (LV) remodeling and heart failure. Since cardiovascular disease remains a worldwide problem, the development of novel effective cell-based therapies is crucial to improve patient outcome post MI. Current therapies strive to limit both the loss of cardiomyocytes and adverse remodeling in order to preserve cardiac systolic function; however, treatment of MI still leaves a significant number of patients with impaired cardiac function that leads to more severe LV dysfunction and adverse remodeling. Remodeling of the ventricle is a result of increased cardiomyocyte apoptosis in the ischemic zones after infarction. While apoptosis influences remodeling, the other form of cellular death that occurs in the heart, necrosis, provokes inflammatory reactions, neoangiogenesis, fibroblast activation, and scar formation. Therefore, limiting myocardial apoptosis and necrosis is of critical importance.

Phase II and III clinical studies indicate that stem cell transplantation is feasible and may have beneficial effects on ventricular remodeling after myocardial infarction. However, to date, most of the cellular based therapies have shown limited efficacy because the majority of transplanted cells are readily lost after transplantation due to poor blood supply, ischemia/reperfusion injury, and inflammatory factors. Disconcerting reports have shown that up to 99% of transplanted cells are lost within the first 24 hours (73). Therefore, enhancing cell viability and reduction of apoptosis of ASCs in an ischemic microenvironment of the infarcted heart is critical for improving the efficiency of cell therapy. In the previous chapter, we have

demonstrated that *in vitro* PDE-5 inhibition of ASCs by sildenafil or genetic knock-down with a silencing vector improved cell viability, attenuated necrosis and apoptosis following simulated ischemia/reoxygenation (SI/RO) through a PKG-dependent mechanism. Moreover, we demonstrated the increased secretion of pro-angiogenic/pro-survival growth factors: VEGF; b-FGF; and IGF-1 from sildenafil and $shRNA^{PDE-5}$ -treated ASCs in response to the ischemic conditions. Transplantation of ASCs along with their secretion of VEGF, bFGF, and IGF-1, and has been shown to upregulate the anti-apoptotic protein, Bcl-2, which results in the decrease in cardiomyocyte apoptosis (58). Similarly, we showed that treatment with conditioned medium from sildenafil-treated ASCs resulted in a cytoprotective effect on cultured adult mouse myocytes following SI/RO through attenuation of necrosis and apoptosis.

In this study, we tested the hypothesis whether PDE-5 inhibition could improve the survival and engraftment of ASCs which may lead to enhanced cardiac function following myocardial infarction in mice. Specifically we addressed the question: Whether *in vivo* transplantation of ASCs after *ex vivo* PDE-5 inhibition improve LV function following myocardial infarction. Our results show that *ex vivo* PDE-5 inhibition of ASCs prior to myocardial transplantation enhances their therapeutic potential as shown by reduced fibrosis, cardiomyocyte apoptosis, improved vascular density and cardiac function in mice following myocardial infarction.

Materials and Methods

Animals

Adult male outbred CD-1 mice (~30g) were supplied by Harlan Sprague Dawley. Upon their arrival, the animals were allowed to readjust to the housing environment for at least 3 days before any experiment. Standard food and water were freely accessible for the animals used in these studies. The care and use of the animals were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Virginia Commonwealth University and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 80-23; Office of Science and Health Reports, Bethesda, MD 20205].

Myocardial Infarction (MI) Protocol

Adult CD-1 mice underwent permanent occlusion of the left descending coronary artery as previously described (97). In brief, the animals were anesthetized with pentobarbital (70 mg/kg *ip*), intubated orotracheally and ventilated on a positive-pressure ventilator. The tidal volume was set at 0.2 ml, and the respiratory rate was adjusted to 133 cycles/min. All surgical procedures were carried out under sterile conditions. A left thoracotomy was performed at the fourth intercostal space, and the heart was exposed by stripping the pericardium. The left descending coronary artery (LAD) was then identified and permanently occluded by a 7.0 silk ligature that was placed around it. After coronary artery occlusion, the air was expelled from the chest. The animals were extubated and then received intramuscular doses of analgesia (buprenex; 0.02mg/kg) and antibiotic (Gentamicin; 0.7 mg/kg; for 3 days).

Transplantation of ASCs

Immediately after ligation, $4x10^5$ ASCs (total of 30µL) were injected at 3 injection sites into anterior and lateral wall of the LV bordering the infarction. The control group was injected with identical volume of PBS at similar sites (Fig. 15). To determine the fate of ASCs post transplantation, the cells were labeled with PKH26 dye using a PKH-26 Cell Linker Kit (Sigma) according to the supplier's instructions. The chests were sutured, and animals were allowed to recover. The hearts were harvested for histological studies 4 weeks post cellular transplantation.

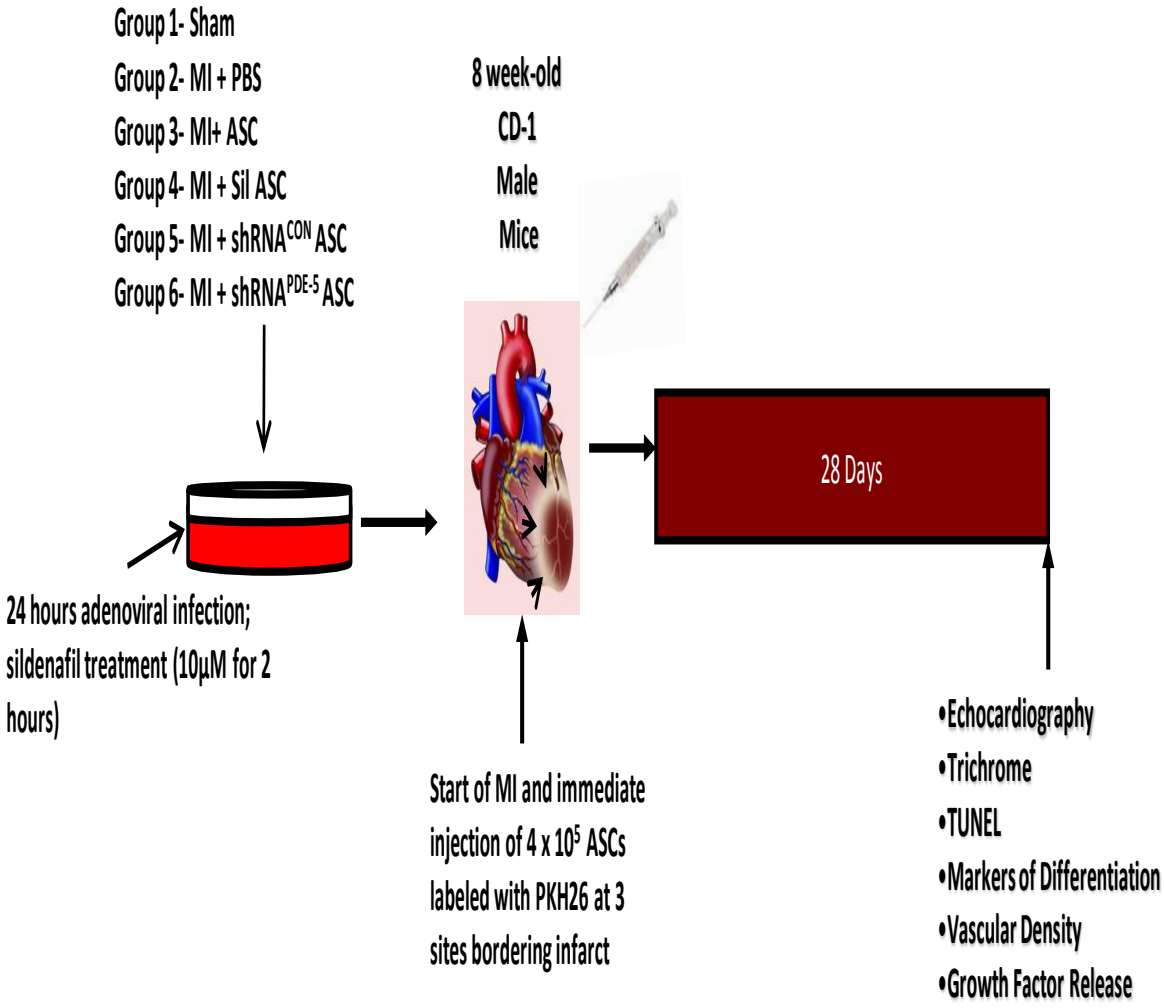

Figure 15. Experimental protocol. *In vivo* protocol, arrows indicate time points for treatment, performance of surgical procedures, and measurement of various parameters for each experimental group. Arrowheads indicate sites of injection of adipose-derived stem cells in the border zone of the infarcted heart.

Echocardiography

Doppler Echocardiography was performed using the Vevo770TM imaging system (VisualSonics Inc., Toronto, Canada) prior to surgery (baseline), and 4 weeks after surgery using techniques similar to those previously reported (97). Mice were anesthetized with pentobarbital sodium (30 mg/kg ip). The mice were placed in the supine position. The chest was carefully shaved, and ultrasound gel was used on the thorax to optimize visibility during the exam. A 30-MHz probe was used to obtain two-dimensional (B-Mode), and cross-sectional (M-mode) imaging from parasternal short-axis view at the level of the papillary muscles. The M-mode cursor was positioned perpendicular to the anterior and posterior wall to measure left ventricular (LV) end-diastolic and end-systolic diameters (LVEDD and LVESD, respectively). M-mode images were then obtained at the level of the papillary muscles below the mitral valve tip. LV fractional shortening (FS) was calculated as follows: FS=(LVEDD–LVESD)/LVEDDx100. Ejection fraction was calculated with the Teichholz formula.

Histology

Following the 4 week post-MI echo, the mice were sacrificed and subsequently, the hearts were excised, trimmed free of the atria, vena cava, and pulmonary vessels, and prepared for histology. In brief, the aorta was attached to a Langendorff apparatus and the heart was perfused in retrograde fashion with saline to remove the residual blood. Tranverse sections of the median third of the LV (n = 4-6/group) were flash frozen in liquid nitrogen and sent to VCU Tissue and Data Acquisition and Analysis Core (TDAAC) Facility for tissue processing and

cryosectioning. Apoptosis was examined using TUNEL assay according to manufacturer's instructions which were described in Chapter 2. The apoptotic rate was expressed as the number of apoptotic cells of all cardiomyocytes per field. The apoptotic rate in the peri-infarct regions was calculated using 10 random fields, which virtually cover the entire peri-infarct area.

Myocardial fibrosis was examined to address prevalence of scar formation within the LV. Heart sections (5 μm) were stained with Masson's trichrome (Sigma). Fibrotic area was computed using computer morphometry (Bioquant) and expressed as a percentage of left ventricle.

Determination of Vascular Density

The vascular density in the peri-infarct region of myocardial tissue was determined as previously described (147). In brief, tissue sections were stained using CD-31 antibody (Millipore) for evaluation of vascular density. Blood vessels were quantified using histomorphometry. Vessels were distinguished from larger arterioles and venules by deriving lumen diameters from area assessments of each vessel. Only vessels with diameters <10 μm and cut in an orthogonal fashion were considered and included in the quantification. For quantification of positively stained vessels, five sections within the peri-infarcted area of each animal were analyzed. Blood vessels were detected at low magnification, x 200.

Data Analysis and Statistics

Data are presented as mean ± S.E. The differences between groups were analyzed with one way analysis of variance followed by Student-Newman-Keuls post hoc test for pair-wise comparison. $P<0.05$ was considered to be statistically significant.

Results

PDE-5 Inhibition Enhances ASC Survival in Infarcted Hearts

To determine the engraftment of ASCs, we examined myocardial tissue sections for the presence of PKH26 positive cells. Donor PKH26 positive cells were identified only in mice that received ASCs after treatment with sildenafil or shRNA^{PDE-5}. PKH26-positive cells were present primarily within the epicardial layer of the infarct border zone region (Fig. 16). There was no evidence of engrafted ASCs in mice receiving control non-preconditioned ASCs. To determine endothelial cell differentiation of ASCs, we immunostained heart sections with endothelial cell specific marker, CD31, and assessed for co-localization of PKH26 positive cells. Animals receiving ASCs treated with sildenafil or shRNA^{PDE-5} showed CD31 positive cells.

