HYPOXIC REGULATION OF ANGIOTENSIN-CONVERTING ENZYME 2 AND MAS RECEPTOR IN HEMATOPOIETIC STEM/PROGENITOR CELLS: A TRANSLATIONAL STUDY

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Vascular disease is the leading cause of mortality and morbidity in the western world, and account for the 1 of every 3 death's in the US, but a cure for vascular disease is yet to be realized. Hematopoietic stem progenitor cells (HSPCs) are mobilized from bone marrow and have the innate propensity to accelerate vascular repair by reendothelialization and revascularization of ischemic areas. The vasoreparative ability of HSPCs is largely due to their capacity to home to the areas of hypoxia and their sensitivity to hypoxia plays a critical role in the vasoreparative functions of these cells. The discovery of vasoreparative potential of HSPCs resulted in a breakthrough approach of cell-based therapies for the treatment of ischemic vascular diseases. However, success of this approach is essentially dependent on the number of cells that could be collected from an individual. Therefore, novel mechanism-based strategies are needed to enhance the outcomes of autologous cell-based therapies in poor mobilizers and older adults. Recent evidence of a potential role of the vasoprotective axis of the renin angiotensin system (RAS) in HSPCs functions offers a breakthrough. Angiotensin-(1-7), the primary mediator of the protective functions which acts on Mas receptor (MasR), is generated by angiotensin converting enzyme-2 (ACE2). In this study, we tested the effects of hypoxia on stimulation of vasoreparative potential of HSPCs and in upregulation of ACE2 and MasR. Importantly, we delineated the molecular mechanism of hypoxic exposure in regulation of ACE2 and MasR in a HIF1α- dependent manner and hypoxic exposure induced shedding of the membrane bound ACE2 in HSPCs. We used luciferase, a reporter assay, cell-based assays, gene/protein expression studies and pharmacological strategies in human and mouse HSPCs to test our hypotheses. To verify the biological significance of hypoxia, we performed *in vivo* studies in mice and humans, which recapitulated the *in vitro* observations on vascular protective

axis of RAS in HSPCs. Collectively, these studies provided mechanistic insights into hypoxic regulation of vascular protective axis of RAS in HSPCs and also provided compelling evidence for the clinical use of hypoxia as a promising approach for enhancing the vasoreparative outcomes of cell-based therapies.

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DEDICATION

I dedicate this work to my family.

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LIST OF ABBREVIATIONS

7-AAD.....7-Amino-Actinomycin D

AA.....Arachidonic Acid

AAV.....Adeno-associated virus

ACE.....Angiotensin Converting Enzyme

ACE2.....Angiotensin Converting Enzyme-2

AFU.....Arbitrary Fluorescence Units

Akt……. Protein Kinase B

Ang.....Angiotensin

ANOVA.....Analysis of Variance

APC.....Allophycocyanin

ASTMI.....Autologous Stem Cell Transplantation in Acute Myocardial Infarction

AT_1R.....Angiotensin II Type 1 Receptor

AT_2R.....Angiotensin II Type 2 Receptor

BFR.....Blood Flow Restriction

BM.....Bone Marrow

BOOST.....Bone Marrow Transfer to Enhance ST-Elevation Infarct Regeneration

BrDU.....5-Bromo-2ʿ-deoxyuridine

CAR.....CXCL12-abundant reticular cells

CD.....Cluster of Differentiation

cDNA.....Complimentary Deoxyribonucleic Acid

cGMP.....Cyclic Guanosine Monophosphate

CVD .....Cardiovascular Disease

CXCL12 .....C-X-C Motif Chemokine 12

CXCR4.....C-X-C Chemokine Receptor Type 4

DC .....Aspartic Acid Decarboxylase

DiI-Ac-LDL .....1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate – Acetylated – Low-Density Lipoproteins

DNA .....Deoxyribonucleic Acid

EDTA .....Ethylene Diamine Tetra Acetic Acid

ELISA .....Enzyme Linked Immunosorbent Assay

FBS .....Fetal Bovine Serum

FITC .....Fluorescein Isothyocyanate

FL .....Fluorescence Parameter

FLK1 .....Fetal Liver Kinase 1

FOCUS-Br .....Intramyocardial Injection of Autologous Aldehyde Dehydrogenase-Bright Stem Cells for Therapeutic Angiogenesis

FOCUS-HF .....Autologous Stem Cells for Cardiac Angiogenesis

G-CSF .....Granulocyte Colony Stimulating Factor

GM-CSF.....Granulocyte-Macrophage Colony Stimulating Factor

HBSS.....Hank's Balanced Salt Solution

HIF .....Hypoxia-Inducible Factor

HLI.....Hindlimb Ischemia

HPC.....Hematopoietic Progenitor Cell

HSC.....Hematopoietic Stem Cell

HSPC.....Hematopoietic Stem/Progenitor Cell

KDR .....Kinase Insert Domain Receptor

KOMP .....Trans-NIH Knock-Out Mouse Project

LateTIME.....Autologous stem cells in treating people who have had a heart attack

LDI.....Laser Doppler Imaging

LDL.....Low-Density Lipoproteins

LI-BFR.....Low Intensity-Blood Flow Restriction

Lin^- .....Lineage Negative

LK .....Lineage Negative and c-Kit Positive

LSK.....Lineage Negative, Stem Cell Antigen-1 Positive and c-Kit Positive

MasR.....Mas Receptor

MasR-KO.....Mas Receptor Knock Out

MMP .....Matrix Metalloproteinase

MNC .....Mononuclear Cell

mRG-D.....Mas-Related G-Protein Coupled Receptor Member D

mRNA.....Messenger Ribonucleic Acid

MSC.....Mesenchymal Stromal Cell

NADPH.....Nicotinamide Adenine Dinucleotide Phosphate

NEP.....Neprilysin

NIH .....National Institutes of Health

PBS .....Phosphate Buffer Saline

PCR.....Polymerase Chain Reaction

PE.....Phycoerythrin

PI3K .....Phosphatidylinositol 3-Kinase

qPCR .....Quantitative Polymerase Chain Reaction

RAS .....Renin Angiotensin System

REGENT .....Regeneration by Intracoronary Infusion of Selected Population of Stem Cells in Acute Myocardial Infarction Trial

REPAIR-AMI .....Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction Trail

RNA .....Ribonucleic Acid

ROS .....Reactive Oxygen Species

RPMI .....Roswell Park Memorial Institute

RT-PCR .....Real-Time Polymerase Chain Reaction

sca-1 .....Stem Cell Antigen-1

SDF .....Stromal Cell-Derived Factor

TAC-HFT .....The Transendocardial Autologous Cells (hMSC or hBMC) In Ischemic Heart Failure Trial

TICAP .....Transcoronary Infusion of Cardiac Progenitor Cells in Patients with Single Ventricle Physiology

TIME .....Autologous stem cells in treating people who have had a heart attack

TOPCARE-AMI .....Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction

VCAM .....Vascular Cell Adhesion Molecule

VEGF .....Vascular Endothelial Growth Factor

VEGFR-2 .....Vascular Endothelial Growth Factor Receptor-2

VLA-4 .....Very Late Antigen-4

vWF .....Von Willebrand Factor

WT ....Wild Type

LIST OF SYMBOLS

BW .....Body Weight

Ca .....Calcium

$CaCl_2$ .....Calcium Chloride

cm.....Centimeter

^oC.....Degree Celsius

ΔCt .....Change in Cycle Threshold

h.....Hour

HCl .....Hydrochloric Acid

kg.....Kilogram

M .....Molar

mA.....Milliampere

Mg .....Magnesium

mg .....Milligram

min .....Minute

mL .....Milliliter

mM.....Millimolar

µg .....Microgram

µL.....Microliter

µM.....Micromolar

NaCl .....Sodium Chloride

ng.....Nanogram

nM.....Nanomolar

NO.....Nitric Oxide

pH.....Potential of Hydrogen

rpm .....Rotations per Minute

V.....Volt

CHAPTER 1. BACKGROUND; OBJECTIVES AND SPECIFIC AIMS; MATERIALS AND METHODS

1.1. Background

1.1.1. Stem cells

Stem cells—body's basic building blocks are undifferentiated cells that can differentiate and give rise to many different cells with specialized functions. The key features of stem cells include unlimited capability to self-renew and produce daughter cells (new stem cells) and/or differentiate to produce specialized cell type such as blood cells or muscle cells in heart. Progenitor cells are the earliest offspring of stem cells which can form a limited number of differentiated cell types in the body, but they have limited self-renewal capacity. Stem cells and progenitor cells have the innate potential to control tissue formation, the maintenance and repair of damaged tissues. Bone marrow is traditionally viewed as the major source of adult stem cells, which are maintained in relatively quiescent state within the hypoxic niches of bone marrow (1). Self-renewal and differentiation of these cells occur in response to selective physiological stimuli. Bone marrow contains hematopoietic stem and progenitor cells, which form all types of blood cells in the body, and the mesenchymal stromal cells (MSCs), that generate bone, cartilage, fat cells, connective tissue and most importantly help in retention of stem and progenitor cells in bone marrow (2).

1.1.2. Evidence for the vasoreparative functions of hematopoietic stem/progenitor cells (HSPCs)

Hematopoietic stem/progenitor cell (HSPCs) have the potential to mobilize out of bone marrow into the blood circulation, induce endothelialization, promote vascular regeneration and tissue repair (3,4). Developmentally, hemangioblasts or hematopoietic stem cells (HSCs) give

rise to hematopoietic progenitor cells (HPCs) which form various blood cells and contribute to the vascular development (5–7). HSPCs are identified based on different set of markers present on the surface of these cells (8) (Fig. 1).

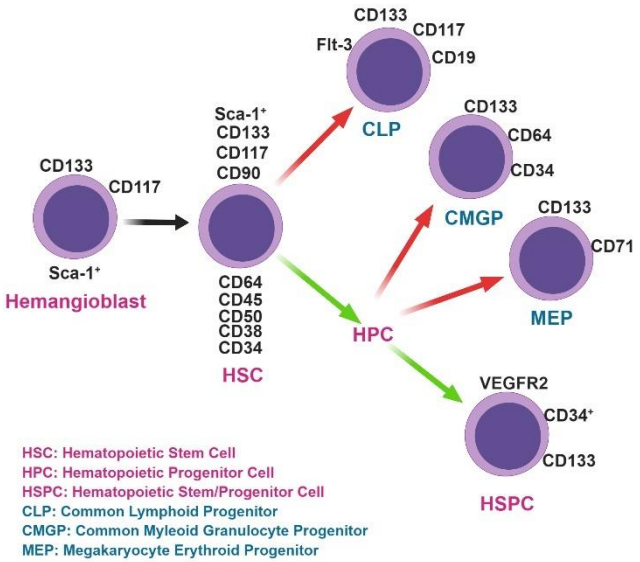

Figure 1. Surface markers for HSPCs derived from bone marrow. Figure adapted from (8,9).

Physiologically endothelium is quiescent and tissue injury or local ischemia releases hypoxia-regulated factors such as VEGF and SDF, which activate endothelial cells to form tip cells. Endothelial cells then migrate to the areas of ischemia resulting to the formation of sprout. Eventually, endothelial sprouts form lumen by internal vacuolization resulting in tubular structures. Formation of vascular network via this process of vasculogenesis /angiogenesis restores the blood flow to the ischemic areas. Seminal discovery by Stump and colleagues was the first to show the endothelialization of implanted dacron axillofemoral grafts by circulating cells and possibility of endothelium formation from circulating cells in the blood stream (10). Wu and colleagues showed evidence of human endothelial cells by endothelial factor VIII/ von Willebrand factor (vWF) and agglutinin on a dacron arterial prosthesis in a 65-year old patient and presented a definitive proof of endothelialization (11).

It was a well-accepted dogma in the scientific community that development of vascular system occurs during embryonic stage via process known as 'vasculogenesis', which is formation of blood vessels by cells, whereas in the neonatal stage blood vessel formation occurs via 'angiogenesis', blood vessel formation from pre-existing blood vessels (5,6). On the other hand, percentage of circulating HSPC was considered nearly zero. Just around 20 years ago, this scientific dogma was challenged by the landmark discovery of Asahara and colleagues (12). In 1997, they first reported that putative cells from peripheral blood can differentiate to an endothelial phenotype *ex vivo* and they identified these cells as vascular progenitor cells. They have also shown that vascular progenitor cells formed tube-like structures on fibronectin-coated plates and also differentiated *in vitro* into endothelial cells. Injection of vascular progenitors into a mouse model of limb ischemia caused these cells to mobilize to the area of ischemia and promote the formation of new vessels (vasculogenesis) at the sites of ischemic injury (12). This transformative discovery fostered a new era in stem cell research and also overturned the long-standing belief that differentiated organs cannot be repaired after ischemic injury. Vascular progenitor cells specifically form endothelial cells upon differentiation and they characteristically express both HSPC markers and endothelial cell surface markers (12,13).

1.1.3. $CD34^+$ cells as vascular progenitor cells

CD34 antigen, which is a sialomucin adhesion molecule, is a most commonly used marker for identification of HSPCs (13,14), and it is expressed on bone marrow and circulating HSPCs (12). Identification of vascular progenitors in peripheral blood by Asahara et al. (12) was based on surface expression of CD34 and an endothelial marker vascular endothelial growth factor receptor (VEGFR, also known as Flk1/KDR). Shi et al. also reported the existence of circulating bone marrow derived vascular progenitors and they showed that a subset of $CD34^+$

cells can differentiate into endothelial lineage expressing endothelial markers such as vWF and Dil-Ac-LDL(15). CD133 (prominin-1/AC133) is a more immature and less lineage-determined marker (16) and is expressed on HSPCs but absent on differentiated endothelial cells. To date, vasoreparative HSPCs from human and mice can be identified based on various cell surface markers (17,18) as shown in Fig. 2. *In vitro* and *in vivo* studies have shown that these surface markers can participate in the vascular repair and support angiogenesis (19–24). Human HSPCs are devoid of differentiated/lineage committed cell markers (CLP, CMGP and MEP as shown in Fig. 1) and are referred as lineage-negative (Lin^-) cells. Human HSPCs having vascular reparative functions are identified as Lin^- cells expressing CD34, CD133 and VEGFR2 and devoid of CD45. However, circulating HSPCs expressing these markers are very scarce ($CD34^+CD133^+VEGFR2^+$: 0.0084% of total circulating mononuclear cells (25)). Alternatively, $Lin^-CD34^{high}CD45^{low}$ are most commonly used markers for enrichment of human HSPCs and were used clinically for autologous cells therapies of ischemic cardiovascular diseases (4,26).

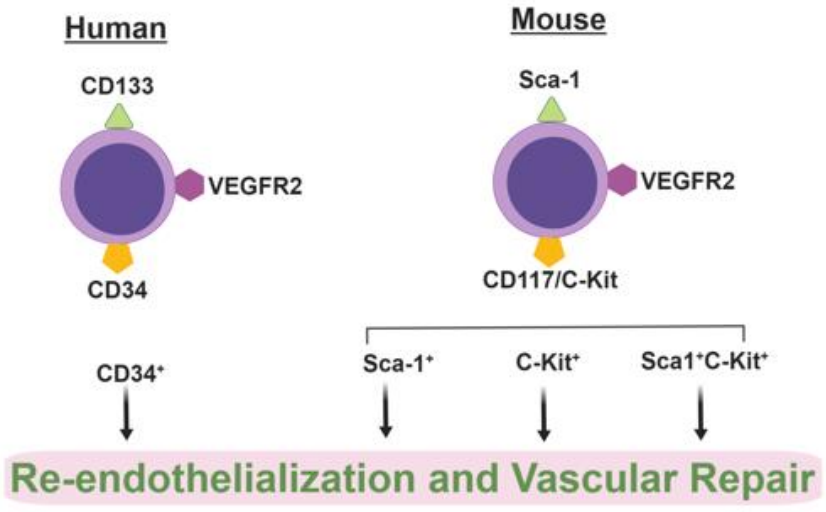

Figure 2. Characteristics of vasoreparative HSPCs in human and mice. Human vasoreparative HSPCs markers are identified as lin^- cells expressing CD133, CD34 and VEGFR2, whereas HSPCs from mice are identified as lin^- cells expressing sca-1 and c-kit.

Most widely used marker for identification of murine HSPCs with vascular reparative functions are $Lin^{-}Sca1^{+}c\text{-}Kit^{+}$ (LSK) (27,28).

1.1.4. Proposed mechanisms of vascular repair by HSPCs

Bone marrow microenvironment/niches are localized regions that play a key role in supporting and maintaining HSPCs and also support their homing and migration. Adult bone marrow is filled with stroma and resident cells such as HSPCs, osteoblasts and osteoclasts, MSCs, CXCL12-abundant reticular (CAR) cells and endothelial cells. MSCs, CAR cells and osteoblasts release adhesion factors such as Stromal-derived factor-1 (SDF) or CXCL12, vascular endothelial growth factor (VEGF), stem cell factor (SCF), and vascular cell adhesion molecule (VCAM) to which HSPCs express receptors such as CXCR4, VEGFR-2, c-Kit, and very light antigen (VLA)-4 (29). These adhesion factors help in the retention of HSPCs in the bone marrow. Quiescence of HSPCs is essential for their maintenance and to prevent their exhaustion and this process is supported by paracrine factors such as SCF, angiopoietin-1 (Ang-1), thrombopoietin (TPO) and IL-7. Most importantly, bone marrow niches are hypoxic, and hypoxia inducible factor-1α (HIF) induces the expression of SDF and VEGF. SDF provides the retention signal by interacting with CXCR-4. Physiologically, egress of HSPCs from bone marrow niches is under influence of nor-adrenaline secretion and activation of sympathetic nervous system (SNS), which passively mobilizes HSPCs from bone marrow. Under steady-state conditions, SNS modulates circadian rhythm and influences the mobilization of HSPCs through clock genes (30). Of note, dopamine-β-hydroxylase knockout mice have shown reduced mobilization of HSPCs which was rescued by β2-adrenoceptor agonists providing the evidence for the role of SNS in the regulation of HSPCs mobilization (31).

In situation of endothelial damage due to ischemia, vascular or tissue injury, the damaged and surrounding cells secrete certain cytokines mainly SDF and VEGF (32,33). As a result, HSPCs are mobilized in large number into the blood circulation (34,35). SDF and VEGF gradient across blood-bone marrow interface is always higher in the bone marrow environment and promote the retention of cells in the bone marrow. SDF and VEGF that are generated by ischemic areas switch the gradient towards circulation and this gradient also direct the cells to ischemic areas where they are generated (36–38). These cells now circulate, migrate and tend to target zones of damaged regions (39,40) and accomplish vascular repair by releasing paracrine factors that stimulate angiogenic functions of local endothelium in the peri-ischemic areas and promote peri-endothelial vascular development by angiogenesis. Alternatively, there are mixed evidences for the direct incorporation and transdifferentiation of HSPC into the newly developing vasculature to induce vasculogenesis. However, widely accepted phenomena is release of paracrine angiogenic factors by which HSPCs repair the damaged endothelium (Fig. 3). One thing to emphasize here is the vasoreparative ability of HSPCs to migrate and home to the areas of ischemia/hypoxia is due to their sensitivity to hypoxia-regulated factors, SDF and VEGF, and HSPCs can only respond to the environmental cues of increased SDF/VEGF if they express abundant CXCR4 and VEGFR (41,42). Clinical study reported by Kalka and colleagues have shown that gene therapy with VEGF augmented levels of circulating HSPCs in humans (43). To date several studies have also demonstrated that exercise can stimulate mobilization of HSPCs in rodents and in humans (44–49). There is a mechanistic similarity between VEGF and exercise induced upregulation of HSPCs levels which involves the stimulation of phosphoinsositol-3-kinase (PI3K)/ Akt-pathway and subsequent activation of eNOS (50–54).

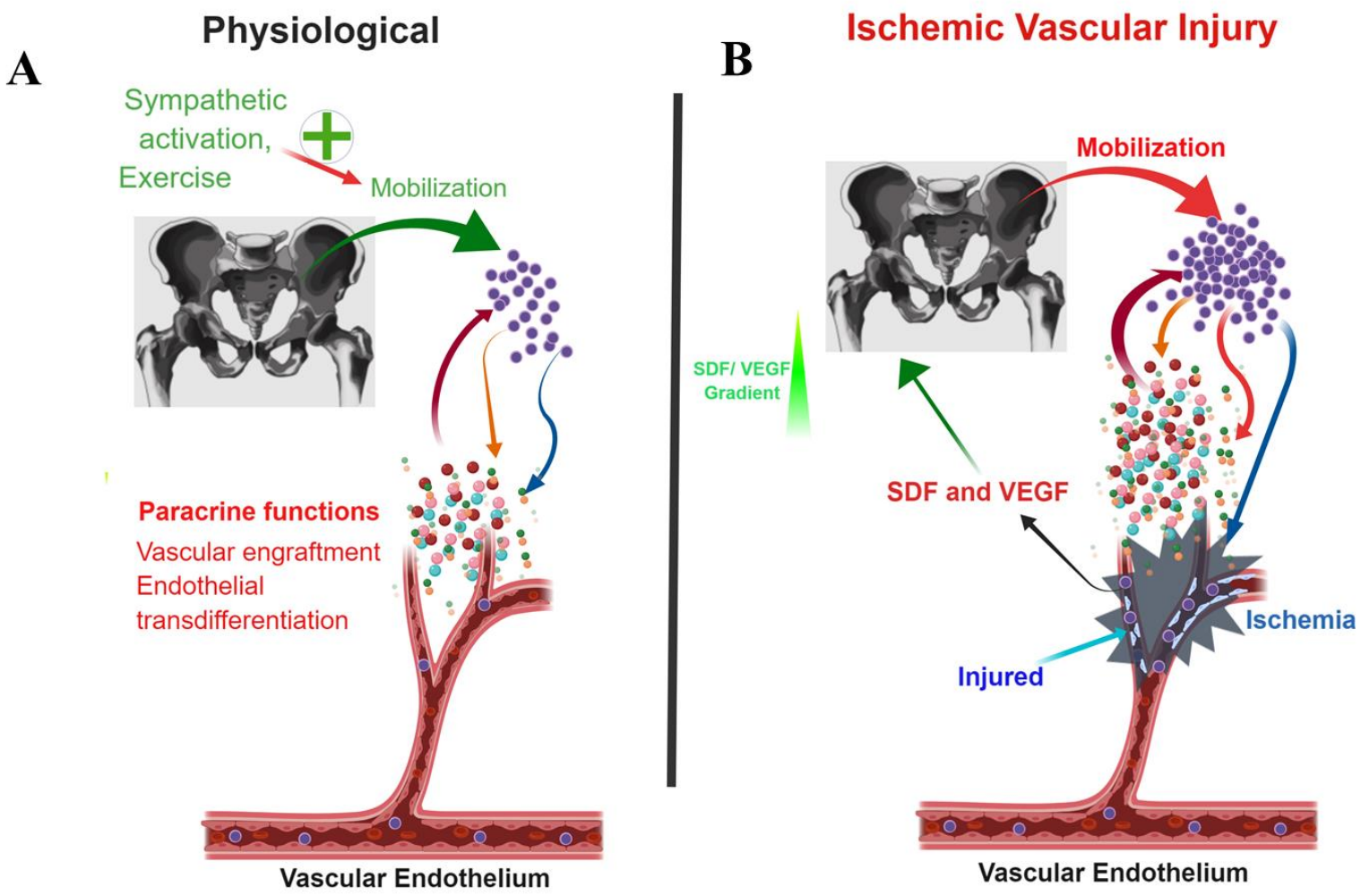

Figure 3. Mechanism of vascular repair by HSPCs. *A.* Physiologically HSPCs are continuously mobilized in small numbers into circulation and release paracrine factors that maintain the endothelial health. *B.* During ischemic injury large numbers of HSPCs are mobilized into circulation that release more paracrine factors and stimulate local endothelium to accomplish vascular repair.

This hypothesis is further supported by the studies showing genetic ablation of eNOS blunted VEGF- or exercise-stimulated HSPCs mobilization (44,55).

Mobilization of endogenous HSPCs can be stimulated by pharmacological mobilizing agents such as G-CSF (56) and plerixafor (57,58). These two agents are extensively used as clinical mobilizers in cell-based therapies to collect HSPCs for bone marrow transplantation and hematopoietic recovery in cancer patients following radiation chemotherapy and have also shown to improve ischemic vascular repair (4,59–62). G-CSF is relatively a slow mobilizer for HSPCs which binds to homodimer G-CSF receptor (63) and stimulates several pathways. It was shown to act by reducing SDF and up-regulating its receptor, CXCR4 (64). Conversely, AMD3100/plerixafor is a fast acting and potent mobilizer which act via inhibiting SDF binding to its receptor CXCR4, thereby releasing HSPCs (29). The cytokine GM-CSF, which is related to G-CSF was also shown to stimulate mobilization and augment HSPCs levels (38). Currently, for HSPCs mobilization, three drugs are approved by Food and Drug Administration (FDA): G-CSF (Filgrastim/Neupogen), GM-CSF (Sargramostim), and AMD3100 (Plerixafor/Mozobil).