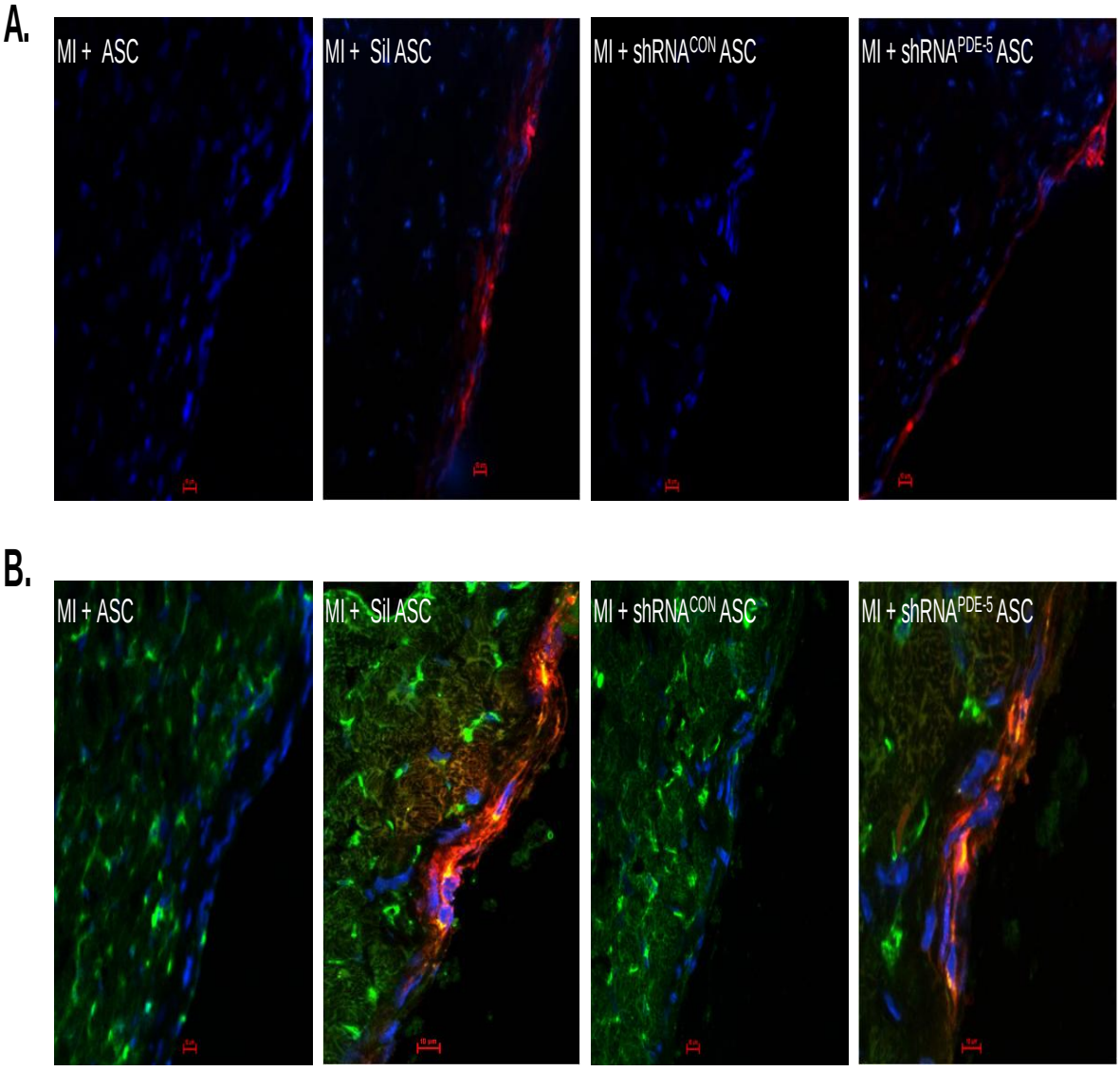

Figure 16. Tracking of PKH26 labeled ASCs following myocardial infarction. ASCs were preconditioned with sildenafil (Sil) or shRNA^{PDE-5} and injected in the LV wall following myocardial infarction. (A) PKH26 labeled ASCs Labeled (red) transplanted Sil or shRNA^{PDE-5} ASCs. DAPI (blue) was used to visualize nuclei. (B) Immunostaining of CD31 (green), PKH26 (red) and DAPI (blue).

Preconditioned ASCs Improve Cardiac Function, Reduce Fibrosis and Apoptosis

Figure 17A shows representative M-mode images 4 weeks after MI. Significant functional loss continued over the following 28 days in saline-treated hearts. Echocardiography recordings show that values for all measures of LV function decreased significantly in hearts treated with PBS post MI as compared with sham. Conversely, hearts injected with ASCs demonstrated a trend toward an increase in function. However, treatment with preconditioned ASCs demonstrated significant improvement in function as compared with non-preconditioned cells or PBS. Specifically, LVEDD, LVESD, EF, and FS were improved significantly in mice receiving sildenafil-treated ASCs as compared with mice receiving non-treated ASCs at 4 weeks after MI (Fig. 17). Administration of ASCs preconditioned with $shRNA^{PDE-5}$ also caused similar enhanced preservation of cardiac function and attenuation of cardiac remodeling as compared to ASCs treated with control vector. There were no significant differences in the echo parameters in mice receiving $shRNA^{CON}$ ASCs and non-treated ASCs (Fig. 17).

The percentage of fibrosis in the LV wall was 55 to 60% lower in hearts receiving ASCs preconditioned with PDE-5 inhibition as compared to non-treated ASCs (7.1±0.7% with sildenafil-treated ASCs; 6.8±1.7% with $shRNA^{PDE-5}$ ASCs vs 14.9±3.8% with non-treated ASCs, $p < .05$, n=5). All cellular treatment groups showed significant reduction in fibrosis compared with PBS controls (31.3±3.8%, Fig. 18). Also, the reduction in fibrosis seen in sildenafil-treated and $shRNA^{PDE-5}$ ASCs correlated with a reduction of cardiomyocyte apoptosis. PBS-treated mice after 4 weeks of permanent occlusion had a resident myocyte apoptotic rate of 3.3±0.32%, which was reduced to 1.9±0.23% in non-treated ASC group. Sildenafil-treated or

shRNA^{PDE-5} ASCs injected mice further reduced apoptosis (0.9±.14% with sildenafil and 0.8±.12% with shRNA^{PDE-5} ASCs, $p<0.05$ vs. PBS control, n=5, Fig. 19).

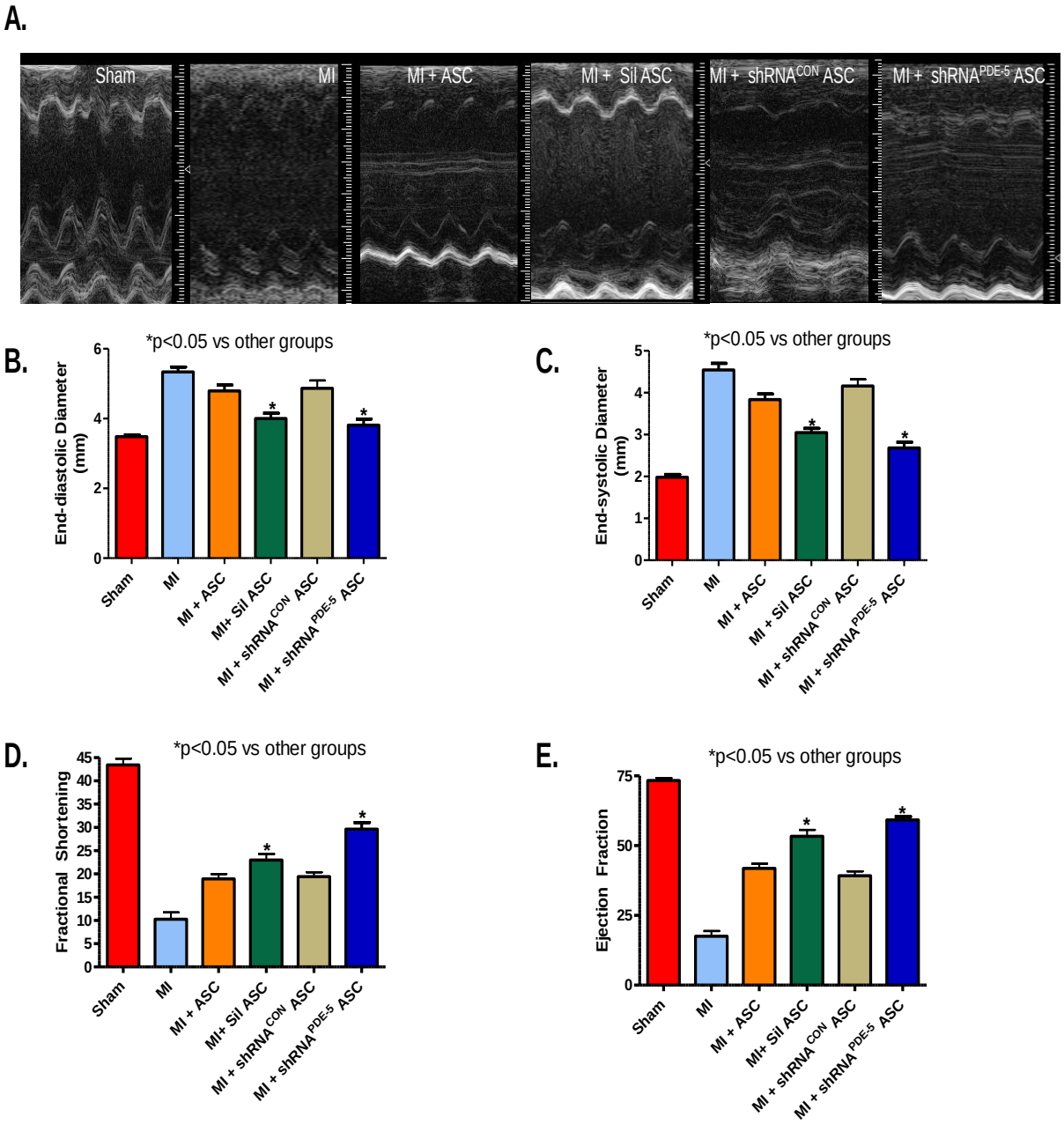

Figure 17. Transplantation of preconditioned ASCs improves cardiac function and remodeling following myocardial infarction. (A) Representative M-mode images showing preservation of LV contractility of hearts treated with preconditioned ASCs as compared with non-treated ASCs control following myocardial infarction (MI). Bar diagram showing quantitative data of hearts treated with preconditioned ASCs as compared with non-treated ASCs control following myocardial infarction. (B) LV end-diastolic diameter; (C) end-systolic diameter; (D) fractional shortening; (E) ejection fraction; (n≥5).

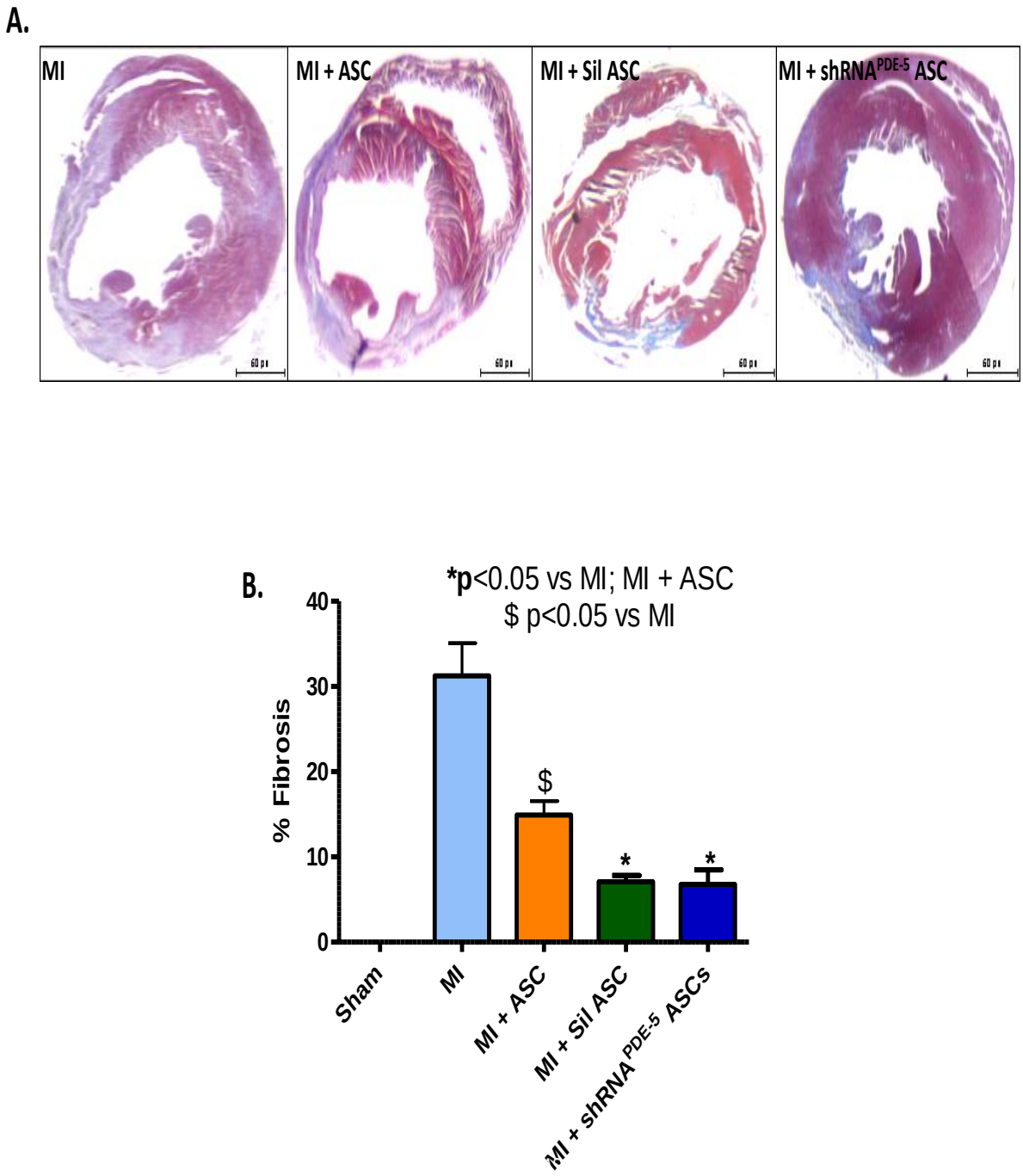

Figure 18. Preconditioned ASCs reduces myocardial fibrosis following myocardial infarction. (A) Representative Masson's trichrome staining of tissue sections and accompanying (B) bar diagram showing quantification of the amount of fibrosis (n=4).