1.1.5. Cell-based therapies for cardiovascular diseases

Conventional therapies do not facilitate vascular generation in case of ischemic vascular diseases which are the leading cause of morbidity and mortality in the world (65). In consideration of unmet needs for vascular diseases and failure of current treatment options to reduce vascular complications, attempts have been made to develop alternate therapeutic approaches. In light of this, when innate vascular repair mechanism fails, and clinically alarming ischemic conditions are presented — cell-based therapy represents a novel and promising approach. Undoubtedly, HSPCs are intimately involved in the complications requiring therapeutic revascularization, vascular growth and vascular repair. Fueled on the basis of success

of preclinical studies with cell-based therapies in the models of ischemia (12,23,66–71), the therapeutic potential of autologous HSPCs for the safety and efficacy in the treatment of ischemic cardiovascular diseases is being actively explored in many recent studies and clinical trials (72–76). A major conceptual breakthrough in the therapeutic use of HSPCs was the discovery of mobilizing agents which augmented the density of HSPCs in the circulation, where they can be easily collected and purified. Unlike conventional pharmacotherapies, autologous cell therapy involves collections of HSPCs directly from patients' bone marrow or in most cases, mobilization of HSPCs using a pharmacological mobilizing agent. This process is followed by collection of peripheral blood mononuclear cells (MNCs) by an apheresis instrument, isolation, purification or enrichment of HSPCs using magnetic cell separation, and finally reinfusing them back into the peri-ischemic region (Fig. 4). Lessons learned from the clinical trials suggest that yield of HSPCs or in other words number of HSPCs that can be collected from circulation is an important determinant of the final therapeutic outcome (4,77). Average number of cells required for autologous transplantation are 5×10^5 HSPCs/kg, or more. However, circulating HSPCs are very low and do not always suffice this requirement for a cell-based therapy (4). Eventually, the success of cell-based therapy is essentially dependent on the number of cells that could be collected from an individual, which often requires long-protocols of mobilization of bone marrow HSPCs. An alternate approach to overcome this limitation and to obtain enough cells is to modify them to enhance therapeutic outcomes, instead of using freshly isolated cells. HSPCs expanded *ex-vivo* in the culture were shown to have enhanced vasoreparative effects (4,26,78).

However, such approach requires long protocols and alters phenotypic characteristics of cells with decline in CD34 expression. Additionally, these cells with altered phenotype do not

offer resemblance to the corresponding cells *in vivo*, therefore enumerating freshly isolated cells in circulation would give a better estimate of vasoreparative HSPCs.

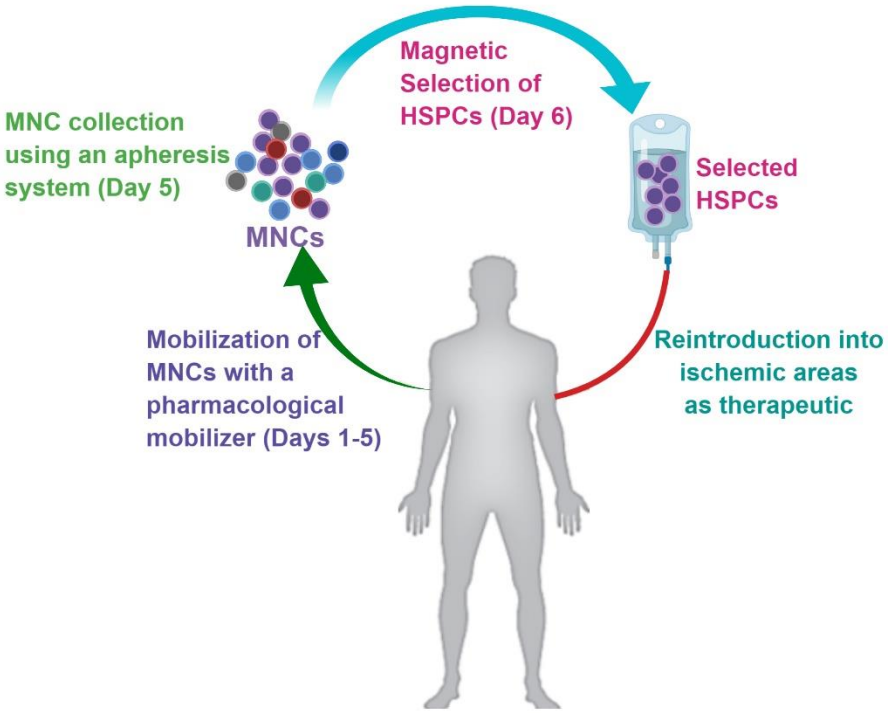

Figure 4. Currently used clinical strategy for the autologous transplantation of HSPCs. Total mononuclear cells (MNCs) were mobilized from bone marrow into the circulation by using a pharmacological mobilizer. MNCs are containing all differentiated cells and a fraction of HSPCs. MNCs are then collected and processed for immunomagnetic enrichment of HSPCs. Finally, HSPCs are injected back into the peri-ischemic regions as therapeutic.

Conversely, CD34 marker can distinguish the HSPCs with marked clinical benefits (4,79). $CD34^+$ cells are popular choice in hematopoietic reconstitution in patients undergoing radiation and chemotherapy treatments and they are used clinically in treating cancers such as lymphoma and leukemia, several blood and immunological diseases (80,81). The repertoire of $CD34^+$ cells was expanded beyond cancer in the field of cardiovascular disease after Asahara's seminal discovery (12), and they offer a huge clinical promise in peripheral, myocardial or cerebral ischemia. A series of reasonably sized clinical studies have reported initial safety and efficacy of autologous bone marrow-derived HSPCs, particularly $CD34^+$ cells for conditions of

myocardial ischemia, infarction and other cardiovascular diseases. Clinical endpoints in these diseases are regeneration of damaged myocardial tissue, reduction in the infarct size, improvement in the LVEF (left ventricular ejection fraction) or regeneration of blood vessels in the ischemic myocardium or peripheral limbs (75). Several studies listed on ClinicalTrials.gov are evaluating the use of isolated MNCs, $CD34^+$ cells, or pharmacological interventions that can improve the functions of HSPCs and have shown positive therapeutic outcomes as described in the Table 1.

Of note, the TOPCARE-AMI and BOOST trials reported improvements directly related to cardiac remodeling with bone marrow HSPCs (82,83). The REPAIR-AMI trial was very encouraging. It was largest clinical trial conducted with myocardial infarction (MI) patients (n=204) and showed that bone marrow derived progenitors significantly improved LVEF (left ventricular ejection fraction) (5.5% vs placebo group) (84). However, administration of total MNCs in the ischemic myocardium require administration of cells at regular intervals and high doses of these cells administered by intramyocardial injections were shown to produce side effects such inflammation and hemorrhage in a preclinical model (26,83,85). Some clinical trials (TIME (86), LateTime (87) and ASTAMI (88)) have also produced contradictory results. These trials did not show improvements in cardiovascular parameters between the placebo and MNCs groups. According to the REGENT trial, the dose of selected HSPCs ($CD34^+CXCR4^+$ cells) for improvement in LVEF in acute MI patients is 100 times less than the dose of unselected population of MNCs (89) reaffirming the potency of HSPCs. Several clinical studies have also evaluated the effectiveness of $CD34^+$ cells in the peripheral arterial disease or critical limb ischemia in human patients. Clinical study by Losordo and colleagues (79) –ACT-34-CLI, provided evidence for the safety of autologous $CD34^+$ cells in the critical limb ischemia (CLI)

patients. This study also showed reduced amputation rates in CD34 cells-treated versus control subjects (90). Corroborative evidences from these studies confirm the inherent capacity of HSPCs for performing vasoreparative functions and their therapeutic effectiveness in the treatment of ischemic vascular diseases.

Table 1. Currently ongoing/completed clinical trials using HSPCs to treat ischemic cardiovascular diseases

Name of the trial	**Phase**	**Condition (n)**	**Type of HSPCs (dose)**	**Primary outcomes**	**ClinicalTrials.gov-NCTID**
ESCAPE	Phase III	Angina, coronary disease (n =250)	Autologous bone marrow MNCs (300 X 10^6 cells)	Lon-term Survival benefit with cells	NCT00841958
FOCUS	Phase II	Coronary artery disease (n = 92)	Autologous bone marrow MNCs (100 X 10^6 cells)	Improved perfusion and functions of myocardium	NCT00824005
TOPCARE-AMI	Phase I	Acute Myocardial Infarction (n =59)	Autologous bone marrow MNCs/ HSPCs (5.5 ± 3.9× 10^6 cells)	Improved LVEF (left ventricular ejection fraction)	N/A(82)
BOOST	Phase I	Coronary Artery Disease (n =60)	Autologous $CD34^+$ cells (3.6 X 10^6 cells)	Improvements in cardiac remodeling and LVEF	NCT00224536 (91)
TICAP	Phase I	Hypoplastic left heart syndrome (n = 14)	Cardiac progenitor cells (0.3 million cells/Kg of BW)	Improved right ventricular ejection fraction	NCT01273857
REPAIR-AMI	Phase I/II	Acute Myocardial Infarction (n =204)	Autologous bone marrow MNCs (198 X 10^6 cells)	Decreased LVEF in LV angiography	NCT00279175

Table 1. Currently ongoing/completed clinical trials using HSPCs to treat ischemic cardiovascular diseases (continued)

Name of the trial	Phase	Condition (n)	Type of HSPCs (dose)	Primary outcomes	ClinicalTrials.gov-NCTID
LateTIME	Phase II	Left ventricular dysfunction (n=120)	Autologous bone marrow MNCs (150 X 10^6 cells)	Improved left ventricular ejection fraction	NCT00684021
Time	Phase II	Dilated cardiomyopathy (n =120)	Autologous bone marrow MNCs (150 X 10^6 cells)	Improved global and regional LV function	NCT00684021
Pre-Serve-AMI	Phase II	ST Segment Elevation Myocardial Infarction (n = 195)	$CD34^+$ cells (14.9 X10^6 cells)	Safety and efficacy of intracoronary infusion of $CD34^+$ cells	NCT01495364
ACT34-CLI	Phase IIb	Critical Limb Ischemia (n=28)	$CD34^+$ cells (10^6 cells/Kg of BW)	Safety and efficacy of intramuscular injection of $CD34^+$ cells	NCT00616980 (90)
Effects of Atorvastatin on EPCs after coronary surgery	Phase IV	Coronary artery bypass surgery (n =60)	$CD34^+VEGFR\text{-}2^+CD133^+CD45^-$ HSPCs (Atorvastatin 40 mg/day for 14 days)	Increased circulating HSPCs	NCT01096875

Autologous cell-therapies are not free from challenges and in this the case major barrier for their use as a regenerative medicine to treat vascular conditions is the low number of cells that can be collected from the peripheral blood. Number of circulating HSPCs and their innate vascular regeneration potential is impaired with aging (92), diabetes (93–96), hypertension (97,98) and in the patients who smoke (99,100). Cells mobilized by G-CSF/plerixafor showed efficacy in pre-clinical ischemic diseases and wound healing models (62,101–104). In the conditions when HSPCs fail to mobilize or mobilization in response to a stimulus such as

ischemia or clinical mobilizing agent is less than adequate, that condition is called 'mobilopathy'. The cohort which shows mobilopathy is known as 'poor mobilizers.' Cell-based therapies frequently fail in poor mobilizers due to difficulty in obtaining enough number of cells for therapy. G-CSF was extensively used as mobilizer in clinical conditions of cardiovascular diseases, however, it was shown to be less beneficial in improving myocardial recovery (105,106) due to decreased adhesion molecules on mobilized HSPCs that might lead to reduced homing capacity (107).

Therefore a conceptual breakthrough is urgently needed to identify novel mechanism-based interventions that can potentiate the reparative functions of HSPCs so even with less number cells which are highly potent we can still achieve equivalent vascular regeneration

1.1.6. Approaches to stimulate the vasoreparative functions of HSPCs

Several approaches have been tried to increase the number of circulating HSPCs. It is already established that $CD34^+$ HSPCs are clinically useful and more practically viable source to pursue for cell-based therapies. However, age or underlying disease condition such as diabetes limits their utility and *ex vivo* manipulation is indeed required to overcome this limitation before their reinfusion. Approaches have been also tried to potentiate their vasoreparative functions. These approaches are mainly *ex vivo* manipulation of cells or maneuvers to prime the cells before transplantation. HSPCs counts were shown to be increased by systemic administration of VEGF (32,38). Accumulating evidence suggest that genetically modifying HSPCs to release pro-angiogenic factors may be therapeutically more effective and potent armamentarium as compared to unmodified cells. In this regard, HSPCs that were genetically engineered to overexpress eNOS in a rabbit model of angioplasty showed inhibition of neointimal hyperplasia (108). In a first-in-human— The Pulmonary Hypertension and Angiogenic Cell Therapy

(PHACeT), phase I clinical trial in the patients with idiopathic pulmonary arterial hypertension (PAH), eNOS gene transfected HSPCs have shown improvements in short-term hemodynamic parameters, sustained improvements in the exercise capacity and quality of life scores (109). Similarly, HSPCs from mice transduced with VEGF gene using adeno-associated viral (AAV) vector showed enhanced angiogenic potential in the *in vivo* and *in vitro* assays compared to non-transduced cells (110). In another such study, when HSPCs from humans transduced with pCDNA encoding for VEGF were injected in the athymic mouse model of ischemia, VEGF-transduced cells demonstrated increased blood flow and neovascularization of ischemic areas (111). Another such approach was based on resetting the telomerase length and delay the process of senescence by transducing HSPCs using human TERT (Telomerase Reverse Transcriptase) which resulted in increased blood flow and capillary density in the mouse model of hindlimb ischemia (HLI) (112). However, one major limitation of such approaches is the possibility of abnormal angiogenesis (113,114). Increased levels of TGF-β1 (Transforming growth factor-β1) was shown to induce cellular senescence and growth arrest (115) so another approach was to modify $CD34^+$ HSPCs *ex vivo* using antisense phosphorodiamidate morpholino oligomers (PMOs) to transiently inhibit TGF-β1 protein expression and this strategy was shown to enhance repair after vascular damage and enhanced the recruitment of healthy and diabetic HSPCs to the sites of retinal injury (116).

A successful outcome of stem cell therapy vastly depends on the milieu of the host. HSPCs reside in the hypoxic niches of bone marrow but are collected from circulation where they experience different gradients of oxygen and eventually may lose their reparative abilities (117). Bone marrow hypoxic niches provide “fertile” environment to these cells and the vaso-reparative ability of HSPCs is majorly due to their innate capacity to sense the low-oxygen

conditions of ischemia and home to the areas of hypoxia. Mobilization of HSPCs in response to ischemic injury and migration to the ischemic areas are important properties of HSPCs that determine their vasoreparative abilities. Undoubtedly, the sensitivity of HSPCs to hypoxia plays an important role in initiation of vascular repair after ischemia and vascular-repair relevant functions of HSPCs. Hypoxic preconditioning has been successfully tried in different cell types (118–121). Gnecchi et al. has shown that genetically modified human MSCs overexpressing Akt1 release paracrine factors, especially under hypoxic conditions, that exerts protective effects on ischemic myocardium (122). Hypoxic preconditioning was shown to increase the surface expression of CXCR4, and VEGF receptors, VEGFR1 and VEGFR2 (123) which enables these cells to the signals of hypoxia (41,124) and may promote the vasoreparative function of HSPCs. Increased CXCR4 expression via hypoxic preconditioning was shown to enhance the reparative functions of progenitor cells in both *in vitro* and *in vivo* angiogenesis assays and augment their recruitment to ischemic heart (123). Similarly, hypoxic preconditioning of MNCs also showed increased expression of CXCR4 and increased retention of cells into ischemic hindlimbs after HLI (125). Transplantation of hypoxia-preconditioned MSCs into peri-ischemic areas of infarcted heart was shown to increase angiogenesis via augmented expression of pro-survival and pro-angiogenic factors (118). In human MSCs, hypoxic exposure augmented proliferation, growth and tissue formation with increased extracellular matrix formation (126). Interestingly, clinical conditions of vasoreparative dysfunction was shown to be associated with desensitization of HSPCs to hypoxia (127,128). A recent study by Mantel et al. has shown the importance of hypoxia for the collection of HSPCs and provided mechanistic evidence for the extra physiologic oxygen shock to HSPCs when they are exposed to normoxia. Ambient air (normoxia) was shown

to induce mitochondrial permeability transition pore (mPTP) opening and increased stress on cell which could be mitigated by collecting and processing the cells under hypoxic conditions (117).

Priming of HSPCs by hypoxic preconditioning could be a better approach to stimulate the vasoreparative potential and requires further investigations.

1.1.7. Renin angiotensin system

Renin Angiotensin System (RAS) is an ever-evolving endocrine system and its role is well characterized in cardiovascular disorders. Classical RAS consists of two opposing arms – Angiotensin Converting Enzyme (ACE)/Angiotensin (Ang)-II/AT1 receptor (AT_1R) axis, a cardiovascular detrimental arm, and ACE2/Angiotensin (Ang)-(1-7)/Mas receptor (MasR) axis, a cardiovascular protective arm (Fig. 5). RAS begins with angiotensinogen, a dodecapeptide, which is synthesized by liver and is activated by renin which is synthesized by juxtaglomerular cells of the kidney to give rise to a decapeptide-angiotensin 1 (Ang I). Diacarboxy exopeptidase, ACE, cleaves two terminal amino acids of Ang I to form an octapeptide and a potent vasoconstrictor, Ang II, which increases peripheral vascular resistance and consequently increases blood pressure. In an alternate pathway, monocarboxy peptidase ACE2 mediates metabolism and synthesis of a heptapeptide, Ang-(1-7) (129,130). Ang I via action of Neprilysin (NEP), ACE, and ACE2 enzymes cleaved to form Ang-(1-7). It is noteworthy that, ACE2-dependent generation of Ang-(1-7) is the most favored pathway. Ang-(1-7) can be further cleaved by ACE or aspartic acid decarboxylase (DC) to form an inactive Ang-(1-5) or Ala^1-Ang-(1-7), respectively (131). Ang II exerts mainly cardiovascular detrimental actions through AT_1R while Ang-(1-7) acts on G-protein coupled receptor- Mas (MasR) and exhibits cardiovascular protective effects. Ala^1-Ang-(1-7) recognizes Mas-related G-protein coupled receptor member D (mRG-D) and its signaling was recently shown to have vasorelaxant effects (132).

It is well documented that hyperactivation of deleterious—ACE/Ang II/AT_1R pathway is linked to cardiovascular maladies such as hypertension, congestive heart failure, stoke, renal failure and other vascular detrimental conditions. As a result these disorders are frequently and successfully targeted by using ACE inhibitors (ACEis) or AT_1 receptor blockers (ARBs) (133). Recently accumulated evidences point that activating ACE2/Ang-(1-7)/MasR system has potential therapeutic benefits (134). It is interesting enough to note that, the beneficial effects of ACEis and ARBs in cardiovascular diseases might also be partly attributed to increased circulating levels of Ang-(1-7), via enhanced hydrolysis of accumulated Ang I (due to effect of ACEi) by ACE2 enzyme or decreased metabolism of Ang-(1-7) (135). Ang- (1-7) is a vasodilator peptide with anti-hypertensive, anti-inflammatory, anti-fibrotic and anti-thrombotic functions (136). Therefore, the balance between ACE and ACE2 is critical in maintaining cardiovascular homeostasis and imbalance in the relative expression of ACE2 and ACE leads to pathological cardiovascular events. This suggested that ACE2 represents an endogenous counter-regulatory mechanism within RAS (137) and strategies which can maintain higher ACE2 in relation to ACE could be cardiovascular protective through enhanced Ang-(1-7)/MasR signaling.

Young et al. first discovered Mas as a proto-oncogene (138) which was believed to modulate intracellular signaling of Ang II through AT_1R (139). However, the tumorigenic potential was later rebutted by subsequent studies. An elegant study by Santos et al. showed that MasR is a specific receptor for Ang-(1-7) and identified Ang-(1-7) as an endogenous agonist of MasR (140). Additionally, the authors have showed that Ang-(1-7) stimulates arachidonic acid release via activation of the phospholipase A2 (PLA_2)/arachidonic acid (AA) pathway which can be blocked by A779, a MasR antagonist (140). Sampaio et al. have shown that Ang-(1-7)/MasR signaling activates phosphatidylinositol 3-kinase (PI3K)/Akt-dependent eNOS, stimulates NO

generation and also attenuates NADPH oxidase activity (141). Ang-(1-7) failed to stimulate NO generation in cells/tissues of MasR- deficient mice (142). Cumulatively, results from these studies mainly point towards vasorelaxant effects of Ang-(1-7). Ang-(1-7) was also demonstrated to act via AT_2R (143) however, these effects might also require interaction with MasR (144,145).

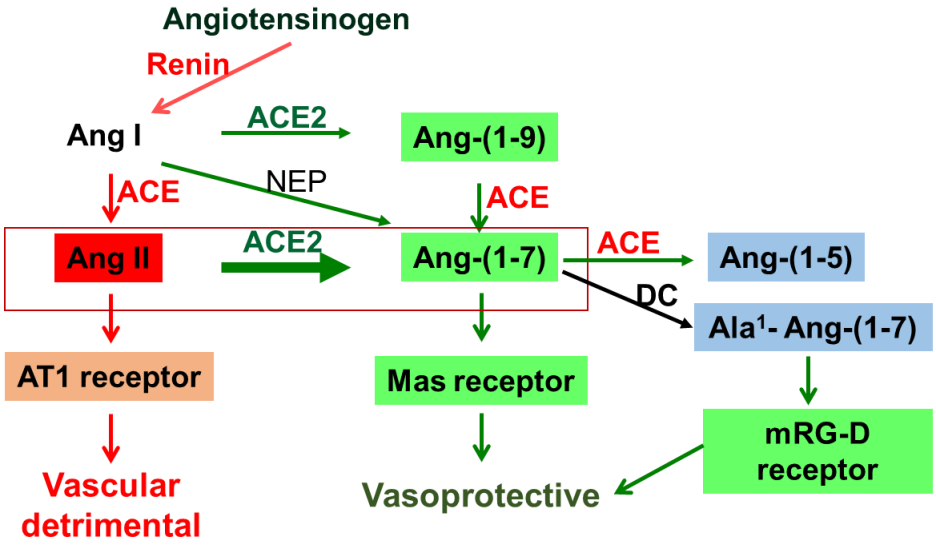

Figure 5. Pathways of various peptides involved in renin angiotensin system (RAS) and their receptors. Ang-(1-7) biosynthesis is highlighted with a red box. Figure adapted from (131).