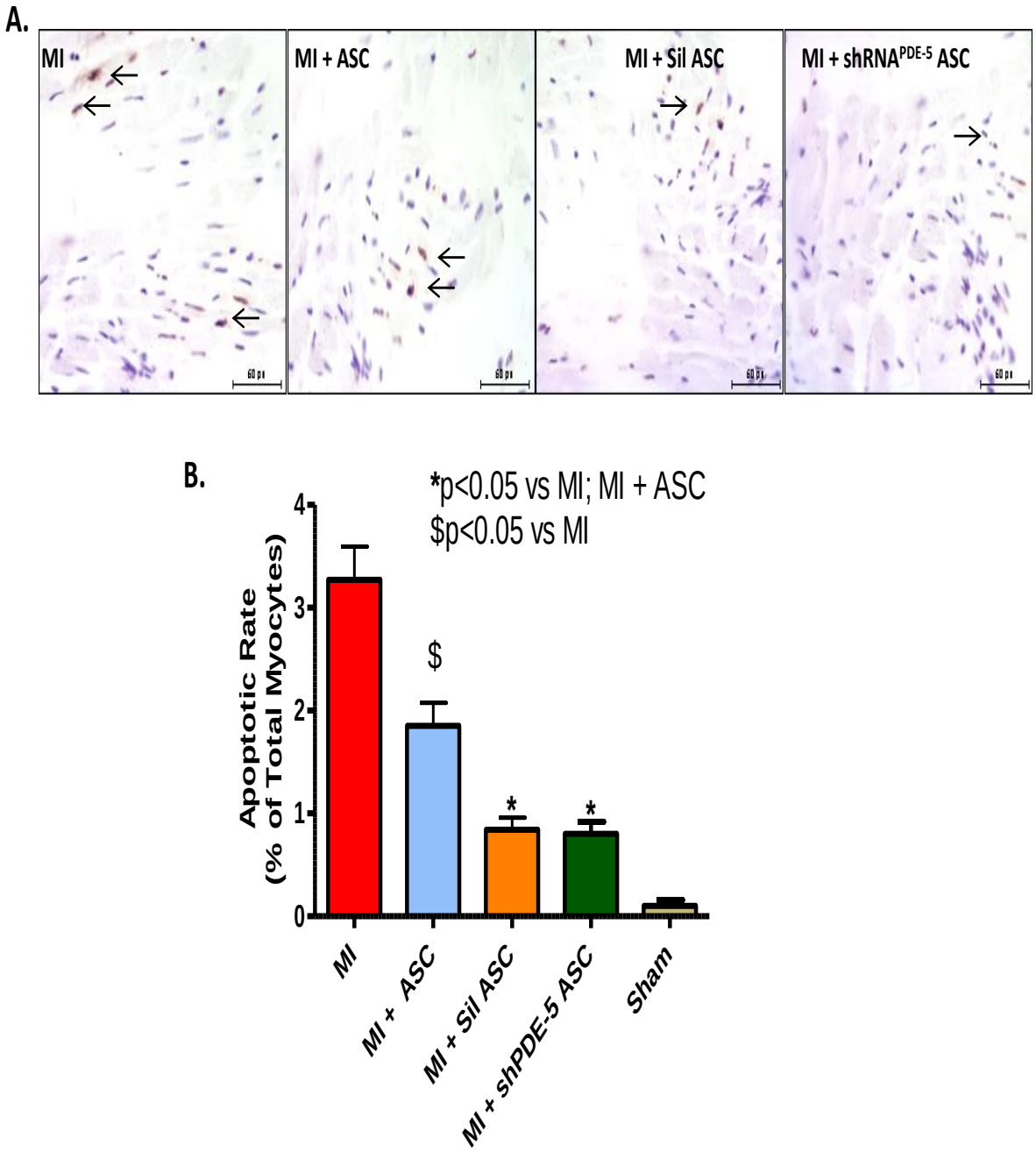

Figure 19. Preconditioned ASCs reduces myocardial apoptosis following myocardial infarction. (A) Representative TUNEL staining of sections and (B) bar diagram showing quatification of TUNEL positive cells; (n=4).

Preconditioned ASCs Increase Vascular Density and Secretion of Growth Factors

Rapid restoration of blood supply to the ischemic region is critical for stabilizing the border region of the infarct and supporting viable and regenerating myocardium. We used CD-31 positive staining to determine the vascular density in the border zone of MI. Transplantation of ASCs significantly increased vascular density as compared with that of PBS-treated hearts (Fig. 20). However, the vascular density in sildenafil and shRNA^{PDE-5} ASCs treated mice was significantly enhanced as compared to non-treated ASCs mice (8.3±1.3 in sildenafil-treated ASCs; 7.8±0.5 shRNA^{PDE-5} ASCs vs 4.5±0.6 vessels/HPF, $p<0.01$, $n=4$) (Fig. 20). Moreover, the plasma levels of b-FGF, Ang-1, and VEGF were significantly increased in mice receiving preconditioned ASCs (with sildenafil-treated or shRNA^{PDE-5} ASCs) as compared to non-treated ASCs ($p<0.001$, $n=5$, Fig. 21).

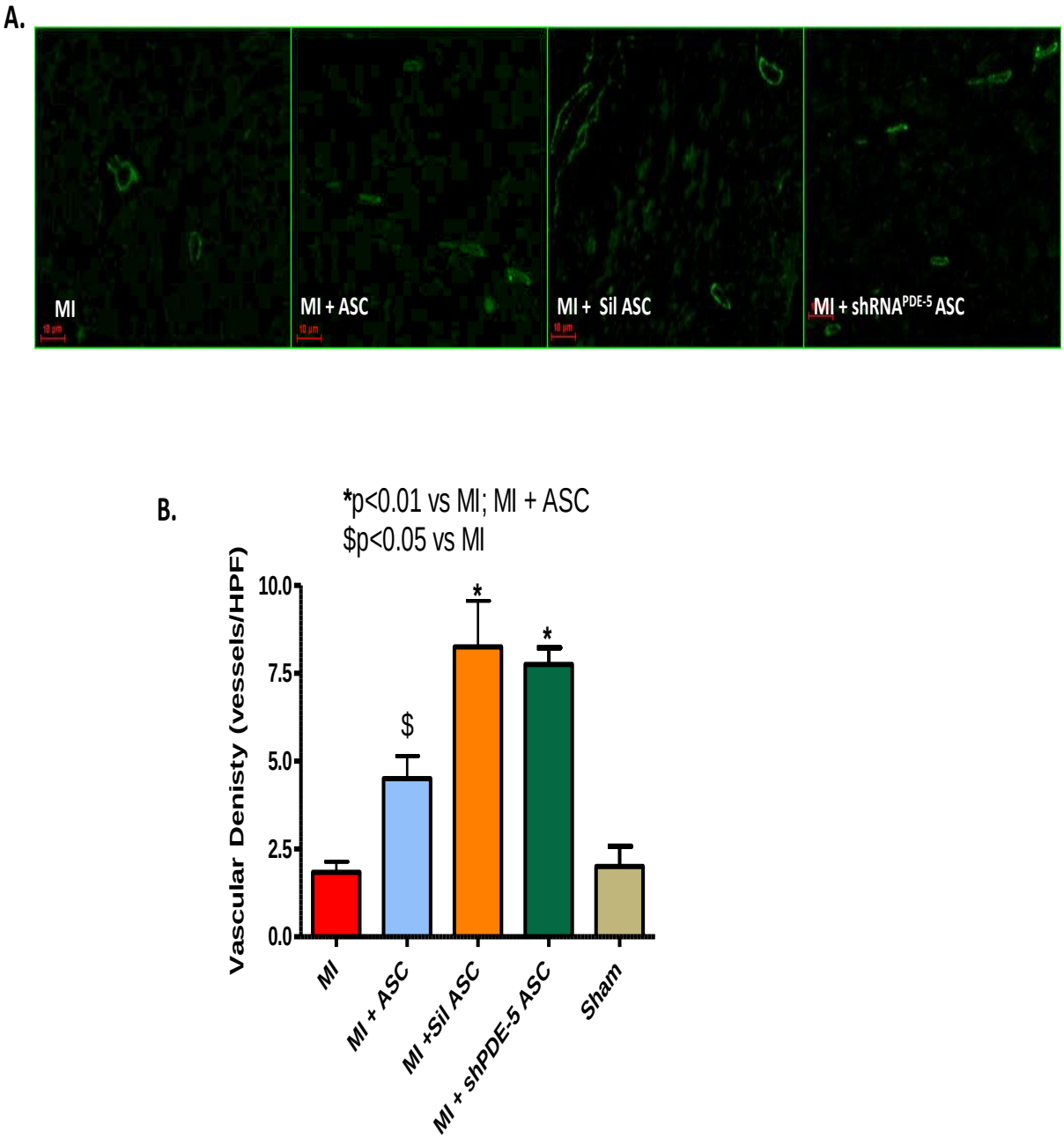

Figure 20. Preconditioned ASCs increases vascular density following myocardial infarction. (A) Representative images and (B) bar graph depicting immunostaining of sections with CD31 to assess vascular density; (n=4).

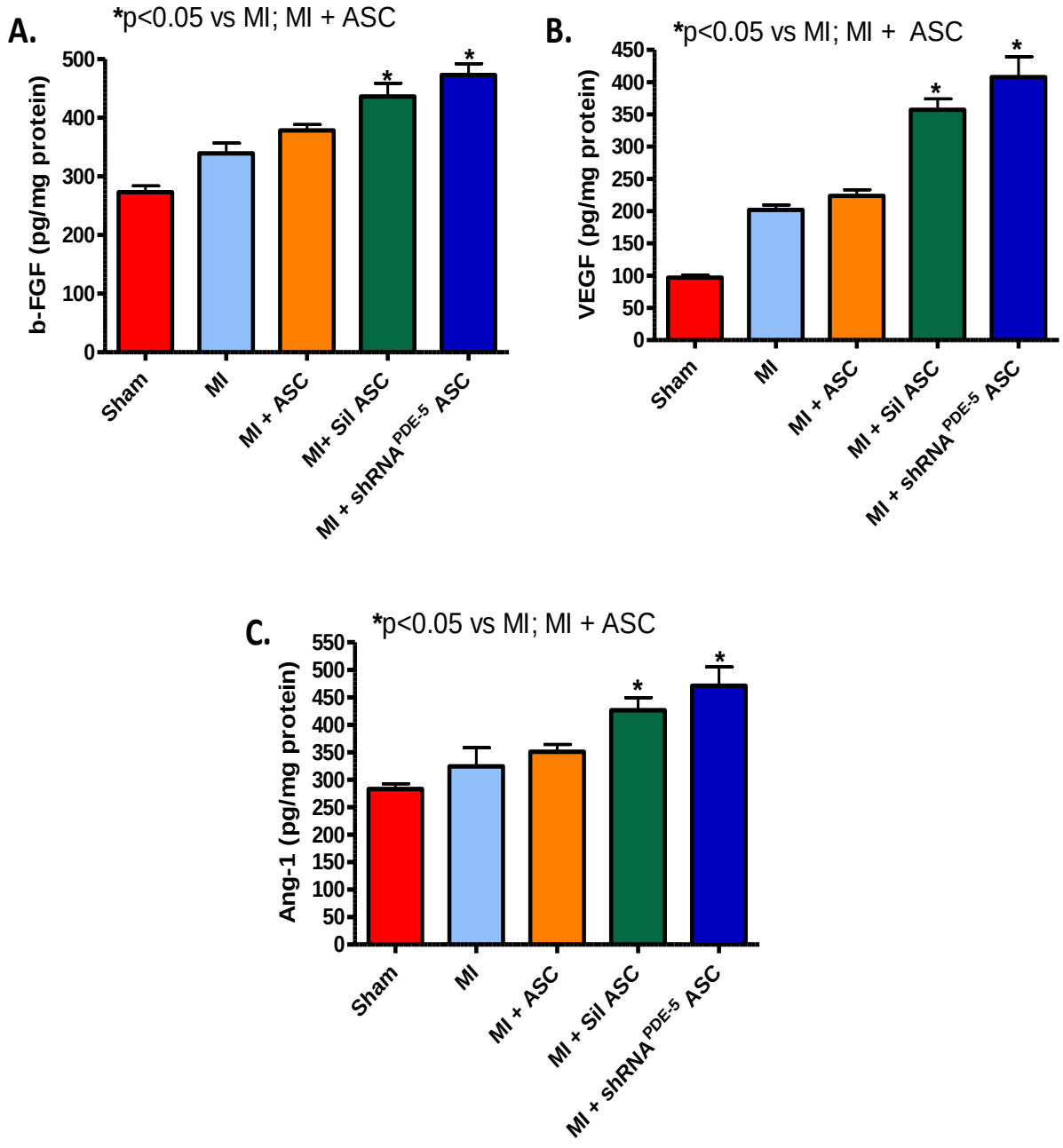

Figure 21. Transplantation of preconditioned ASCs enhances release of growth factors following myocardial infarction. Bar diagram showing blood levels of (A) b-FGF, (B) VEGF and (C) Ang-1 from the sham and infarcted hearts treated with PBS (MI); non-preconditioned ASCs (MI+ASC); preconditioned with sildenafil (MI+Sil ASCs) or PDE-5 silencing vector, $shRNA^{PDE-5}$ (MI+ $shRNA^{PDE-5}$) 4 weeks after MI. Note that preconditioned ASCs enhanced release of b-FGF, VEGF and Ang-1 as compared to control hearts; (n=5).