1.1.8. Evidence for the expression and functions of renin angiotensin system in HSPCs

There is an increasing body of evidence suggesting that apart from global circulating RAS, modulation of local RAS in various tissues and organs including bone marrow helps in maintaining the homeostasis (146). Several lines of evidence support the notion that the imbalance in ACE and ACE2 would be detrimental to the regenerative functions of HSPCs. Thus far, studies have provided strong evidence that modulation of local RAS maybe critical for the regulatory role of angiotensin peptides in the hematopoietic functions of HSPCs (146–150). Activation of local RAS in HSPCs plays an important role in mobilization, proliferation, differentiation, recruitment to areas of injury, paracrine functions and vascular repair (151,152). Experimental studies with ACEis and/or ARBs support the notion that the pathological axis of

RAS is deleterious to regenerative functions of HSPCs. Treatment with these pharmacological agents are associated with increases in the circulating levels of HSPCs in patients with cardiovascular diseases which may be due to reduced senescence of HSPCs (97,153,154)

Previous reports from our lab and others have provided direct evidence for the vasoprotective functions of ACE2/Ang-(1-7)/MasR in mediating protective functions of HSPCs (151,155–157). Importantly, a study from our lab showed that ACE2 is expressed more than ACE in human $CD34^+$ cells and mice Lin^- cells (158). Study by Wang and colleagues in mice provided direct evidence that the Ang-(1-7) mediated cardiac recovery following ischemia occurred via enhanced recruitment of HSPCs to the areas of myocardial injury (159). Activation of MasR by Ang-(1-7) was shown to enhance the vasoreparative potential of dysfunctional $CD34^+$ cells derived from older adults with diabetes (155) and induced migration of HSPCs by enhancing NO release. A study by Chen et al. in an experimental model of stroke has shown that overexpression of ACE2 in HSPCs enhanced their protective functions, reduced occurrences of ischemic brain injury and promoted recovery via modulation of NADPH-ROS and eNOS-NO signaling pathways (160). Along similar lines, we have previously shown that ACE2 gene transfer via lentiviral approach ameliorates vascular dysfunction of HSPCs in a mouse model of HLI (161). Pharmacological activation of ACE2 by diminazene aceturate (DIZE) showed improvement in angiogenic functions of progenitor cells in an animal model of pulmonary hypertension (162). Based on these encouraging preliminary findings it is tempting to hypothesize that endogenous ACE2 expression in HSPCs could be a reliable prognostic biomarker for ischemic vascular complications. Seminal studies by Rodgers et al. was the first to show that Ang-(1-7) can accelerate hematopoietic recovery in mice which were subjected to non-lethal irradiation by enhancing the proliferative and differentiation functions of HSPCs to form

hematopoietic cells (150). Similarly, Ang-(1-7) also accelerated hematopoietic recovery in breast cancer patients after chemotherapy (147,163). MasR expression was found on HSPCs (150,156,164) and Ang-(1-7) treatment reversed the vasoreparative dysfunction of murine HSPCs (165) and $CD34^+$ cells from diabetic individuals (155). Activation of MasR via Ang-(1-7) was shown to restore the migration of HSPCs to SDF and VEGF (165,166). Overexpression of Ang-(1-7) by lentiviral gene expression improves vasoreparative functions, *in vitro* and homing efficiency to areas of ischemia, *in vivo*. Thus, there are ample evidences strongly supporting that activation of ACE2/Ang-(1-7)/MasR axis is an attractive target to enhance the regenerative outcomes of HSPCs because of multiple benefits associated with the activation of ACE2: i) restoration of physiological environment in the damaged host tissue, ii) attenuation of deleterious axis of RAS, iii) stimulation of regenerative functions of HSPCs and iv) enhanced HSPC mobilization from bone marrow. Therefore, activation of the vasoprotective axis of RAS in HSPCs could be therapeutically beneficial in ischemic vascular diseases where vascular remodeling and endothelial regeneration are desired therapeutic outcomes. As mentioned above, because of the intrinsic ability of HSPCs to preferentially migrate and home to the areas of ischemia, their *ex vivo* modification to produce vasoactive molecules which could be delivered to the sites of tissue repair could enhance the therapeutic outcomes of cell-based therapies. In addition to activation of ACE2/Ang-(1-7)/MasR axis, hypoxic preconditioning is another promising and novel approach to enhance the regenerative outcomes of HSPCs. However, the effects of hypoxic intervention on four members of RAS (ACE, ACE2, AT_1R and MasR) in HSPCs are not known.

1.2. Objective and specific aims

The discovery of HSPCs resulted in a breakthrough approach for the treatment of ischemic vascular diseases (3,4,12,75) and several clinical trials are underway evaluating their therapeutic potential (72–74,79). Major caveats for the autologous HSPCs-based therapies is advanced age and long-term complications such as hypertension, obesity or diabetes that attenuate the regenerative abilities of these cells. The success of this approach is essentially dependent on the number of cells that could be collected from an individual, and often requires long-protocols of mobilization of bone marrow HSPCs. However, many individuals eligible for cell-based therapies do not respond adequately to mobilizing agents (167–169)**. Therefore, novel mechanism-based strategies are urgently needed to enhance the outcomes of autologous cell-based therapies in poor mobilizers.**

Activation of ACE2/Ang-(1-7)/MasR axis was shown to stimulate vascular repair-relevant functions of human and murine HSPCs (147,151,155–157,162). Higher expression of ACE2 and MasR in HSPCs has been shown to prevent the development of vascular disease (155). Therefore, overexpression of ACE2 and MasR in HSPCs would be a promising approach to increase neovascularization outcomes of cell-based therapies. It is known that hypoxia modulates the reparative functions and expression of angiogenic genes in HSPCs, however the effect of hypoxia on ACE2 and MasR is unknown.

1.2.1. Objective

To test hypoxic preconditioning as an approach to enhance the vaso-reparative potential of HSPCs and provide mechanistic evidence for the activation of the protective axis of RAS via hypoxic stimulation.

1.2.2. Hypothesis

We hypothesized that ACE2 and MasR expression are hypoxia-regulated in HSPCs, and that the hypoxic-stimulation of vasoreparative functions are largely mediated by ACE2/Ang-(1-7)/MasR axis in HSPCs.

1.2.3. Specific aims

To test this hypothesis, we proposed the following specific aims, which are depicted in the project overview (Fig. 6).

1.2.3.1. Specific aim 1: To test the hypothesis that ACE2 is hypoxia-regulated in vascular progenitor cells (HSPCs)

In this aim, we determined whether hypoxia or its target molecules stimulates the expression and activity of ACE2 and also delineated a mechanism for hypoxia-induced shedding of ACE2.

1.2.3.2. Specific aim 2: To test the hypothesis that MasR is hypoxia-regulated in vascular progenitor cells (HSPCs)

In this aim, we determined whether hypoxia or its target molecules stimulates the expression of MasR.

1.2.3.3. Specific aim 3: To test the hypothesis that regional hypoxia *in vivo* upregulates ACE2 and MasR in vascular progenitor cells (HSPCs)

In this aim, we determined if the *in vitro* findings could be recapitulated in *in vivo* conditions. This aim was carried out in mouse and human models of ischemic injury and regional hypoxia, respectively.

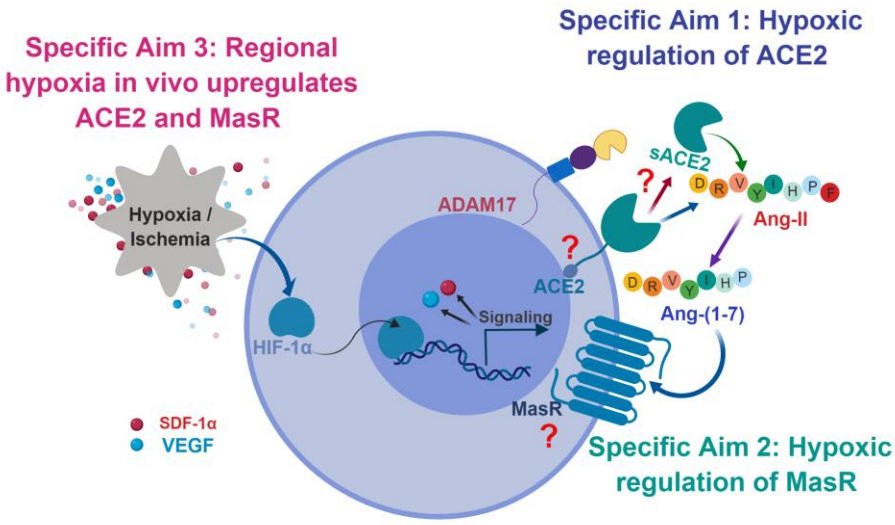

Figure 6. Project overview.

1.3. Materials and methods

1.3.1. Characteristics of subjects

Our research study was approved by the Institutional Biosafety Committee of North Dakota State University. Leucocyte samples were derived from healthy human volunteers (n=145) including both males and females of age ranging from 28 to 75 years at the United Blood Services (Fargo, ND). All the volunteers were healthy Caucasian individuals and non-smokers. Leucocytes were collected in Leucoreduction chamber (LRS chambers) following apheresis by using TrimaAccel system (80440). Freshly obtained LRS cones were used for the isolation of $CD34^+$ cells as described in the previous publication from our lab (156). We used freshly isolated $CD34^+$ cells for studies conducted to answer specific aims 1 and 2. Studies in specific aim 3 were approved by the Institutional Review Board of North Dakota State University (IRB#HE18137). This study was performed in collaboration with Dr. Kyle Hackney and his graduate students Logan Pitts and Sean Mahoney. Details about participant characteristics are captured in the section 1.3.12.

1.3.2. Isolation of $CD34^+$ cells

LRS cones were processed to isolate peripheral blood mononuclear cells (MNCs) by Ficoll-Paque (GE Healthcare Waukesha, WI, USA) density-gradient centrifugation (800 g, 30 min), according to the manufacturer's protocol. Differentiated MNCs were collected from buffy coats and plasma was excluded by washing the cells three times with working buffer (1X PBS with 2% FBS and 1 mM EDTA) and centrifuged at 120g for 10 min. Cell count was performed by using hemocytometer and 100 million MNCs were enriched for lineage negative (Lin^-) cells by using a human progenitor cell enrichment kit (19356; StemCell Technologies, Vancouver, BC, Canada) as per supplier's instructions. This is a negative selection process and by using this process We removed unwanted lineage cells (Lin^+) by targeting them with tetrameric antibody complexes recognizing CD2, CD3, CD11b, CD11c, CD14, CD16, CD19, CD20, CD24, CD56, CD61, CD66b glycophorin A and dextran-coated magnetic particles. We further enriched Lin^- cells for $CD34^+$ cells by using a positive selection kit (18056; StemCell Technologies, Vancouver, BC, Canada). This kit contains antibodies to human Fc receptor to minimize nonspecific binding and tetrameric antibody complexes recognizing CD34. Then, CD34 antibody labelled cells were targeted with dextran-coated magnetic nanoparticles and separated using EasySep magnet (18000; StemCell Technologies, Vancouver, BC, Canada). After enrichment and cell counting, we confirmed the purity of enriched $CD45^{low}CD34^+$ cells by flow cytometry (Accuri C6, BD Biosciences). For flow cytometry, we stained the cells by incubating with a allophycocyanin(APC) conjugated anti-human lineage cocktail (363601; Biolegend, 1:500, Ex/Em: 650/660 nm), phycoerythrin (PE) conjugated anti-human CD45 (304039; Biolegend, 1:500, Ex/Em: 565/578 nm), Fluorescein isothiocyanate (FITC) conjugated anti-human CD34 antibodies (343604; Biolegend; 1 in 250, Ex/Em: 490/525 nm) or isotype control antibodies (1 in

500) (Biolegend, SanDiego, CA, USA) in the presence of FcR blocking reagent (130-059-901; Miltenyi Biotech, 1:100) for 45 min at 4°C. Dead cells were excluded using 7-AAD viability staining solution (420404; Biolegend). We routinely assessed the purity of these cells after enrichment. On average purity of the $CD34^+$ cells in all experiments was more than 95% (Fig. 7).

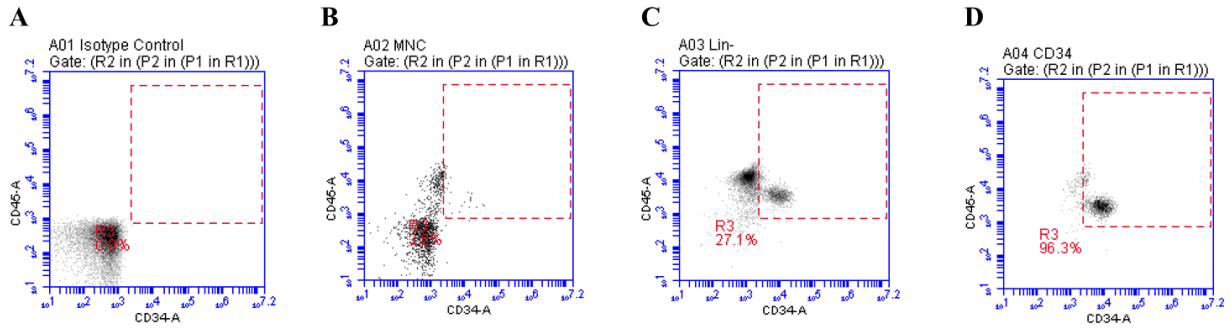

Figure 7. Representative dot plots of $CD34^+$ cells before and after immunomagnetic enrichment. Lin^- cells or $CD34^+$ cells from MNCs were enriched by immunological selection as described in the text. *A.* Cell populations treated with isotype controls or with antibodies conjugated with different fluorophores. Percent of $CD45^{low}CD34^+$ cells in the *B.* Total MNCs prior to enrichment, *C.* in Lin^- cells and *D.* after enrichment of $CD34^+$ cells.

1.3.3. Culture of $CD34^+$ cells, morphology and exposure to hypoxia

Freshly isolated cells were plated in round-bottom 96-well plates (Nunc) with no more than 30,000 cells per well in serum free StemSpan SFEM medium (09650; StemCell Technologies). These cells are suspension cells and they show distinct morphology as opposed to adherent cells when observed under the microscope (Fig. 8). Cells were either maintained in a CO_2 incubator at 37°C in 20% O_2 (normoxia) or were exposed to hypoxic conditions (1% O_2-94% N_2 and 5% CO_2). Hypoxic conditions were accomplished by using a hypoxia chamber (ProOx C21, BioSpherix, Lacona, NY, USA) (Fig. 9). The hypoxia chamber was maintained at 37°C and was sealed and purged with nitrogen for 1 hour to achieve 1% O_2, 5% CO_2, and -balance N_2. We first optimized levels (0.1%, 1%, 3-10%) and duration of hypoxia (between 0-24 hr) in preliminary experiments and by measuring ACE2 activity as a readout as discussed in the

next chapter. We exposed up to 400,000 cells to hypoxia or normoxia, and then either used them freshly for flow cytometry, migration or proliferation, activity assays, luciferase assay or preserved them at -80 °C for Western blotting and real-time PCR. Cell supernatants after normoxic or hypoxic exposure were also collected and kept frozen at -80 °C, and analyzed later for measuring secretory enzyme activities and for measuring protein expression by Western blotting.

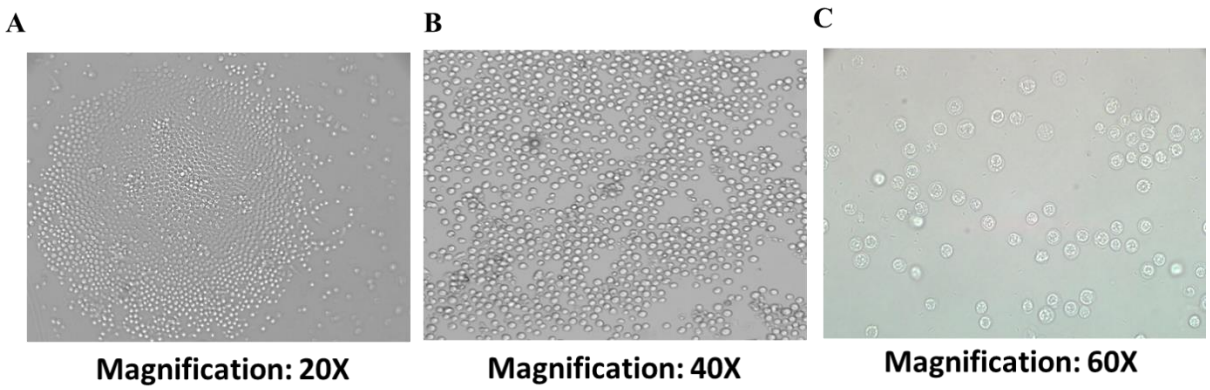

Figure 8. Distinctive morphology of $CD34^+$ cells. Cells in the round bottom 96-well plate observed with bright-field microscopy at magnification of *A.* 20x, *B.* 40x and *C.* 60x.

1.3.4. Cell migration assay

Migration of cells was determined using a QCM™ chemotaxis 5 µM, 96-well cell migration assay (ECM512; EMD Millipore) as described in Singh et al (156). Briefly, 20,000 cells were plated in the cell-migration chamber in the serum-free basal medium HBSS (Hyclone) and were assayed by plating them in duplicates. Cells were kept in normoxic or hypoxic conditions without any chemoattractant or in the presence of SDF (100 nM) or VEGF (30 nM). Cells were allowed to migrate through a 5 µM membrane for five hours in either condition. After five hours of migration, cells were gently dislodged from the membrane using cell detachment buffers, lysed and quantified using the CyQuant GR fluorescent dye on a Spectramax plate

reader at excitation of 480 nm and emission of 520 nm (Molecular Devices, San Jose, CA, USA). Response of this assay was quantified as arbitrary fluorescence units (AFUs) and response to different treatment groups SDF, VEGF or hypoxia were expressed as percent increase in AFU (corresponding to migrated cells) compared to untreated cells kept in normoxic condition.

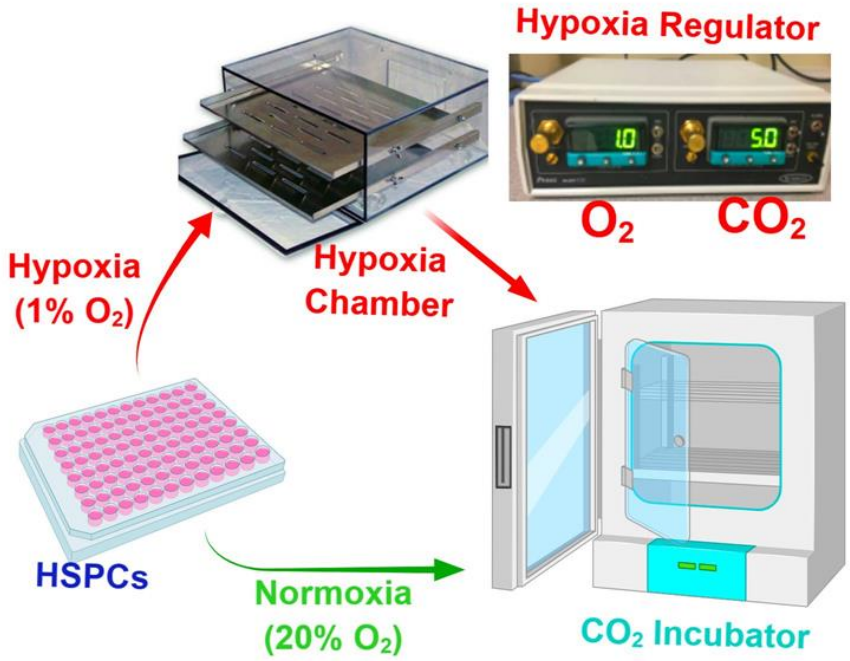

Figure 9. Experimental setup of the hypoxia chamber.

1.3.5. Cell proliferation assay

Proliferation of cells was determined by using colorimetry using a bromodeoxyuridine (BrdU) incorporation assay as per the manufacturer's instructions (Roche Bioscience) (156). The assay was performed using 10,000 cells, in 96-well round bottom dishes, in duplicate, under normoxic or hypoxic conditions for 48 hours untreated or treated with SDF/VEGF. In this assay BrdU is incorporated into the proliferating cells, and measured from cell lysates at an absorbance of 370 nm (Spectramax plate reader). Cell proliferation was expressed as fold increase as compared to cell proliferation measured in the presence of mitomycin (1 µM).

1.3.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

Cells which were preserved at -80°C were thawed and RNA was extracted from the cells using Trizol reagent (ThermoFisher). Briefly, cell pellet was mixed with Trizol and lysed gently by mechanical agitation followed by mixing in an order with chloroform, IPA and ethanol and finally washing the pellet using RNAase free water. After isolation, the concentration and purity of RNA was determined by using a spectrophotometer (NanoDrop Technologies). cDNA was synthesized from RNA by taking 100 ng of RNA sample and reverse transcribing it by using a qScript cDNA synthesis kit (Quantabio) according to manufacturer's protocol. Synthesized cDNA was immediately processed for real-time RT-PCR using double-stranded DNA dye-SYBR green protocol. For SYBR green reaction 20 µM of forward and reverse primers were mixed together and added to 1µL of cDNA, iQ SYBR green supermix (Bio-Rad) and nuclease free water (Growcells). Primer sequences for the genes of interest are listed in Table 2. Primers for ACE2, ACE, AT_1R, and MasR were synthesized by Invitrogen. B-actin or GAPDH were used as internal housekeeping controls and all other genes were normalized to these controls by quantifying the *Ct* value of the gene of interest relative to the *Ct* value for control. Real-time PCR reactions were performed in 20µL reaction mixture with default thermal cycler conditions (95°C for 10 min (hot-start PCR), followed by 40 cycles of 95°C for 10 s (denaturation), 60°C for 20 s (annealing), and 72°C for 30 s (extension)) in a quantitative PCR system (Stratagene Mx3000P).

1.3.7. ACE and ACE2 enzyme activities

Cells were first lysed by using lysis buffer (20 mM Tris,100 mM EDTA and 0.5% Triton X-100 and a protease inhibitor cocktail (Thermo Fisher)). Protein concentration in the cell

lysates was determined by BCA (bicinchoninic acid) assay. Activities of two main enzymes of RAS: ACE and ACE2 in the cell-lysates and cell-supernatants were determined by using intramolecularly quenched enzyme-specific fluorogenic substrates (Mca-RPGFSAFK (Dnp)-OH and 7-McaYVADAPK (Dnp), respectively for ACE and ACE2;R&D Systems, Minneapolis) as described in our previous work (158). Recombinant human ACE (rhACE) and ACE2 (rhACE2) were used to plot the standard curve. Fluorescence activity was read using a plate reader at an ex:320 nm and em:405 nm. Enzyme-specific inhibitors, captopril for ACE and MLN-4760 for ACE2 were used to define enzyme activities, and enzyme-sensitive fluorescence was expressed as arbitrary fluorescence units/µg of protein/hr.

Table 2. List of primers used for real-time PCR studies

Gene (NCBI Reference)	**Primer Sequence**
ACE2- Human (NM_021804)	**Forward**: 5′- CATTGGAGCAAGTGTTGGATCTT -3′ **Reverse:** 5′- GAGCTAATGCATGCCATTCTCA -3′
ACE- Human (NM_000017)	**Forward:** 5′- GCAAGGAGGCAGGCTATGAG -3’ **Reverse:** 5′- CGGGTAAAACTGGAGGATGG -3’
AT_1R- Human (MIM 106165)	**Forward:** 5′-GACGCACAATGCTTGTAGCCA -3’ **Reverse:** 5′-CTGCAATTCTACAGTCACGTATG -3’
MasR- Human (MIM165180)	**Forward:** 5′-GAGGAGGATGAGGGTGTCTATAGGT-3′ **Reverse:** 5′-GTGATCAGCTCCAGGTTTGACTT-3′
β-actin-Human (MIM 102630)	**Forward:** 5′-GACAGGATGCAGAAGGAGATTACT -3’ **Reverse:** 5′-TGATCCACATCTGCTGGAAGGT -3’
GAPDH- Human (MIM138400)	**Forward:** 5’-GGATTTGGTCGTATTGGG-3’ **Reverse:** 5’-GGAAGATGGTGATGGGATT-3’
ACE2-Murine (BC026801)	**Forward:** 5′-GGATACCTACCCTTCCTACATCAGC-3′ **Reverse:** 5′-CTACCCCACATATCACCAAGCA-3′
ACE-Murine (BC040404)	**Forward:** 5′-CAGAATCTACTCCACT GGCAAGGT-3′ **Reverse:** 5′-TCGTGAGGAAGCCAGGATGT-3′
AT_1R-Murine (NM_177322)	**Forward:** 5’-CTT TTC TGG GTT GAG TTG GTC T-3’ **Reverse:** 5’-GGC TGG CAT TTT GTC TGG ATA-3’
MasR-Murine (NM_008552.5)	**Forward:** 5’- GTC CTC TAC TTG CTG TAC GAG -3’ **Reverse:** 5’- GTT GGC GCT GTT GAT G -3’
β-actin-Murine (NM-007393.5)	**Forward:** 5’- CCA TCA TGA AGT GTG ACG TTG -3’ **Reverse:** 5’- CAA TGA TCT TGA TCT TCA TGG TG -3’

1.3.8. Western blotting

Cells and cell supernatants were collected and frozen at -80 °C after exposure to normoxic or hypoxic conditions. For western blotting cell pellets were thawed and were lysed in radioimmuno precipitation assay (RIPA) buffer (Tris (pH 7.4) 10 mM, NaCl 140 mM, EDTA 1 mM, NaF 1 mM, SDS 0.10%, sodium deoxycholate 0.50%, NP-40 0.1%, Triton X-100 1%, and protease inhibitor cocktail (Thermo Fisher)). Protein concentrations in cell lysates and cell supernatants were determined by BCA with bovine serum albumin (BSA, Thermo Fisher) as a standard. Loading buffer (Amresco) with equal volumes of cell lysates and cell supernatants were mixed and boiled for 5 min in an analog heat block containing boiling water. 30µg of protein samples were loaded on 10% pre-cased gels (SurePage, Genescript) and proteins were separated by using SDS-PAGE electrophoresis at 100V until dye-front reaches the end of the gel. After separation, proteins were electroblotted to nitrocellulose membranes (Bio-Rad) by using a wet-transfer method at 300mA for 90 minutes. The blot was blocked using 5% (w/v) dried milk in Tris-buffered saline containing 0.5% (v/v) Tween-20 for 1 hour with gentle agitation on plate rocker. Membranes were then washed and incubated with the primary antibody which recognizes an epitope within ACE2 ectodomain (ab87436; Abcam), an epitope within the cytoplasmic tail (ab15348; Abcam), and domain non-specific antibody (sc-20998; Santa Cruz Biotechnology). Membranes were also incubated with the primary antibody for an epitope within the ectodomain of ACE (ab28311; Abcam), an epitope within the cytoplasmic tail of ACE (GTX11737; Genetex), Mas receptor (SC-135063; Santa Cruz Biotechnology), and AT1 receptor (ab124734; Abcam). B-actin (mab8929; R&D Systems) was used as a loading control for cell lysates. Memcode (Thermo Fisher) was used to stain the membrane to verify equal protein loading on membrane and quantify the total protein per lane. Membranes were incubated with primary

antibodies overnight at 4°C on a rocker set at low speed. Secondary antibodies HRP-conjugated goat anti-mouse IgGs (405306; Biolegend) and donkey anti-rabbit IgGs (406401; Biolegend) were diluted to 1:20,000 and membranes were incubated with them for 1 hour with gentle rocking at room temperature. Primary antibodies were diluted in 5% milk in TBST (Tris-buffered saline containing (0.5% v/v) Tween-20) and secondary antibodies were diluted in TBST. Protein bands on membranes were visualized by using enhanced chemiluminescence reagent (ECL, K15045-D50; Advansta) according to manufacturer's instructions and developed on X-ray films (Phenix research products). Intensities of protein bands were quantified using Image J software (NIH).