Discussion

Current treatment of MI still leaves a significant number of patients with impaired cardiac function that leads to more severe LV dysfunction as the ventricle remodels. Thus, cell therapy has generated much excitement as a novel therapy that might provide additive benefits over conventional treatment to restore or prevent further LV dysfunction after MI. Although there have been several reports that ASCs improve cardiac function post-MI effects have been limited (69-72). This study investigated the feasibility of *ex vivo* preconditioning by PDE-5 inhibition as a strategy to enhance the efficacy of stem cell therapy post-MI. As described in Chapter 2, PDE-5 inhibition in ASCs confers a powerful preconditioning-like effect against SI/RO limiting apoptosis and necrosis thus increasing survivability. The present study shows significant improvement in cardiac function 4 weeks after transplantation of preconditioned ASCs as demonstrated by significant preservation of FS and EF as compared to other treatment groups. Moreover, the superior functional improvement compared with non-preconditioned ASC injected group was associated with enhanced vascular density (Fig. 20), decrease in fibrosis and resident cardiomyocyte apoptosis (Fig. 18, 19). The observed benefits correlated with increased plasma levels of pro-angiogenic growth factors including VEGF, b-FGF, and Ang-1.

Various studies have reported the beneficial effects of stem cell therapy without long-term engraftment, and a link between donor cell-derived factors and LV recovery following cellular transplantation (67, 148-150). In the present study, we also observed a very limited number of PKH26 positive ASCs cells that were identified only in hearts which received preconditioned ASCs. PKH26-positive cells were present primarily in the epicardium of the border zone regions after 4 weeks (Fig. 16). The observation that only a limited number of

preconditioned cells were present 4 weeks post-MI is consistent with other studies that suggest that improving the effectiveness of graft patency with stem cells is dependent on increased concentrations of NO (151). We observed an increase in NO generation following sildenafil treatment (Chapter 2). Furthermore, other studies have showed a rapid loss of transplanted cells after 24 h and 7 d following direct intramyocardial injection post-MI. Moreover similar to our study, it was reported that the numbers of cells present in the post-MI heart were almost negligible by 2 weeks. However, cellular transplantation still attenuated LV dysfunction and remodeling, reduced myocyte apoptosis and augmented myocardial neovascularization, despite poor engraftment of transplanted cells (152). Another study by MacLellan et al (51) demonstrated the ability of ASCs, delivered acutely following LAD ischemia/reperfusion, to improve cardiac function independent of engraftment. This is in agreement with Limbourg et al. who also showed that hematopoietic stem cells can improve cardiac function post-MI, even in the absence of sustained engraftment (153). Interestingly, similar reductions in fibrotic tissue have been observed when either the supernatant from the modified cells or specific secreted proteins used to modify the mesenchymal stem cell were injected into the infarct zone (66). These observations support the paracrine hypothesis of myocardial repair, and suggest not only the potential for pharmacological or genetic enhancement of stem cell therapies, but also suggest that high degree of benefit observed with cell therapy is independent of cellular engraftment. As cellular differentiation does not appear to play a major role in the therapeutic effect of ASCs in this model system, it is logical to hypothesize that the paracrine release of cytokines and growth factors by the transplanted ASCs in the first few days or weeks after injection is responsible for the observed effects. Consistent with our results, it is possible that a small number of specifically

conditioned or genetically altered cells may be able to exploit paracrine pathways to maximize the biological and clinical effects after cell delivery (9, 58 148, 154-159). Paracrine factors released by modified or preconditioned ASCs potentially mediate multiple mechanisms such as myocardial cell survival, remodeling, contractility, increased blood flow to ischemic tissue, regulation of inflammatory response, and recruiting endogenous stem cells to regenerate injured tissue. The lack of significant engraftment despite tremendous reduction in cardiac fibrosis seen in the preconditioned ASC treatments suggests that a paracrine mechanism from enhanced secretion of growth factors may play an important beneficial role in repairing the damaged myocardium.

This proposed mechanism has been suggested by other investigators examining stem cell therapy administered acutely post-MI. Angiogenic and cytoprotective growth factors such as VEGF and Ang-1 have been shown to significantly improve cardiac function through increased angiogenesis and decreases in infarct size when administered acutely or delivered as a gene construct post-MI (21, 57, 59). Similarly, Li et al. showed that increased capillary density following ASC treatment in a rat model of MI that correlated with higher VEGF mRNA and protein levels (57). Experiments have compared the effects of individual angiogenic genes, Ang-1 and VEGF with ASC therapy in a murine model of MI. While both Ang-1 and VEGF significantly improved cardiac performance, ASCs were superior in alleviating diastolic function and improving capillary density. In our present study, we observed that reduction in fibrosis following transplantation of preconditioned ASC was associated with increased angiogenesis in the infarcted areas in post-MI hearts as demonstrated by increased capillary and arteriole density within the infarct border zones (Fig. 20). Our results showed that the observed improved vascular

density after transplantation of preconditioned ASCs was correlated with increased secretion of growth factors with angiogenic potential; b-FGF, IGF-1 and VEGF (Chapter 2). While myocardial hypoxia during infarction has been demonstrated to induce expression of angiogenic growth factors including VEGF, the increased plasma levels of VEGF, Ang-1, and b-FGF we observed following treatment with preconditioned ASCs suggests the increased duration of ASCs within the ischemic microenvironment possibly allows them to function as "paracrine pumps" to sustain increased levels of growth factors which are known to lead to increased angiogenesis, suppression of cardiac fibrosis, and attenuation of cardiomyocyte apoptosis.

A number of studies have shown that apoptosis in cardiomyocytes contributes to the progression of heart failure after MI (97, 160), and chronic cardiac remodeling with chamber dilation and impaired systolic function is associated with increased myocyte apoptosis in the infarct border zone after MI (161). Our results demonstrate that transplantation of preconditioned ASCs abrogated resident cardiomyocyte apoptosis seen in non-treated ischemic hearts. Furthermore, the extent of apoptosis was significantly lower from preconditioned ASC treated hearts compared with control ASC treated hearts at 28 days post-MI. Overexpression of Ang-1, VEGF, or b-FGF has been shown to improve cell survival, neovascularization, and cardiac function by limiting the remodeling process in the scar while decreasing apoptosis of myocytes in the peri-infarct region (21, 58, 78). Transplantation of ASCs along with their secretion of VEGF, bFGF, and IGF attenuated cardiomyocyte apoptosis *in vitro* and *in vivo* (21). Preconditioning of ASCs through PDE-5 inhibition exerts a similar cardioprotective effect. The present results are consistent with the limitation of necrosis and apoptosis of adult cardiomyocytes treated with conditioned medium and preconditioned ASCs subjected to SI/RO

(Chapter 2) suggesting that the protective effect is attributed to increased duration and release of paracrine factors in the ischemic myocardium.

Besides the increased duration of pro-survival growth factors, another explanation for the cardioprotection seen following transplantation of preconditioned ASCs, could be due to the induction of NO generation following PDE-5 inhibition observed in Chapter 2. We speculate that increased duration of preconditioned ASCs concomitant with increased NO release could allow for diffusion of NO into the ischemic region and possibly preserve cardiomyocytes through the opening of mitoK_{ATP} channels, preventing Ca^{2+} overload, and reducing oxidative stress. Moreover, we have shown that sildenafil attenuated apoptosis as well as necrosis in cardiomyocytes subjected to simulated ischemia and reoxygenation through nitric oxide (NO)-dependent pathway (75). It is also likely that the reduction of fibrotic tissue seen after cellular treatment with preconditioned ASCs could be the result of increased generation of NO that could then diffuse into the ischemic zone and restore the cGMP/PKG pathway. We have reported previously that sildenafil-induced increases in NO is involved in infarct size reduction after 24 h, even in a model of permanent LAD occlusion as the infarct sparing effect of sildenafil were blocked with NOS inhibitor, L-NAME (91). Although, there is no clear mechanism for the observed reduction of apoptosis and fibrosis following preconditioned ASC therapy, it is likely that increased release of NO along with pro-survival growth factors confers a cardioprotective mechanism through enhanced paracrine signaling. However, further studies are needed to investigate the role of NO in our model.

In conclusion, we have shown that *ex vivo* preconditioning of ASCs by PDE-5 inhibition prior to transplantation into mice post-MI ameliorates LV remodeling, preserves LV function,

and reduced cardiomyocyte apoptosis and fibrosis possibly by improving stem cell survival and paracrine effects. Sildenafil is already being tested clinically in patients with heart failure and preserved ejection fraction (i.e. EF>50%) in the ongoing NIH multicenter trial **(RELAX: Evaluating the Effectiveness of Sildenafil at Improving Health Outcomes and Exercise Ability in People With Diastolic Heart Failure; NCT00763867**). We believe that the easy availability of ASCs from humans during surgery combined with the preconditioning strategy of blocking PDE-5 using clinically approved PDE-5 inhibitors may hold great promise for initiation of clinical trials in heart failure patients.

Funding Sources: This study was supported by grants from National Institutes of Health (HL51045, HL79424 and HL93685) to Rakesh C. Kukreja, National Scientist Development Grant from the American Heart Association (10SDG3770011) to Fadi N. Salloum, and Predoctoral Fellowship from the American Heart Association (09PRE2250905) to Nicholas N. Hoke.

Chapter 4

Conclusion and Future Directions

Ischemic heart disease (IHD) remains a leading cause of morbidity and mortality despite recent advances in pharmacotherapy and improved acute care. Increased incidence of risk factors such as hypertension, obesity, diabetes, and smoking over the last several decades have all contributed to this increased prevalence of IHD. Therefore a conceptual breakthrough is needed to develop novel targets and strategies to prevent and cure IHD. In recent years, cardiac repair by stem cells has gained tremendous attention and imagination because of its initial success and safety potential. However, there are several major hurdles such as improvement in the number of reparative cells homing to injured areas, selection of ideal cell type(s), enhancement of regenerative and differentiation capacity of transplanted cells and optimization of extracellular milieu for engraftment and differentiation that would significantly impact long-term beneficial effects on cardiac functions. In the present study, we have used an innovative strategy of preconditioning the adipose stem cells (ASC) through inhibition of phosphodiesterase-5 (PDE-5) by sildenafil or genetic knockdown with a silencing vector. Our results showed that preconditioned ASC developed significant resistance against ischemic injury *in vitro* with both of these approaches. Furthermore, transplantation of preconditioned ASC following a chronic

model of myocardial infarction (MI) resulted in amelioration of left ventricular (LV) remodeling, preserved LV function, reduced cardiomyocyte apoptosis and fibrosis possibly by improving stem cell survival through paracrine effects. We also like to mention that sildenafil is an interesting drug because it is widely used for treatment of erectile dysfunction and other cardiovascular disorders including pulmonary hypertension in patients. Moreover, there is an ongoing NIH multicenter trial (RELAX: Evaluating the Effectiveness of Sildenafil at Improving Health Outcomes and Exercise Ability in People With Diastolic Heart Failure; NCT00763867) in patients with heart failure. Another clinical trial on sildenafil has recently been initiated (REVERSE-DMD, NCT01168908) to treat Duchenne's patients with cardiac disease, which is currently recruiting patients at the Johns Hopkins Medical Institutions. Because of the clinical safety and efficacy of sildenafil, we believe that this drug would be an ideal candidate to trigger cGMP-dependent surivival pathway for preconditioning of ASC and their eventual use in patients for myocardial repair

We also like to stress that despite our promising results, further studies need to be done to investigate the role of PDE-5 inhibition in enhancing stem cell therapy. In particular the effect of preconditioned ASC transplantation should be examined in a model of ischemia/reperfusion injury model in addition to the permanent ischemia model used in the current investigation. This is because the majority of patients with acute myocardial infarction undergo spontaneous or therapeutic reperfusion. Reperfusion is known to cause tissue damage when blood supply returns to the tissue following ischemia. The restoration of circulation following the absence of oxygen and nutrients from blood results in a surge of free radicals in the form of reactive oxygen species and oxidative stress causing subsequent oxidative damage and inflammation.

Consequently, it is of utmost importance to determine whether ASC are effective when coronary occlusion is followed by reperfusion, an event that dramatically alters the milieu of the myocardial interstitium and of the myocardium itself.

In addition to the physiological studies, there is critical need to examine new molecular mechanisms by which preconditioning of ASC leads to improvement in cardiac function and preservation of myocardial structure. We will study the role of PKG in mediating the cytoprotective effect by using strategies such as overexpression of PKG-1α in ASC using adenoviral/lentiviral approaches prior to transplantation in the heart. We also plan to study the role frizzled related protein 2 (Sfrp2) as a survival molecule in ASCs preconditioned with PDE-5 inhibition/PKG1α overexpression. Recent studies have identified sfrp2 as a key survival molecule which acts via the canonical Wnt/β-catenin pathway (162). We anticipate that our current study and future efforts would lead to expanding the use of sildenafil and other clinically used PDE-5 inhibitors in regenerative medicine.