1.3.9. Flow cytometry for protein expression

Flow cytometry was used to quantify the expression of MasR and AT_1R on cells (Accuri C6, BD Biosciences). Freshly isolated cells were first treated with FcR blocking reagent (Miltenyi Biotech) for 5 min at 4°C and washed with cell-staining buffer (Biolegend). The cells were then washed (x3) with washing buffer by centrifugation at 120 g for 10 minutes. The antibodies for MasR and AT_1R were fluorescently conjugated using Zenon-Alexa Fluor 488 Rabbit IgG labelling kit (Z-25302; ThermoFisher) for MasR antibody (SC-54682; Santa Cruz Biotechnology) and Zenon-R-Phycoerythrin Rabbit IgG labeling kit (Z-25355; ThermoFisher) for AT_1R antibody (ab124734; Abcam) as per the manufacturer's protocol. Briefly, l µg of primary antibodies was mixed with the Zenon human IgG labelling reagent followed by blocking the reaction with Zenon blocking reagent at room temperature for 10 min. Antibody-Zenon labelling complexes were applied to cells and incubated for 30 minutes at 4°C and fluorescence was measured using flow cytometer (Accuri C6, BD Biosciences).

1.3.10. Lentiviral transduction of cells using luciferase vector and micro RNA

We used a luciferase reporter assay to assess the effect of hypoxia on transcriptional regulation of cells. Lentiviral (Lv) vectors containing luciferase reporter for ACE2, MasR and their respective control vectors were custom made by Applied Biological Materials Inc., (Richmond, BC, Canada). The pGL3-Basic vector backbone was used as backbone vector and the vector maps for control and ACE2/MasR Lv vectors are shown in Fig. 10. Human ACE2 or MasR promoters were cloned into the backbone vectors between SacI(5') and BgIII(3') for ACE2 and between NotI(5') and XhoI(3') for MasR. Cloned sequences for ACE2/MasR were then inserted into multiple cloning site (MCS) using restriction enzymes and sub-cloned into pLenti-luciferase-UTR (driven by *firefly* luciferase) reporter vector under the control of cytomegalovirus (CMV) and SV40 enhancer promoter. Promoter regions for ACE2 was -1119/+103 and for MasR was -974/+160 (numbers indicate bases upstream of start codon). Putative HIF1α binding sites and hypoxia response elements (HREs) were in the promoter region of ACE2 at -1541/-1537, -870/-866, +543/+547, +487/+491 and +149/+153, whereas in human MasR promoter three putative sites were at -921/-917, -340/-335 and +187/+191. Control-Lv vector containing a scrambled gene sequence with CMV-driven expression of *firefly* luciferase was used as a control in all experiments.

To further confirm selectivity of hypoxic preconditioning in regulating ACE2 and MasR, we performed a luciferase reporter assay by using a Lv vector that consists of a Micro RNA (miR) that selectively downregulates either ACE2 or MasR expression in response to hypoxia. Based on sequence alignment analysis (blast-NCBI-NIH) and by using binding prediction algorithms for miR, we selected miR-421 for ACE2 and miR-143 for MasR. Putative binding

sites for miR-421 and, miR-143 in the 3' -UTR of the ACE2 and MasR transcript, respectively, were revealed by target sequence alignment analysis (Fig. 11).

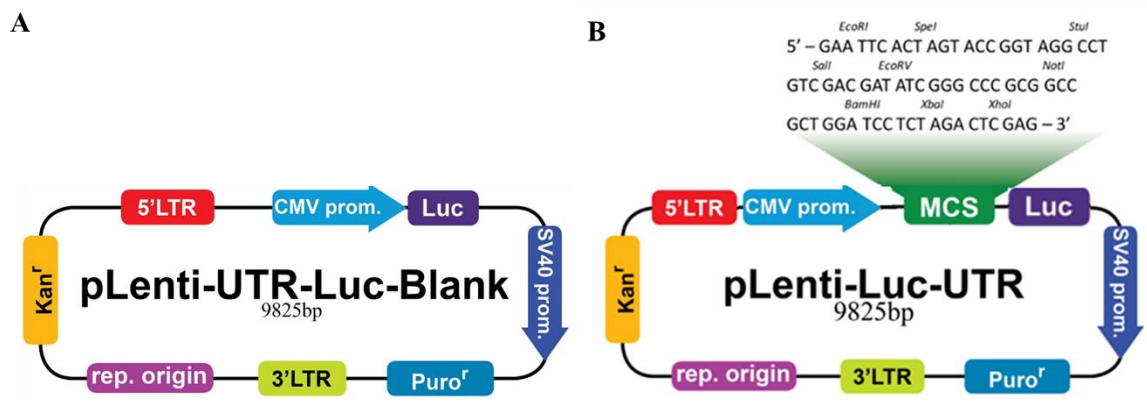

Figure 10. Map of the lentiviral vectors expressing luciferase and genes of interest. *A.* Map of the lentiviral construct with the location of luciferase gene. *B.* location of ACE2 or MasR with respect to luciferase gene, under the control of cytomegalovirus (CMV) promoter. Adapted from applied biological materials, 3' UTR reporters.

CMV driven-Lv vectors that were described above were redesigned to contain both pre-miRNA and luciferase inserts under the same CMV promoter with the termination site for transcription process after pre-miRNA. Thus when the pre-miR was expressed in response to hypoxia, it would prevent the expression of either ACE2 or MasR.

Figure 11. Target sequence prediction for miRNAs. Targets of *A.* miRNA-421 and *B.* miRNA-143 identified by carrying out sequence alignment analysis (blast-NCBI-NIH). Binding sites for miRNA-421 and miRNA-143 were found in the 3'-UTR of ACE2 and MasR transcripts, respectively.

Transduction of Lv was carried by using Lipofecatmine^{TM} 3000 (Invitrogen), according to manufacturer's instructions. After 48-hr of transduction, cells were collected and the excess

Lv particles and lipofectamine were removed by diluting with PBS and centrifugation at 120g. Then, the cells are subjected to hypoxia or normoxia as described above. Luciferase activity after transduction and hypoxic/normoxic exposure was measured using a luciferase assay kit (G287; Applied Biological Materials) as per manufacturer's instructions. Briefly, cells were harvested and washed and transferred in the micro well plate and luminescence was measured by using SpectraMax luminometer (Molecular Devices) at excitation of 480 and emission of 560 nm.

1.3.11. Isolation and enumeration of bone marrow lin$^-$c-kit$^+$ (LK) cells from mice

C57Bl/6 NHsd (wild type) (Envigo) were used in this study. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at North Dakota State University. All experiments were carried out in accordance with guidelines and regulations approved by IACUC. Mice were euthanized after isoflurane (Terrell, USA) anesthesia by cardiac puncture and intra cardiac bleeding. After euthanizing the mice, both tibias and femurs from hind limbs were collected in phosphate buffered saline (PBS). Tips of the bones were cut, and marrow was flushed using ice-cold PBS and filtered through 40 µM filter to remove the bone fragments and other contaminant particles and get mono cell suspension. After filtration, erythrocytes were lysed using 0.8% ammonium chloride with 2 mM EDTA and cell were then washed x3 using PBS to get bone marrow MNCs. Cells were counted using trypan blue dye and further processed for isolation of lin$^-$ cells using negative selection immunomagnetic enrichment kit (Stemcell Technologies Inc.). Similar to the procedure described above for human lin$^-$ cells, bone marrow MNCs were suspended in the recommended medium by manufacturer at 1 X10^8 cells/ mL and incubated with cocktail containing antibodies for CD5, CD11b, CD19, CD45R, 7–4, Ly-6G/C (Gr-1), and TER119.

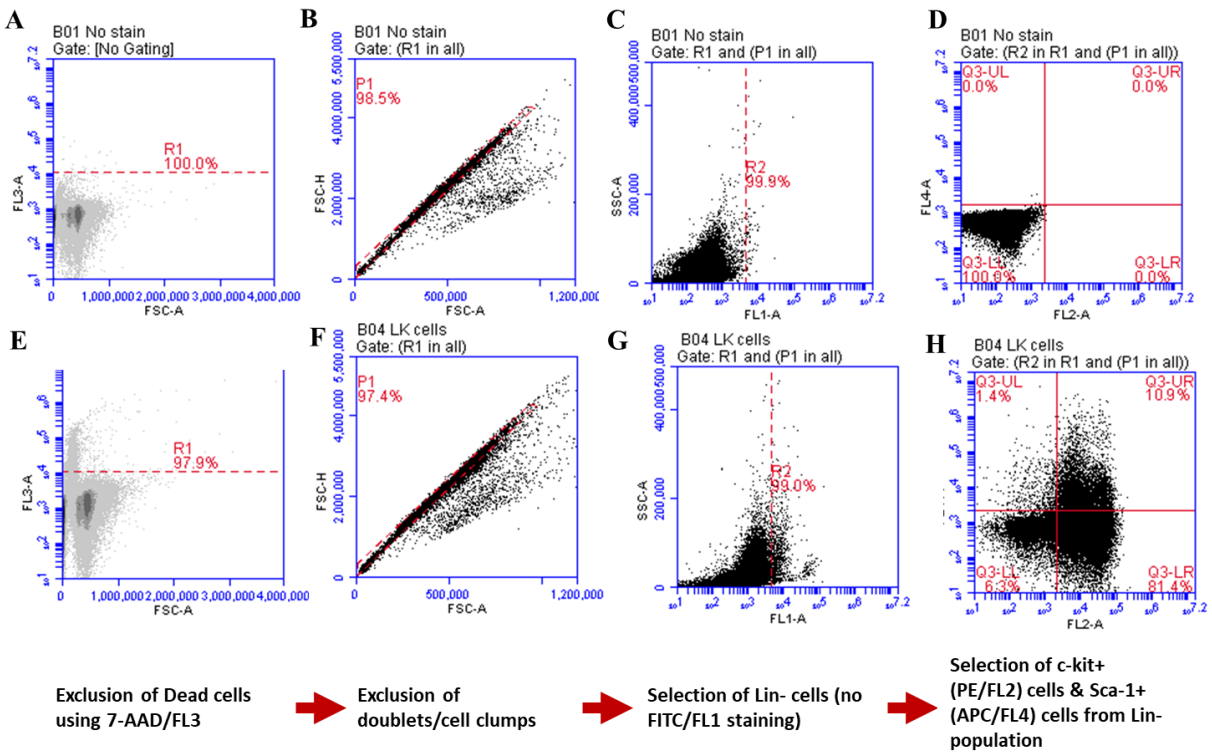

Figure 12. Representative dot plots of flow cytometric analysis of LK cells. Cells are enriched by immunomagnetic selection as described in the text. *A-D*. Sample treated with isotype controls, and *E-H*. sample treated with fluorescent-conjugated antibodies. Sequential gating strategy involves exclusion of dead cells (stained with 7-AAD), exclusion of doublets or cell clumps (area vs height), selection of Lin− cells (exclusion of differentiated cells, stained with FITC) and then selection of LK cells (stained with PE and APC).