List of References

List of References

1. Center for Disease Control: http://www.cdc.gov/Features/HeartMonth/#References
2. R.A. Kloner and R.B. Jennings, Consequences of brief ischemia: stunning, preconditioning, and their clinical implications: part 1. Circulation. 2001 Dec 11;104(24):2981-9.
3. Kilgore KS, Friedrichs GS, Homeister JW, and Lucchesi BR. The complement system in myocardial ischaemia/reperfusion injury. Cardiovasc Res. 1994 Apr;28(4):437-44.
4. Bolli R.: Mechanism of myocardial "stunning". Circulation. 1990 Sep;82(3):723-38.
5. Meng H, Pierce GN: Protective effect of 5-(N,N-dimethyl)-amiloride on ischemia reperfusion injury in hearts. Am J Physiol. 1990 May;258(5 Pt 2):H1615-9.
6. Anversa P, Li P, Zhang X, Olivetti G, Capasso JM. Ischaemic myocardial injury and ventricular remodelling. Cardiovasc Res. 1993 Feb;27(2):145-57.
7. Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction: experimental observations and clinical implications. Circulation. 1990 Apr;81(4):1161-72.
8. Cohn JN, Ferrari R, Sharpe N. Cardiac remodeling—concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling. Behalf of an International Forum on Cardiac Remodeling. J Am Coll Cardiol. 2000 Mar 1;35(3):569-82.

9. Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F, Mu H, Melo LG, Pratt R, Ingwall J, Dzau V. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J. 2006 Apr;20(6):661-9.

10. Casteilla L, Planat-Bénard V, Cousin B, Silvestre JS, Laharrague P, Charrière G, Carrière A, Pénicaud L. Plasticity of adipose tissue: a promising therapeutic avenue in the treatment of cardiovascular and blood diseases? Arch Mal Coeur Vaiss. 2005 (98), 922-926.

11. Oedayrajsingh-Varma M, van Ham S, Knippenberg M, Helder M, Klein-Nulend J, Schouten T, Ritt M, van Milligen F. Adipose tissue-derived mesenchymal stem cell yield and growth characteristics are affected by the tissue-harvesting procedure. Cytotherapy 2006 (8), 166-177.

12. Dicker A, Le Blanc K, Aström G, van Harmelen V, Götherström C, Blomqvist L, Arner P, Rydén M. Functional studies of mesenchymal stem cells derived from adult human adipose tissue. Exp Cell Res. 2005(308), 283-290.

13. Zuk P, Zhu M, Ashjian P, De Ugarte D, Huang J, Mizuno H, Alfonso Z, Fraser J, Benhaim P, Hedrick M. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002(12), 4279-4295.

14. Zuk P, Zhu M, Mizuno H, Huang J, Futrell J, Katz A, Benhaim P, Lorenz H, Hedrick M. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001(2), 211-228.

15. Planat-Benard V, Menard C, Andre M, Puceat M, Perez A, Garcia-Verdugo J, Penicaud L, Casteilla L. Spontaneous cardiomyocyte differentiation from adipose tissue stroma cells. Circ Res. 2004(94), 223–229.

16. Leri A, Kajstura J, Anversa P. Cardiac stem cells and mechanisms of myocardial regeneration. Physiol Rev. 2005(85), 1373-1416.

17. Mann D, Bristow M: Mechanisms and models in heart failure:the biochemical model and beyond. Circulation 2005(111), 2837-2849.

18. Massie B. 15 years of heart-failure trials: what have we learned? Lancet 1998(352), S129-S133.

19. Moon M, Kim S, Kim Y, Kim S, Lee J, Bae Y, Sung S, Jung J. Human adipose tissue-derived mesenchymal stem cells improve postnatal neovascularization in a mouse model of hindlimb ischemia. Cell Physiol Biochem. 2006(17), 279-290.

20. Nagaya N, Kangawa K, Itoh T, Iwase T, Murakami S, Miyahara Y, Fujii T, Uematsu M, Ohgushi H, Yamagishi M, Tokudome T, Mori H, Miyatake K, Kitamura S. Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. Circulation 2005(112), 1128-1135.

21. Nishida S, Nagamine H, Tanaka Y, Watanabe G. Protective effect of basic fibroblast growth factor against myocyte death and arrhythmias in acute myocardial infarction in rats. Circ J. 2003(67), 334-339.

22. Yoon YS, Wecker A, Heyd L, Park JS, Tkebuchava T, Kusano K. Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. J Clin Invest. 2005(115), 326-338.

23. De Ugarte D, Morizono K, Elbarbary A. Comparison of multi-lineage cells from human adipose tissue and bone marrow. Cells Tissues Organs 2003(174), 101–109.

24. Zhu Y, Liu T, Song K, Fan X, Ma X, Cui Z. Adipose-derived stem cell: a better stem cell than BMSC. Cell Biochem Funct. 2008(6), 664-675.

25. Smith P, Adams W, Lipschitz A. Autologous human fat grafting: Effect of harvesting and preparation techniques on adipocyte graft survival. Plast Reconstr Surg 2006(117), 1836 –1844.

26. Pittenger M, Mackay A, Beck S, Jaiswal R, Douglas R, Mosca J, Moorman M, Simonetti D, Craig S, Marshak D. Multilineage potential of adult human mesenchymal stem cells. Science. 1999(284), 143-147.

27. Ryden M, Dicker A, Gotherstrom C, Astrom G, Tammik C, Arner P, Le Blanc K. Functional characterization of human mesenchymal stem cell derived adipocytes. Biochem Biophys Res Commun. 2003(311), 391–397.

28. Young H, Steele T, Bray R, Detmer K, Blake L, Lucas P, Black A. Human pluripotent and progenitor cells display cell surface cluster differentiation markers cd10, cd13, cd56, and mhc class-i. Proc Soc Exp Biol Med. 1999(221), 63–71.

29. Wagner W, Wein F, Seckinger A. Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. Exp Hematol 2005(33), 1402–1416.

30. Fraser J, Schreiber R, Zuk P, Hendrick M. Adult stem cell therapy for the heart. Int J Biochem Cell Biol 2004(36), 658.

31. McIntosh K, Zvonic S, Garrett S, Mitchell JB, Floyd ZE, Hammill L, Kloster A, Halvorsen YD, Ting JP, Storms RW, Goh B, Kilroy G, Wu X, Gimble JM. The immunogenicity of human adipose derived cells: temporal changes in vitro. Stem Cells 2006(24), 1245–1253.

32. Oedayrajsingh-Varma M, van Ham S, Knippenberg M, Helder M, Klein-Nulend J, Schouten T, Ritt M, van Milligen F. Adipose tissue-derived mesenchymal stem cell yield and growth characteristics are affected by the tissue-harvesting procedure. Cytotherapy 2006(8), 166-177.

33. Gronthos S, Franklin D, Leddy H, Robey P, Storms R, Gimble J. Surface protein characterization of human adipose tissue-derived stromal cells. J Cell Physiol. 2001(189), 54-63.

34. Puissant B, Barreau C, Bourin P. Immunomodulatory effect of human adipose tissue-derived adult stem cells: Comparison with bone marrow mesenchymal stem cells. Br J Haematol 2005(129), 118 –129.

35. Rodriguez A, Elabd C, Amri E, Ailhaud G, Dani C. The human adipose tissue is a source of multipotent stem cells. Biochimie. 2005 (87), 125-128.

36. Gaustad KG, Boquest AC, Anderson BE, Gerdes AM, Collas P. Differentiation of human adipose tissue stem cells using extracts of rat cardiomyocytes. Biochem Biophys Res Commun. 2004(314), 420 – 427.

37. Schächinger V, Erbs S, Elsässer A, Haberbosch W, Hambrecht R, Hölschermann H, Yu J, Corti R, Mathey DG, Hamm CW, Süselbeck T, Werner N, Haase J, Neuzner J, Germing A, Mark B, Assmus B, Tonn T, Dimmeler S, Zeiher AM; REPAIR-AMI

Investigators. Improved clinical outcome after intracoronary administration of bone-marrow-derived progenitor cells in acute myocardial infarction: final 1-year results of the REPAIR-AMI trial. Eur Heart J. 2006(27), 2775-2783.

38. Song YH, Gehmert S, Sadat S, Pinkernell K, Bai X, Matthias N, Alt E. VEGF is critical for spontaneous differentiation of stem cells into cardiomyocytes. Biochem Biophys Res Commun. 2007(354), 999 –1003.

39. Rangappa S, Entwistle J, Wechsler A, Kresh J. Cardiomyocyte-mediated contact programs human mesenchymal stem cells to express cardiogenic phenotype. J Thorac Cardiovasc Surg. 2003(126), 124 –132.

40. Yamada Y, Wang XD, Yokoyama S, Fukuda N, Takakura N. Cardiac progenitor cells in brown adipose tissue repaired damaged myocardium. Biochem Biophys Res Commun. 2006(342), 662-670.

41. Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H, Ishino K, Ishida H, Shimizu T, Kangawa K, Sano S, Okano T, Kitamura S, Mori H. Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nat Med. 2006(12), 459 – 465.

42. Planat-Benard V, Silvestre J, Cousin B, André M, Nibbelink M, Tamarat R, Clergue M, Manneville C, Saillan-Barreau C, Duriez M, Tedgui A, Levy B, Pénicaud L, Casteilla L. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation 2004(109), 656-663.

43. Urbich C, Dimmeler S. Endothelial progenitor cells functional characterization. Trends Cardiovasc Med 2004(14), 318 –322.

44. Martinez-Estrada OM, Munoz-Santos Y, Julve J, Reina M, Vilaro S. Human adipose tissue as a source of flk-1 cells: new method of differentiation and expansion. Cardiovasc Res. 2005(65), 328 –333.

45. Cao Y, Sun Z, Liao L, Meng Y, Han Q, Zhao RC. Human adipose tissue-derived stem cells differentiate into endothelial cells in vitro and improve postnatal neovascularization in vivo. Biochem Biophys Res Commun. 2005(332), 370 –379.

46. Miranville A, Heeschen C, Sengenes C, Curat C, Busse R, Bouloumie A. Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. Circulation 2004(110), 349 –355.

47. Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove C, Bovenkerk J, Pell C, Johnstone B, Considine R, March K. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. Circulation 2004(109), 1292–1298.

48. Zhang D, Gai L, Liu H, Jin Q, Huang J, Zhu X. Transplantation of autologous adipose-derived stem cells ameliorates cardiac function in rabbits with myocardial infarction. Chin Med J (Engl). 2007(120), 300-307.

49. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine D. Bone marrow cells regenerate infarcted myocardium. Nature 2001(410), 701-705.

50. Valina C, Pinkernell K, Song Y, Bai X, Sadat S, Campeau R, Le Jemtel T, Alt E. Intracoronary administration of autologous adipose tissue-derived stem cells improves left ventricular function, perfusion, and remodelling after acute myocardial infarction. Eur Heart J. 2007(21), 2667-2677.

51. Schenke-Layland K, Strem B, Jordan M, Deemedio M, Hedrick M, Roos K, Fraser J, Maclellan W. Adipose Tissue-Derived Cells Improve Cardiac Function Following Myocardial Infarction. J Surg Res. 2009 (153),217-23.

52. Strem BM, Zhu M, Alfonso Z, Daniels EJ, Schreiber R, Beygui R, MacLellan WR, Hedrick MH, Fraser JK. Expression of cardiomyocytic markers on adipose tissue-derived cells in a murine model of acute myocardial injury. Cytotherapy 2005 (7), 282–291.

53. Yu L, Kim M, Park T, Cha K, Kim Y, Quan M, Rho M, Seo S, Jung J. Improvement of cardiac function and remodeling by transplanting adipose tissue-derived stromal cells into a mouse model of acute myocardial infarction. Int J Cardiol 2010(139), 166-72.

54. Wang M, Tsai B, Crisostomo P, Meldrum D. Pretreatment with adult progenitor cells improves recovery and decreases native myocardial proinflammatory signaling after ischemia. Shock 2006(5), 454-459.

55. Katz A, Zang Z, Shang H, Chamberlain A, Berr S, Roy R, Khurgel M, Epstein F, French B. Serial MRI assessment of human adipose-derived stem cells (HASCS) in a murine model of reperfused myocardial infarction. Adipocytes. In press.

56. Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F, Mu H, Melo LG, Pratt R, Ingwall J, Dzau V. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J. 2006(6), 661-669.

57. Zhou L, Ma W, Yang Z, Zhang F, Lu L, Ding Z, Ding B, Ha T, Gao X, Li C. VEGF165 and angiopoietin-1 decreased myocardium infarct size through phosphatidylinositol-3 kinase and Bcl-2 pathways. Gene Ther. 2005(3), 196-202.

58. Mangi A, Noiseux N, Kong D, He H, Rezvani M, Ingwall J, Dzau V. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat Med. 2003(9), 1195-1201.

59. Anderson C, Heydarkhan-Hagvall S, Schenke-Layland K, Yang J, Jordan M, Kim J, Brown D, Zuk P, Laks H, Roos K, Maclellan W, Beygui R. The role of cytoprotective cytokines in cardiac ischemia/reperfusion injury. J Surg Res. 2008(148), 164-171.

60. Zhu X, Zhang X, Xu L, Zhong X, Ding Q, Chen Y. Transplantation of adipose-derived stem cells overexpressing hHGF into cardiac tissue. Biochem Biophys Res Commun. 2009 (379), 1084-90.

61. Uemura R, Xu M, Ahmad N, Ashraf M. Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling. Circ Res. 2006(98), 1414-1421.

62. Satoh M, Minami Y, Takahashi Y, Nakamura M. Immune modulation: role of the inflammatory cytokine cascade in the failing human heart. Curr Heart Fail Rep. 2008(2), 69-74.

63. Guo J, Lin G, Bao C, Hu Z, Hu M. Anti-inflammation role for mesenchymal stem cells transplantation in myocardial infarction. Inflammation 2007(4), 97-104.

64. Ohnishi S, Yanagawa B, Tanaka K, Miyahara Y, Obata H, Kataoka M, Kodama M, Ishibashi-Ueda H, Kangawa K, Kitamura S, Nagaya N. Transplantation of mesenchymal stem cells attenuates myocardial injury and dysfunction in a rat model of acute myocarditis. J Mol Cell Cardiol. 2007(42), 88-97.

65. Varda-Bloom N, Leor J, Ohad D, Hasin Y, Amar M, Fixler R, Battler A, Eldar M, Hasin D. Cytotoxic T lymphocytes are activated following myocardial infarction and can recognize and kill healthy myocytes in vitro. J Mol Cell Cardiol. 2000(32), 2141-2149.

66. Ohnishi S, Sumiyoshi H, Kitamura S, Nagaya N. Mesenchymal stem cells attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions. FEBS Lett. 2007(581), 3961-3966.

67. Iso Y, Spees J, Serrano C, Bakondi B, Pochampally R, Song Y, Sobel B, Delafontaine P, Prckop D. Multipotent human stromal cells improve cardiac function after myocardial infarction in mice without long-term engraftment. Biochem Biophys Res Commun. 2007(354), 700-706.

68. Palmer J, Hartogensis W, Patten M, Fortuin F, Long C. Interleukin-1 beta induces cardiac myocyte growth but inhibits cardiac fibroblast proliferation in culture. J Clin Invest. 1995(95), 2555-2564.

69. Mazo M, Planat-Bénard V, Abizanda G, Pelacho B, Léobon B, Gavira JJ, Peñuelas I, Cemborain A, Pénicaud L, Laharrague P, Joffre C, Boisson M, Ecay M, Collantes M, Barba J, Casteilla L, Prósper F. Transplantation of adipose derived stromal cells is associated with functional improvement in a rat model of chronic myocardial infarction. Eur J Heart Fail. 2008 May;10(5):454-62.

70. Zhang DZ, Gai LY, Liu HW, Jin QH, Huang JH, Zhu XY. Transplantation of autologous adipose-derived stem cells ameliorates cardiac function in rabbits with myocardial infarction. Chin Med J (Engl). 2007 Feb 20;120(4):300-7.

71. Tomita S, Mickle DA, Weisel RD, Jia ZQ, Tumiati LC, Allidina Y, Liu P, Li RK. Improved heart function with myogenesis and angiogenesis after autologous porcine bone marrow stromal cell transplantation. J Thorac Cardiovasc Surg. 2002 Jun;123(6):1132-1140.

72. Kim BO, Tian H, Prasongsukarn K, Wu J, Angoulvant D, Wnendt S, Muhs A, Spitkovsky D, Li RK. Cell transplantation improves ventricular function after a myocardial infarction: a preclinical study of human unrestricted somatic stem cells in a porcine model. Circulation. 2005 Aug 30;112(9 Suppl):I96-104.

73. Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation. 2002 Jan 1;105(1):93-8.*

74. Wang JA, Chen TL, Jiang J, Shi H, Gui C, Luo RH, Xie XJ, Xiang MX, Zhang X. Hypoxic preconditioning attenuates hypoxia/reoxygenation-induced apoptosis in mesenchymal stem cells. Acta Pharmacol Sin. 2008 Jan;29(1):74-82.

75. Das A, Xi L, Kukreja RC. Phosphodiesterase-5 inhibitor sildenafil preconditions adult cardiac myocytes against necrosis and apoptosis. *J Biol Chem. 2005 Apr 1;280(13):12944-55.*

76. Pasha Z, Wang Y, Sheikh R, Zhang D, Zhao T, Ashraf M. Preconditioning enhances cell survival and differentiation of stem cells during transplantation in infarcted myocardium. Cardiovasc Res. 2008 Jan;77(1):134-42.

77. Jiang S, Haider HKh, Idris NM, Salim A, Ashraf M. Supportive interaction between cell survival signaling and angiocompetent factors enhances donor cell survival and promotes angiomyogenesis for cardiac repair. Circ Res. 2006 Sep 29;99(7):776-84.

78. Yau TM, Kim C, Ng D, Li G, Zhang Y, Weisel RD, Li RK. Increasing transplanted cell survival with cell-based angiogenic gene therapy. Ann Thorac Surg. 2005 Nov;80(5):1779-86.

79. Pérez NG, Piaggio MR, Ennis IL, Garciarena CD, Morales C, Escudero EM, Cingolani OH, Chiappe de Cingolani G, Yang XP, Cingolani HE. Phosphodiesterase 5A inhibition induces Na+/H+ exchanger blockade and protection against myocardial infarction. Hypertension. 2007 May;49(5):1095-103.

80. Vachiery JL, Huez S, Gillies H, Layton G, Hayashi N, Gao X, Naeije R. Safety, tolerability and pharmacokinetics of an intravenous bolus of sildenafil in patients with pulmonary arterial hypertension. Br J Clin Pharmacol. 2011 Feb;71(2):289-92.

81. Jing ZC, Yu ZX, Shen JY, Wu BX, Xu KF, Zhu XY, Pan L, Zhang ZL, Liu XQ, Zhang YS, Jiang X, Galiè N; for the Efficacy and safety of VArdenafiL in the treatment of pUlmonary Arterial hyperTensION (EVALUATION) Study Group. Vardenafil in Pulmonary Arterial Hypertension: A Randomized, Double-blind, Placebo-controlled Study. Am J Respir Crit Care Med. 2011

82. Lu XL, Xiong CM, Shan GL, Zhu XY, Wu BX, Wu GH, Liu ZH, Ni XH, Cheng XS, Gu Q, Zhao ZH, Zhang DZ, Li WM, Zhang C, Tian HY, Guo YJ, Guo T, Liu HM, Zhang WJ, Gu H, Huang SA, Chen JY, Wu WF, Huang K, Li JJ, He JG. Impact of sildenafil

therapy on pulmonary arterial hypertension in adults with congenital heart disease. Cardiovasc Ther. 2010 Dec;28(6):350-5.

83. Corbin, J.D., Francis, S.H. Pharmacology of phosphodiesterase-5 inhibitors. Int. J. Clin. Pract. 2002(56), 453– 459.

84. Corbin, J.D., Francis, S.H., Webb, D.J. Phosphodiesterase type 5 as a pharmacologic target in erectile dysfunction. Urology 2002(60) (Suppl. 2), 4–11.

85. Rotella, D.P. Phosphodiesterase 5 inhibitors: current status and potential applications. Nat. Rev., Drug Discov. 2002(1), 674– 682.

86. Wallis, R.M., Corbin, J.D., Francis, S.H., Ellis, P. Tissue distribution of phosphodiesterase families and the effects of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings in vitro. Am. J. Cardiol. 1999(83), 3C–12C (suppl.).

87. Lin CS, Lin G, Xin ZC, and Lue TF. Expression, distribution and regulation of phosphodiesterase 5. Curr Pharm Des 2006(12): 3439-3457.

88. Senzaki, H., Smith, C.J., Juang, G.J., Isoda, T., Mayer, S.P., Ohler, A., Paolocci, N., Tomaselli, G.F., Hare, J.M., Kass, D.A. Cardiac phosphodiesterase 5 (cGMP-specific) modulates beta-adrenergic signaling in vivo and is down-regulated in heart failure. FASEB J. 2001(15), 1718–1726.

89. Haider HKh, Lee YJ, Jiang S, Ahmed RP, Ryon M, Ashraf M. Phosphodiesterase inhibition with tadalafil provides longer and sustained protection of stem cells. Am J Physiol Heart Circ Physiol. 2010 Nov;299(5):H1395-404.

90. Ockaili R, Salloum F, Hawkins J, Kukreja RC. Sildenafil (Viagra) induces powerful cardioprotective effect via opening of mitochondrial K(ATP) channels in rabbits. Am J Physiol Heart Circ Physiol. 2002 Sep;283(3):H1263-1269.

91. Salloum F, Yin C, Xi L, Kukreja RC. Sildenafil induces delayed preconditioning through inducible nitric oxide synthase-dependent pathway in mouse heart. Circ Res. 2003 Apr 4;92(6):595-597.

92. Kukreja RC, Ockaili R, Salloum F, Xi L. Sildenafil-induced cardioprotection in rabbits. Cardiovasc Res. 2003 Dec 1;60(3):700-1.

93. Bremer YA, Salloum F, Ockaili R, Chou E, Moskowitz WB, Kukreja RC. Sildenafil citrate (viagra) induces cardioprotective effects after ischemia/reperfusion injury in infant rabbits. Pediatr Res. 2005 Jan;57(1):22-7.

94. Salloum FN, Takenoshita Y, Ockaili RA, Daoud VP, Chou E, Yoshida K, Kukreja RC. Sildenafil and vardenafil but not nitroglycerin limit myocardial infarction through opening of mitochondrial K(ATP) channels when administered at reperfusion following ischemia in rabbits. J Mol Cell Cardiol. 2007 Feb;42(2):453-8.

95. Fisher PW, Salloum F, Das A, Hyder H, Kukreja RC. Phosphodiesterase-5 inhibition with sildenafil attenuates cardiomyocyte apoptosis and left ventricular dysfunction in a chronic model of doxorubicin cardiotoxicity. Circulation. 2005 Apr 5;111(13):1601-10.

96. Koka S, Das A, Zhu SG, Durrant D, Xi L, Kukreja RC. Long-acting phosphodiesterase-5 inhibitor tadalafil attenuates doxorubicin-induced cardiomyopathy without interfering with chemotherapeutic effect. J Pharmacol Exp Ther. 2010 Sep 1;334(3):1023-30.

97. Salloum FN, Abbate A, Das A, Houser JE, Mudrick CA, Qureshi IZ, Hoke NN, Roy SK, Brown WR, Prabhakar S, Kukreja RC. Sildenafil (Viagra) attenuates ischemic cardiomyopathy and improves left ventricular function in mice. Am J Physiol Heart Circ Physiol. 2008 Mar;294(3):H1398-406.

98. Das A, Xi L, Kukreja RC. Protein kinase G-dependent cardioprotective mechanism of phosphodiesterase-5 inhibition involves phosphorylation of ERK and GSK3beta. J Biol Chem. 2008 Oct 24;283(43):29572-85.

99. Arnold WP, Mittal CK, Katsuki S, Murad F. Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. Proc Natl Acad Sci 1977(74):3203–3207.

100. Katsuki S, Arnold W, Mittal C, Murad F. Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. J Cyclic Nucleotide Res 1977(3):23–35.

101. Katsuki S, Murad F. Regulation of adenosine cyclic 3',5'-monophosphate and guanosine cyclic 3¢,5¢-monophosphate levels and contractility in bovine tracheal smooth muscle. Mol Pharmacol 1977(13):330–341.

102. Guo, Y., Jones, W.K., Xuan, Y.T., Tang, X.L., Bao, W., Wu, W.J., Han, H., Laubach, V.E., Ping, P., Yang, Z., Qiu, Y., Bolli, R. The late phase of ischemic preconditioning is abrogated by targeted disruption of the inducible NO synthase gene. Proc. Natl. Acad. Sci. U. S. A. 1999(96), 11507–11512.

103. Imagawa, J., Yellon, D.M., Baxter, G.F. Pharmacological evidence that inducible nitric oxide synthase is a mediator of delayed preconditioning. Br. J. Pharmacol. 1999(126), 701–708.

104. Wang, Y., Guo, Y., Zhang, S.X., Wu, W.-J., Wang, J., Bao, W., Bolli, R. Ischemic preconditioning upregulates inducible nitric oxide synthase in cardiac myocyte. J. Mol. Cell. Cardiol. 2002(34), 5 –15.

105. Hofmann F. The biology of cyclic GMP-dependent protein kinases. J Biol Chem 2005(280):1–4.

106. Lohmann SM, Walter U. Tracking functions of cGMP-dependent protein kinases (cGK). Front Biosci 2005(10):1313–1328.

107. Lincoln TM, Wu X, Sellak H, Dey N, Choi CS. Regulation of vascular smooth muscle cell phenotype by cyclic GMP and cyclic GMP-dependent protein kinase. Front Biosci 2006(11):356–367.

108. Kass DA, Champion HC, Beavo JA. Phosphodiesterase type 5: expanding roles in cardiovascular regulation. Circ Res 2007(101):1084–1095.

109. Hofmann, F., Ammendola, A., and Schlossmann, J. Rising behind NO: cGMP-dependent protein kinases. J. Cell Sci. 2000(113), 1671-1676.

110. Komalavilas, P., Shah, P. K., Jo, H., and Lincoln, T. M. Activation of mitogen-activated protein kinase pathways by cyclic GMP and cyclic GMP-dependent protein kinase in contractile vascular smooth muscle cells. J. Biol. Chem. 1999(274), 34301-34309.

111. Lincoln, T. M., and Cornwell, T. L. Intracellular cyclic GMP receptor proteins. FASEB J. 1993(7), 328-338.

112. Lincoln, T. M., Dey, N., and Sellak, H. Invited review: cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression.J. Appl. Physiol. 2001(91), 1421-1430

113. Kukreja, R. C., Salloum, F., Das, A., Ockaili, R., Yin, C., Bremer, Y. A., Fisher, P. W., Wittkamp, M., Hawkins, J., Chou, E., Kukreja, A. K., Wang, X., Marwaha, V. R., and Xi, L. Pharmacological preconditioning with sildenafil: Basic mechanisms and clinical implications.Vasc. Pharmacol. 2005(42), 219-232.

114. Das A, Smolenski A, Lohmann SM, Kukreja RC. Cyclic GMP-dependent protein kinase Ialpha attenuates necrosis and apoptosis following ischemia/reoxygenation in adult cardiomyocyte. J Biol Chem. 2006 Dec 15;281(50):38644-52.

115. Krumenacker JS, Murad F. NO-cGMP signaling in development and stem cells. Mol Genet Metab 2006(87):311–314.

116. Krumenacker JS, Katsuki S, Kots A, Murad F. Differential expression of genes involved in cGMP-dependent nitric oxide signaling in murine embryonic stem (ES) cells and ES cell-derived cardiomyocytes. Nitric Oxide 2006(14):1–11.

117. Wong JC, Fiscus RR. Essential roles of the nitric oxide (no)/cGMP/protein kinase G type-Iα (PKG-Iα) signaling pathway and the atrial natriuretic peptide (ANP)/cGMP/PKG-Iα autocrine loop in promoting proliferation and cell survival of OP9 bone marrow stromal cells. J Cell Biochem. 2011 Mar;112(3):829-39.

118. Charles N, Ozawa T, Squatrito M, Bleau AM, Brennan CW, Hambardzumyan D, Holland EC. 2010. Perivascular nitric oxide activates notch signaling and promotes stem-like character in PDGF-induced glioma cells. Cell Stem Cell 6:141–152.

119. Yoneyama M, Kawada K, Shiba T, Ogita K. Endogenous nitric oxide generation linked to ryanodine receptors activates cyclic GMP / protein kinase G pathway for cell proliferation of neural stem/progenitor cells derived from embryonic hippocampus. J Pharmacol Sci. 2011;115(2):182-95.

120. Mujoo K, Sharin VG, Bryan NS, Krumenacker JS, Sloan C, Parveen S, Nikonoff LE, Kots AY, Murad F. Role of nitric oxide signaling components in differentiation of embryonic stem cells into myocardial cells. Proc Natl Acad Sci U S A. 2008 Dec 2;105(48):18924-9.

121. Madhusoodanan KS, Murad F. NO-cGMP signaling and regenerative medicine involving stem cells. Neurochem Res. 2007 Apr-May;32(4-5):681-94.Müller-Ehmsen J, Whittaker P, Kloner RA, Dow JS, Sakoda T, Long TI, Laird PW, Kedes L. Survival and development of neonatal rat cardiomyocytes transplanted into adult myocardium. J Mol Cell Cardiol. 2002 Feb;34(2):107-16.

122. Pagani FD, DerSimonian H, Zawadzka A, Wetzel K, Edge AS, Jacoby DB, Dinsmore JH, Wright S, Aretz TH, Eisen HJ, Aaronson KD. Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation. J Am Coll Cardiol. 2003 Mar 5;41(5):879-88.

123. Hodgetts SI, Beilharz MW, Scalzo AA, Grounds MD. Why do cultured transplanted myoblasts die in vivo? DNA quantification shows enhanced survival of donor male myoblasts in host mice depleted of CD4+ and CD8+ cells or Nk1.1+ cells. Cell Transplant. 2000 Jul-Aug;9(4):489-502.

124. Hu X, Yu SP, Fraser JL, Lu Z, Ogle ME, Wang JA, Wei L. Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. J Thorac Cardiovasc Surg. 2008 Apr;135(4):799-808.

125. Das R, Jahr H, van Osch GJ, Farrell E. The role of hypoxia in bone marrow-derived mesenchymal stem cells: considerations for regenerative medicine approaches. Tissue Eng Part B Rev. 2010 Apr;16(2):159-68.

126. Shintani S, Kusano K, Ii M, Iwakura A, Heyd L, Curry C, Wecker A, Gavin M, Ma H, Kearney M, Silver M, Thorne T, Murohara T, Losordo DW. Synergistic effect of combined intramyocardial CD34+ cells and VEGF2 gene therapy after MI. Nat Clin Pract Cardiovasc Med. 2006 Mar;3 Suppl 1:S123-8.

127. Haider HK, Jiang S, Idris NM, Ashraf M. IGF-1-overexpressing mesenchymal stem cells accelerate bone marrow stem cell mobilization via paracrine activation of SDF-1alpha/CXCR4 signaling to promote myocardial repair. Circ Res. 2008 Nov 21;103(11):1300-8.

128. Gopal, V.K., Francis, S.H., Corbin, J.D. Allosteric sites of phosphodiesterase-5 (PDE5). A potential role in negative feedback regulation of cGMP signaling in corpus cavernosum. Eur. J. Biochem. 2001 (268), 3304– 3312.

129. Qiu Y, Ping Tang XL, Manchikalpudi S, Rizvi A, Zhang J, Takano H, Wu WJ, Teschner S, and Bolli R. Direct evidence that protein kinase C plays an essential role in the development of late preconditioning against myocardial stunning in conscious rabbits and that epsilon is the isoform involved. J Clin Invest 1998(101): 2182–2198.

130. Zhang M, Koitabashi N, Nagayama T, Rambaran R, Feng N, Takimoto E, Koenke T, O'Rourke B, Champion HC, Crow MT, Kass DA. Expression, activity, and pro-hypertrophic effects of PDE5A in cardiac myocytes. Cell Signal. 2008 Dec;20(12):2231-6.

131. Xiao, R-P., Avdonin, P., Zhou, Y-Y., Cheng, H., Akhter, S.A., Eschenhagen, T., Lefkowitz, R.J., Koch,, W.J., and Lakatta, E.G. Coupling of beta2-adrenoceptor to Gi proteins and its physiological relevance in murine cardiac myocytes. Circ. Res. 1999(84), 43-52.

132. Zhou, Y-Y., Wang, S-Q., Zhu, W., Chruscinski, A., Kobilka, B., Ziman, B., Wang, S., Lakatta, E.G., Cheng, H., and Xiao, R-P. Culture and adenoviral infection of adult mouse cardiac myocytes: methods for cellular genetic physiology. Am. J. Physiol. Heart Circ. Physiol. 2000(279), H429-H436.

133. Cai L, Johnstone BH, Cook TG, Tan J, Fishbein MC, Chen PS, March KL. Human adipose tissue-derived stem cells induce angiogenesis and nerve sprouting following myocardial infarction, in conjunction with potent preservation of cardiac function. Stem Cells. 2009 Jan;27(1):230-7.

134. Sauer H, Sharifpanah F, Hatry M, Steffen P, Bartsch C, Heller R, Padmasekar M, Howaldt HP, Bein G, Wartenberg M. NOS inhibition synchronizes calcium oscillations in human adipose tissue-derived mesenchymal stem cells by increasing gap junctional coupling. J Cell Physiol. 2010 Nov 10.

135. Linscheid P, Seboek D, Zulewski H, Scherberich A, Blau N, Keller U, Müller B. Cytokine-induced metabolic effects in human adipocytes are independent of endogenous nitric oxide. Am J Physiol Endocrinol Metab. 2006 Jun;290(6):E1068-77.

136. Kang Y, Park C, Kim D, Seong CM, Kwon K, Choi C. Unsorted human adipose tissue-derived stem cells promote angiogenesis and myogenesis in murine ischemic hindlimb model. Microvasc Res. 2010 Dec;80(3):310-6.

137. Huang YC, Ning H, Shindel AW, Fandel TM, Lin G, Harraz AM, Lue TF, Lin CS. The effect of intracavernous injection of adipose tissue-derived stem cells on hyperlipidemia-associated erectile dysfunction in a rat model. J Sex Med. 2010 Apr;7(4 Pt 1):1391-400.

138. Foresta C, Lana A, Cabrelle A, Ferigo M, Caretta N, Garolla A, Palù G, Ferlin A. PDE-5 inhibitor, Vardenafil, increases circulating progenitor cells in humans. Int J Impot Res. 2005 Jul-Aug;17(4):377-80.

139. Strong TD, Gebska MA, Burnett AL, Champion HC, Bivalacqua TJ. Endothelium-specific gene and stem cell-based therapy for erectile dysfunction. Asian J Androl. 2008 Jan;10(1):14-22.

140. Kukreja RC, Ockaili R, Salloum F, Yin C, Hawkins J, Das A, Xi L. Cardioprotection with phosphodiesterase-5 inhibition--a novel preconditioning strategy. J Mol Cell Cardiol. 2004 Feb;36(2):165-73.

141. Keilbach A, Ruth P, Hofmann F. Detection of cGMP dependent protein kinase isozymes by specific antibodies. Eur J Biochem. 1992 Sep 1;208(2):467-73.

142. Lincoln TM, Dey N, Sellak H. cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. J Appl Physiol. 2001;91:1421–1430.

143. Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, Pell CL, Johnstone BH, Considine RV, March KL. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. Circulation. 2004 Mar 16;109(10):1292-8.

144. Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F, Mu H, Melo LG, Pratt RE, Ingwall JS, Dzau VJ.. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J 2006;20:661–669.

145. Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res 2008;103:1204–1219.

146. Uemura R, Xu M, Ahmad N, Ashraf M.. Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling. Circ Res 2006;98:1414–1421.

147. Zhao T, Zhang D, Millard RW, Ashraf M, Wang Y. Stem cell homing and angiomyogenesis in transplanted hearts are enhanced by combined intramyocardial SDF-1{alpha} delivery and endogenous cytokine signaling. Am J Physiol Heart Circ Physiol. 2009; 296:H976-986.

148. Zhang M, Mal N, Kiedrowski M, Chacko M, Askari AT, Popovic ZB, Koc ON, Penn MS. SDF-1 expression by mesenchymal stem cells results in trophic support of cardiac myocytes following myocardial infarction. FASEB J. 2007;21:3197–3207.

149. Mirotsou M, Zhang Z, Deb A, Zhang L, Gnecchi M, Noiseux N, Mu H, Pachori A, Dzau V. Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. Proc Natl Acad Sci USA. 2007; 104: 1643-8.

150. Gnecchi M, He H, Liang OD, Melo LG, Morello F, Mu H, Noiseux N, Zhang L, Pratt RE, Ingwall JS, Dzau VJ. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med. 2005; 11:367-8.

151. Seeger FH, Zeiher AM, Dimmeler S. Cell-enhancement strategies for the treatment of ischemic heart disease. Nat Clin Pract Cardiovasc Med. 2007 Feb;4 Suppl 1:S110-3.

152. See F, Seki T, Psaltis PJ, Sondermeijer HP, Gronthos S, Zannettino AC, Govaert KM, Schuster MD, Kurlansky PA, Kelly DJ, Krum H, Itescu S. Therapeutic Effects of Human STRO-3-Selected Mesenchymal Precursor Cells and their Soluble Factors in Experimental Myocardial Ischemia. J Cell Mol Med. 2010 Dec 14. doi: 10.1111/j.1582-4934.2010.01241.x.

153. Limbourg FP, Ringes-Lichtenberg S, Schaefer A, Jacoby C, Mehraein Y, Jäger MD, Limbourg A, Fuchs M, Klein G, Ballmaier M, Schlitt HJ, Schrader J, Hilfiker-Kleiner D, Drexler H. Hematopoietic stem cells improve cardiac function after infarction without permanent cardiac engraftment. Eur J Heart Fail 2005;7:722.

154. Schenk S, Mal N, Finan A, Zhang M, Kiedrowski M, Popovic Z, McCarthy PM, Penn MS. Monocyte chemotactic protein-3 is a myocardial mesenchymal stem cell homing factor. Stem Cells. 2007;25:245–251.

155. Askari A, Unzek S, Goldman CK, Ellis SG, Thomas JD, DiCorleto PE, Topol EJ, Penn MS. Cellular, but not direct, adenoviral delivery of vascular endothelial growth factor results in improved left ventricular function and neovascularization in dilated ischemic cardiomyopathy. J Am Coll Cardiol. 2004;43:1908 –1914.

156. Spiegelstein D, Kim C, Zhang Y, Li G, Weisel RD, Li RK, Yau TM. Combined transmyocardial revascularization and cell-based angiogenic gene therapy increases transplanted cell survival. Am J Physiol Heart Circ Physiol. 2007;293:H3311–H3316.

157. Kim C, Li RK, Li G, Zhang Y, Weisel RD, Yau TM. Effects of cell-based angiogenic gene therapy at 6 months: persistent angiogenesis and absence of oncogenicity. Ann Thorac Surg. 2007;83:640–646.

158. Salva MZ, Himeda CL, Tai PW, Nishiuchi E, Gregorevic P, Allen JM, Finn EE, Nguyen QG, Blankinship MJ, Meuse L, Chamberlain JS, Hauschka SD. Design of tissue-specific regulatory cassettes forhigh-level rAAV-mediated expression in skeletal and cardiac muscle. Mol Ther. 2007;15:320 –329.

159. Bian J, Popovic ZB, Benejam C, kiedrowski M, Rodriguez LL, Penn MS. Effect of cell-based intercellular delivery of transcription factor GATA4 on ischemic cardiomyopathy. Circ Res. 2007;100:1626 –1633.

160. MacLellan WR, Schneider MD. Death by design. Programmed cell death in cardiovascular biology and disease. Circ Res 1997(81): 137–144.

161. Sam Sawyer DB, Chang DL, Eberli FR, Ngoy S, Jain M, Amin J, Apstein CS, Colucci WS. Progressive left ventricular remodeling and apoptosis late after myocardial infarction in mouse heart. Am J Physiol Heart Circ Physiol 2000(279): H422–H428.

162. Mirotsou M, Zhang Z, Deb A, Zhang L, Gnecchi M, Noiseux N, Mu H, Pachori A, Dzau V. Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. Proc Natl Acad Sci U S A. 2007;104:1643-8.

VITA

1. Born: May 24, 1983, Richmond, Virginia

2. Citizenship: U.S.A

3. EDUCATION

Degree	Subject	Institution	Years
B.S.	Biochemistry	Va. Polytechnic Institute and State University Blacksburg, VA	2001-2005
Ph.D.	Biochemistry	Virginia Commonwealth University School of Medicine, Richmond, VA	2005-2011

GRADUATE EDUCATION/RESEARCH
Virginia Commonwealth University School of Medicine, Department of Biochemistry and Molecular Biology
Doctor of Philosophy in Biochemistry, **May 12, 2011**
Concentration: Molecular and Cellular Cardiology
Dissertation Title: **Phosphodiesterase-5 Inhibition: A Novel Strategy to Improve Stem Cell Therapy in the Heart**
Research Mentor: **Rakesh C. Kukreja, PhD**

SPECIAL AWARDS, FELLOWSHIPS AND OTHER HONORS

Awards Virginia Polytechnic Institute and State University Undergraduate Research Award 2005

Fellowships American Heart Association Predoctoral Fellowship 2009-2011

SCHOLARY, RESEARCH OR TEACHING EXPERIENCE

Undergraduate Students Trained

1. Thasin Shahanaz, B.S. student, Department of Biology, 2010

Cardiology Fellow Trainees in Basic Research

1. P Brody Wehman, M.D., Internal Medicine/Cardiology, 2009
2. Christopher Thomas, M.D., Internal Medicine/Cardiology, 2010

BIBLIOGRAPHY

1. Salloum FN, Abbate A, Das A, Houser J, Mudrick CA, Qureshi IZ, **Hoke NN**, Roy SK, Brown WR, Prabhakar S, and Kukreja RC. Sildenafil (Viagra) attenuates ischemic cardiomyopathy and improves left ventricular function in mice. *Am J Physiol Heart Circ Physiol.* 294: H1398-H1406; MARCH 2008.

2. Abbate A, Salloum FN, Vecile E, Das A, **Hoke NN**, Straino S, Houser J, Qureshi IZ, Ownby ED, Gustini E, Biondi-Zoccai GL, Biasucci LM, Severino A, Capogrossi MC, Vetrovec GW, Crea F, Baldi A, Kukreja RC and Dobrina A. Anakinra, Anakinra, a recombinant human interleukin-1 receptor antagonist, inhibits apoptosis in experimental acute myocardial infarction. *Circulation.* 117(20):2670-83. MAY 2008.

3. Abbate A, Bussani R, Sinagra G, Barresi E, Pivetta A, Perkan A, **Hoke NN**, Salloum FN, Kontos MC, Biondi-Zoccai GL, Vetrovec GW, Sabbadini G, Baldi F, Silvestri F, Kukreja RC and Baldi A. Right ventricular cardiomyocyte apoptosis in patients with acute myocardial infarction of the left ventricular wall. *Am J Cardiol.*102(6):658-62. SEP 2008.

4. Salloum FN, Chau VQ, Varma A, **Hoke NN**, Toldo S, Biondi-Zoccai GGL, Crea F, Vetrovec GW and Abbate A. Anakinra in experimental acute myocardial infarction--does dosage or duration of treatment matter? *Cardiovasc Drugs Ther.* APRIL 2009.

5. Salloum FN, **Hoke NN**, Seropian IM, Varma A, Ownby ED, Houser JE, Van Tassell BW, Abbate A. Parecoxib inhibits apoptosis in acute myocardial infarction due to permanent coronary ligation but not due to ischemia-reperfusion. *J Cardiovasc Pharmacol*. 53(6):495-8. JUNE 2009

6. **Hoke NN**, Salloum FN, Loesser-Casey KE, Kukreja RC. Cardiac regenerative potential of adipose tissue-derived stem cells. *Acta Physiol Hung*. 96(3):251-65. SEPT 2009.

7. Abbate A, Sinagra G, Bussani R, **Hoke NN**, Merlo M, Varma A, Toldo S, Salloum FN, Biondi-Zoccai GG, Vetrovec GW, Crea F, Silvestri F, Baldi A. Apoptosis in patients with acute myocarditis. *Am J Cardiol*. 104(7):995-1000. OCT 2009.

8. Salloum FN**,** Chau VQ, **Hoke NN**, Abbate A, Varma A, Ockaili RA, Toldo S, Kukreja RC. Phosphodiesterase-5 inhibitor, tadalafil, protects against myocardial ischemia/reperfusion through protein-kinase g-dependent generation of hydrogen sulfide. *Circulation*. 20(11 Suppl):S31-6. SEP 2009.

9. Van Tassell BW, Varma A, Salloum FN, Das A, Seropian IM, Toldo S, Smithson L, **Hoke NN**, Chau VQ, Robati R, Abbate A. Interleukin-1 Trap attenuates cardiac remodeling following experimental acute myocardial infarction in mice. *J Cardiovasc Pharmacol*. NOV 2009.

10. Van Tassell BW, Seropian IM, Toldo S, Salloum FN, Smithson L, Varma A, **Hoke NN**, Gelwix C, Chau V, Abbate A. Pharmacologic inhibition of Myeloid Differentiation Factor 88 (MyD88) prevents left ventricular dilation and hypertrophy after experimental acute myocardial infarction in the mouse. *J Cardiovasc Pharmacol*. 55(4):385-90. APRIL 2010.

11. Bogaard HJ, Natarajan R, Mizuno S, Abbate A, Chang PJ, Chau VQ, **Hoke NN**, Kraskauskas D, Kasper M, Salloum FN, Voelkel NF. Adrenergic receptor blockade reverses right heart remodeling and dysfunction in pulmonary hypertensive rats. *Am J Respir Crit Care Med.* 182(5):652-60. SEP 2010.

12. Das A, Durrant D, Mitchell C, Mayton E, **Hoke NN**, Salloum FN, Park MA, Qureshi I, Lee R, Dent P, Kukreja RC. Sildenafil increases chemotherapeutic efficacy of doxorubicin in prostate cancer and ameliorates cardiac dysfunction. *Proc Natl Acad Sci U S A.* 107(42):18202-7. OCT 2010.

13. Chau VQ, **Hoke NN**, Abbate A, Salloum FN, Kukreja RC. Mitigation of the Progression of Heart Failure with Sildenafil Involves Inhibition of RhoA/Rho-Kinase Pathway. ACCEPTED TO AJP 2011.

14. Salloum FN, Das A, **Hoke NN**, Chau VQ, Ockaili RA, Stasch JP, Kukreja RC. Cinaciguat - a novel activator of soluble guanylate cyclase, protects against ischemia/reperfusion injury: Role of hydrogen sulfide. SUBMITTED TO JACC 2011.

15. **Hoke NN,** Salloum FN, Woolbright BL, Kass DA, Das A, Kukreja RC. Transplantation of Preconditioned Human Adipose Derived Stem Cells by Phosphodiesterase-5 Inhibition Improves Myocardial Function after Infarction in Mice. SUBMITTED TO STEM CELL 2011.

Abstracts

1. Salloum FN, Abbate A, Brown WR, Ockaili RA, **Hoke NN**, and Kukreja RC. Phosphodiesterase-5 Inhibitors Reduce Myocardial Infarction, Apoptosis and Improve Post-Ischemic Ventricular Function in Female Mice. *J AM Col Cardiol* 51 (10): 178A-178A Suppl. A MAR 11 2008.

2. **Hoke NN**, Salloum FN, Houser J, Baldi A, Kukreja RC, and Abbate A. Caspase-cleaved Cytokeratin-18: an early marker of apoptosis in cardiomyocytes. *J AM Col Cardiol* 51 (10) Suppl. A MAR 11 2008.

3. Abbate A, Salloum FN, Straino S, Das A, Houser J, **Hoke NN**, Varma A, Qureshi IZ, Dobrina A, Vecile E, Vetrovec GW, Baldi A, and Kukreja RC. Anakinra, Recombinant Human Interleukin-1 Receptor Antagonist, Inhibits Apoptosis in Acute Myocardial Infarction. *J AM Col Cardiol* 51 (10) Suppl. A MAR 11 2008.

4. Abbate A, Dobrina A, Vecile E, Salloum FN, Das A, **Hoke NN**, Vetrovec GW, and Kukreja RC. Interleukin-1 Receptor Antagonist Inhibits Caspase-1 Mediated Cardiomyocyte Apoptosis. *Accepted for presentation at the European Society of Cardiology 2008 Annual Sessions.*

5. Abbate A, Salloum FN, Houser J-E, Ownby ED, Qureshi IZ, **Hoke NN**, Vetrovec GW and Kukreja RC. Parecoxib, a Cyclo-Oxygenase-2 Inhibitor, Reduces Myocardial Apoptosis in Mice with Permanent Coronary Ligation but not in Mice with Ischemia-Reperfusion Injury. *Accepted for presentation at the European Society of Cardiology 2008 Annual Sessions.*

6. Abbate A, Sinagra G, Bussani R, **Hoke NN**, Toldo S, Barresi E, Salloum FN, Silvestri F, Vetrovec GW, Kukreja RC, Baldi A. Can Apoptosis Predict Functional Recovery in Acute Myocarditis? *Circulation* Supplement II-1033: 118 (18): 5936; 2008.

7. Salloum FN, Ockaili RA, Abbate A, **Hoke NN**, Chau VQ, Lall S, Toldo S, Kukreja RC. Long Acting Erectile Dysfunction Drug Tadalafil Limits Myocardial Ischemia/Reperfusion Injury and Preserves Left Ventricular Function through Protein Kinase G Dependent Pathway. *Circulation* Supplement II-706: 118 (18): 2320; 2008.

8. Salloum FN, Chau VQ, Houser J, Varma A, **Hoke NN**, Abbate A and Kukreja RC. Long-Acting Erectile Dysfunction Drug Tadalafil Protects the Heart against Ischemia/ Reperfusion Injury through Hydrogen Sulfide Signaling. *Accepted for presentation at the American College of Cardiology 2009 Annual Conference.*

9. Salloum FN, **Hoke NN**, Varma A, Van Tassell B, Toldo S, Chau VQ, Abbate A. 14-Day Treatment with Anakinra Provides Superior Protection against Myocardial Remodeling Versus 7-Day Treatment in Experimental Acute Myocardial Infarction. *Accepted for presentation at the American College of Cardiology 2009 Annual Conference.*

10. Salloum FN, **Hoke NN**, Varma A, Van Tassell B, Toldo S, Abbate A. Safety and Efficacy of High-Dose Anakinra in Experimental Acute Myocardial Infarction. *Accepted for presentation at the American College of Cardiology 2009 Annual Conference.*

11. Salloum FN, **Hoke NN**, Varma A, Van Tassell B, Toldo S, Chau VQ, Vecile E, Dobrina A, Abbate A. Endogenous Interleukin-1 Receptor Antagonist Protects Against Severe Adverse Cardiac Remodeling After Acute Myocardial Infarction. *Accepted for presentation at the American College of Cardiology 2009 Annual Conference.*

12. Varma A, Salloum FN, **Hoke NN**, Kukreja RC. A Novel Soluble Guanylate Cyclase Activator, BAY 58-2667, Reduces Infarct Size, Improves Fasting Glucose Levels and Attenuates Inflammation in Diabetic Mice. *Accepted for presentation at the American Heart Association 2009 Annual Conference.*

13. Varma A, Salloum FN, **Hoke NN**, Kukreja RC. Chronic Daily Therapy with Tadalafil Improves Multiple Cardiovascular Risk Factors in Obese, Diabetic Mice. *Accepted for presentation at the American Heart Association 2009 Annual Conference.*

14. Varma A, Salloum FN, **Hoke NN**, Kukreja RC. Daily Therapy with the Phospholipase A2 Inhibitor, PX-18, Improves the Chronic Inflammatory State in Obese Mice and Reduces Infarct Size following Ischemia/Reperfusion. *Accepted for presentation at the American Heart Association 2009 Annual Conference.*

15. **Hoke NN**, Salloum FN, Chau VQ, Das A, Wehman PB, Kukreja RC. Adenoviral transfer of PKGIα attenuates apoptosis and necrosis in adipose derived stem cells. *Accepted for presentation at Experimental Biology 2010 Annual Conference.*

16. Salloum FN, Das A, Chau VQ, **Hoke NN**, Ockaili RA, Stasch PJ, Kukreja RC. Bay 58-2667, a Novel NO-Independent Activator of Soluble Guanylate Cyclase, Protects Against Ischemia/Reperfusion Injury: Role of Hydrogen Sulfide Signaling. *Accepted for presentation at Experimental Biology 2010 Annual Conference.*

17. Salloum FN, Chau VQ, **Hoke NN**, Abbate A, Kukreja RC. Mitigation of Heart Failure Progression with Sildenafil Involves Inhibition of Rho-A/Rho Kinase Pathway. *Accepted for presentation at Experimental Biology 2010 Annual Conference.*

18. Salloum FN, **Hoke NN**, Das A, Sturz GR, Thomas CS, Kukreja RC. MicroRNA-21 mediates cardioprotection with PKGI-α over-expression through upregulation of hydrogen sulfide. *Accepted for presentation at the American Heart Association 2010 Annual Conference.*

19. **Hoke NN**, Salloum FN, Woolbright BL, Das A, Kukreja RC. Sildenafil Increases the Efficacy of Adipose Derived Stem Cell Transplantation following Myocardial Infarction.

Accepted for presentation at VCU's Watt's Day 2010 and Experimental Biology 2011 Annual Conference.