These cells were called lin^+ cells and were then labeled by magnetic tetrameric antibody complexes that recognize biotin and dextran-coated magnetic particles. Lin^+ cells which were now bound to magnetic particles were then separated by using EasySep magnet by negative selection, thus giving single cell suspension containing only lin^- cells. This whole procedure of lin- cells isolation was performed at 4 °C. These lin^- were subsequently enriched for c-Kit^+ cells by using a positive immunomagnetic selection kit (Stemcell Technologies). Purity of isolated LK cells was confirmed using flow cytometry as per the method described before (170) (Fig. 12). LK cells were plated in Roswell Park Memorial Institute (RPMI) 1640

media (GE Healthcare) in round bottom, 96-well plate at 30,000 cells/200 µL per well. These plates were then kept in normoxic or hypoxic conditions as described above. After exposure to nomoxia/hypoxia, cells were used in *in vitro* assays for migration or proliferation, or cells and cell supernatants were used for qRT-PCR or Western blotting.

1.3.12. Mouse hind-limb ischemia

Hind-limb ischemia (HLI) surgery was carried out in mice under isoflurane (Terrell, USA) anesthesia as described before by Vasam et al. (170) (Fig. 13). After depilation and disinfection of hindlimbs, a small incision was made using a surgical scissor in the skin of right inguinal region. Femoral artery was separate from neurovascular bundle, ligated and cut at proximal and distal ends with 7-0 silk-braided suture. The incision was closed with 7-0 vicryl suture and mice were allowed to recover. Peripheral blood was collected under anesthesia on day 2 following HLI and circulating Lin^- cells were isolated by using cell isolation kit (StemCell Technologies).

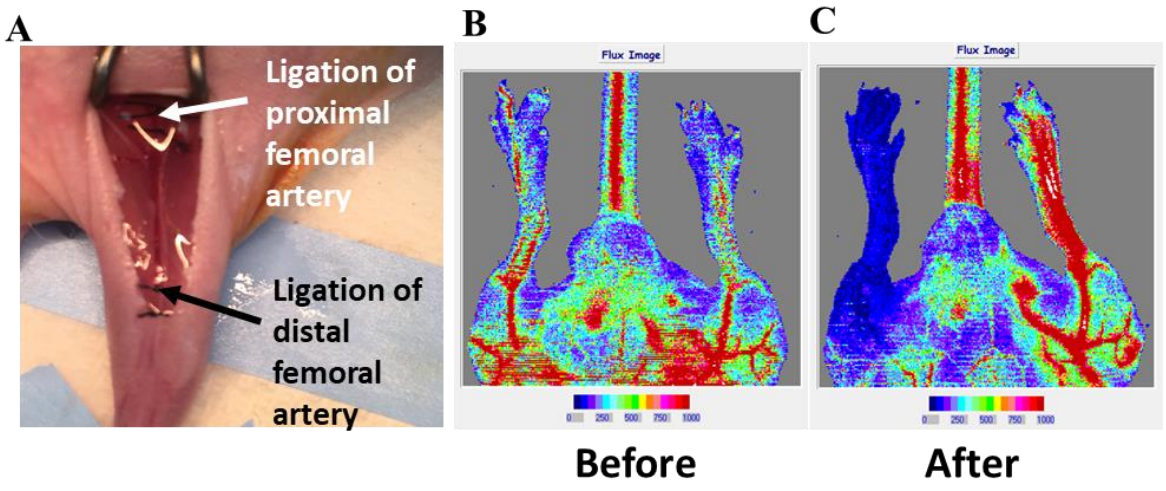

Figure 13. Femoral artery location in hind limb and the sites for ligation and excision. *A*: Location of femoral artery in the hind limb ligated sites on the femoral artery after the surgery. *B* and *C.* Blood flow in the hind limb monitored using laser doppler imaging showing blood flow before and after surgery.

Cells were tested for expression of ACE, ACE2, AT_1R and MasR, and activities of ACE and ACE2. Protein expression of ACE2 and MasR were detected by using flow cytometry. Primary antibody of ACE2 (ab87436; abcam) was labelled with Zenon Rabbit IgG Allophycocynin labelling kit (Z-25351; ThermoFisher), whereas primary antibody for MasR (SC-390453; Santa Cruz Biotechnology) was labelled with Zenon Alexa Fluor 488 labelling kit (Z-25002; ThermoFisher).

Some of the preliminary studies were performed in MasR-KO (Knock Out) mice. Wild type (WT) and MasR-KO mice of C57BL/6 background were generated by the KOMP (trans-NIH Knock-Out Mouse Project) and obtained from the KOMP repository. These mice were in bred in the animal facility of the Department of Pharmaceutical Sciences, College of Health Profession at NDSU.

1.3.13. Low-intensity blood flow restriction exercise (LI-BFR)

1.3.13.1. Characteristics of participants

Healthy young male participants aged 22-26 years were recruited in the study (Table 3: participant characteristics). This study was designed as within subject-crossover trial. Participants were experienced with both resistance and cardiovascular exercise and exercised minimum 3 days per week for the past 6 months. This study was approved by the Institutional Review Board of North Dakota State University (IRB#HE18137). This study was done in collaboration with Dr. Kyle Hackney in the Department of Health, Nutrition, and Exercise Sciences at NDSU.

Table 3. Participant's characteristics

Parameter	**mean ± SD**
Age (years) (n=5)	22 ± 1.58
Height (cm)	176.16 ± 7.40
Weight (kg)	73.884 ± 9.95
BMI (kg/m^2)	23.136 ± 2.86
Limb circumference (cm)	55.1 ± 4.14
Systolic blood pressure (mmHg)	118.8 ± 7.29
Diastolic blood pressure (mmHg)	75 ± 7.55

1.3.13.2. Inclusion/exclusion criteria

Participants completed a physical activity readiness questionnaire (PAR-Q), health history surveys and provided written informed consents. Our health-questionnaires also determined that participants were free of clinical conditions such as history of cancer, stroke, musculoskeletal, cardiovascular, neuromuscular or other disease. Participants were having low or free of any underlying deep vein thrombosis risk factors (0-1 risk factors), or medications or supplements that would increase the risk of an adverse event. We excluded participants having the history of blood clotting disorders, exertional rhabdomyolysis, who bruised easily. Participants were also free from any injuries to the lower body that could interfere with the exercise sessions.

1.3.13.3. Study design

1.3.13.3.1. Familiarization session

In this visit, subjects completed a formalization/informational session where descriptive measurements such as height, weight and blood pressure were taken (Table 3). Subjects were familiarized with BFR exercise. The participants were instructed on the correct technique for

performing exercise with BFR cuff. The familiarization session was conducted 48 hours before starting the experimental session.

1.3.13.3.2. Exercise session

Low-intensity (LI) blood flow restriction exercise (LI-BFR) was carried out by using Delphi personalized tourniquet system (PTS) (Delphi Medical Innovations Inc., Vancouver, BC, Canada). After 48 hours of recovery without other exercise, subjects were randomized to complete either the control session first followed by LI-BFR exercise sessions or vice-versa. Protocol consisted of four sets knee extension-flexion exercise (30/15/15/15 repetitions) with a 30 second rest between sets of knee extension and flexion. The intensity of knee extension and flexion exercise was set at 10% of maximal strength of participants' (one repeat maximum (1 RM)) by using Biodex 4 dynamometer in Control (LI-free blood flow) and BFR (LI-80% total limb occlusion pressure). To determine total limb occlusion pressure, the cuff pressure was slowly increased until there was no blood flow and then submaximal of that pressure (80%) was set for the LI-BFR exercise session. Estimated total limb occlusion pressure was 216±36 mmHg and LI-BFR exercise pressure at 80% of limb occlusion pressure was 172±29 mmHg. One repetition maximum strength testing consisted of several submaximal attempts to get used to the weight being lifted, followed by increase of 5-10 pounds until subject could no longer lift the weight. BFR cuff remained inflated for all exercise sets and during the 30 seconds rest period but was deflated while transitioning between exercises. There was a two-minute transition between the exercise with the cuff deflate. Each participant had undergone control and BFR sessions at one-week interval and 50 mL of blood samples were collected 30 minutes after undergoing each session. Blood draws were performed by a trained nurse in the Student Health Services at North

Dakota State University. Muscle oxygen saturation was determined by using a non-invasive muscle oxygen (MOXY) sensor using the technique of near infrared spectroscopy. The MOXY sensor were placed on the muscle of participant and secured with the tape (Fig. 14). Heart rate was monitored by using Bluetooth HR monitor. The general setup of an individual participating in this study is shown in Fig. 14 (with permission from participant). Blood collected from each participant at the end of either control or BFR session was taken to the research lab and processed for enumeration of $Lin^{-}CD45^{low}CD34^{+}$ cells as described above in the section 1.3.2. Representative flow cytometric dot plots for enumeration of $Lin^{-}CD45^{low}CD34^{+}$ for control and BFR session are shown in Fig. 15. Isolated cells were also analyzed for ACE and ACE2 enzyme activities (section 1.3.7), mRNA (section 1.3.6) and protein expression (section 1.3.8) and plasma samples were analyzed for SDF and VEGF ELISA assay.

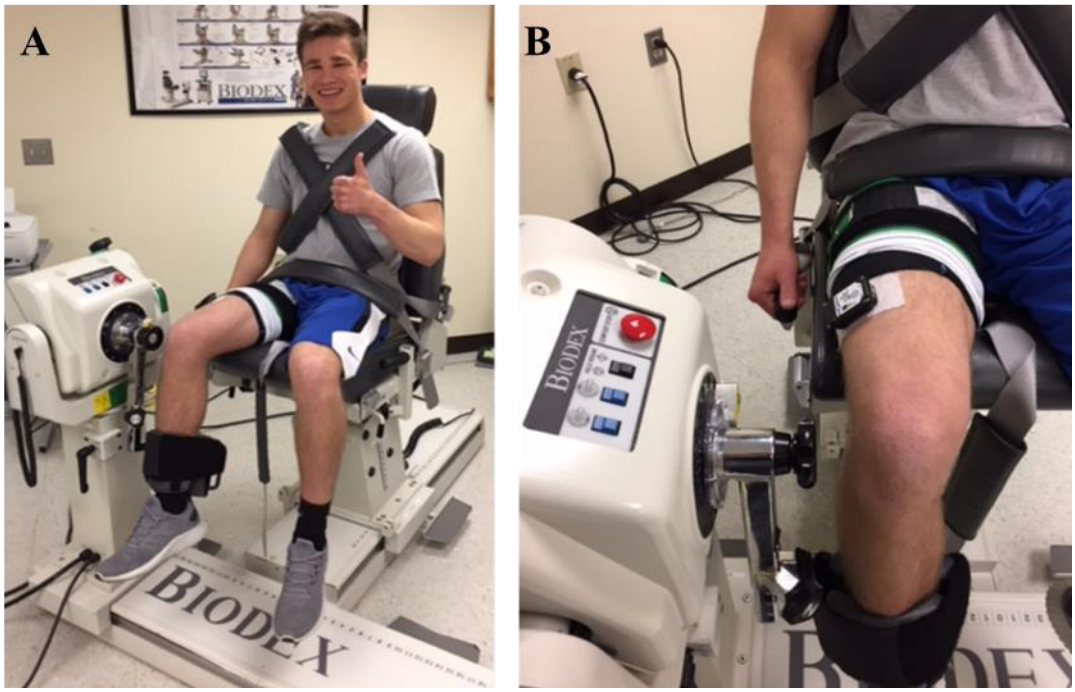

Figure 14. General **s**etup of participant on Biodex. *A* (Left)- General setup of participant on Biodex, *B.* Specific setup of MOXY sensor on vastus lateralis located distal to Delphi cuff inflation on upper right thigh. (shown with permission from the participant).

1.3.14. Biochemical analysis

Human plasma SDF and VEGF were measured by using Quantikine ELISA kit (R&D Systems) as per the manufacturer's protocol. Briefly, standard curves for human SDF/VEGF were first created by serially diluting the supplied reference standard. Protein concentration in the plasma samples was measured using BCA protein assay and then samples were loaded in the ELISA plates along with standards and substrate solution. Fluorescence signal was measured using a plate reader at excitation of 540 nm and emission of 570 nm.

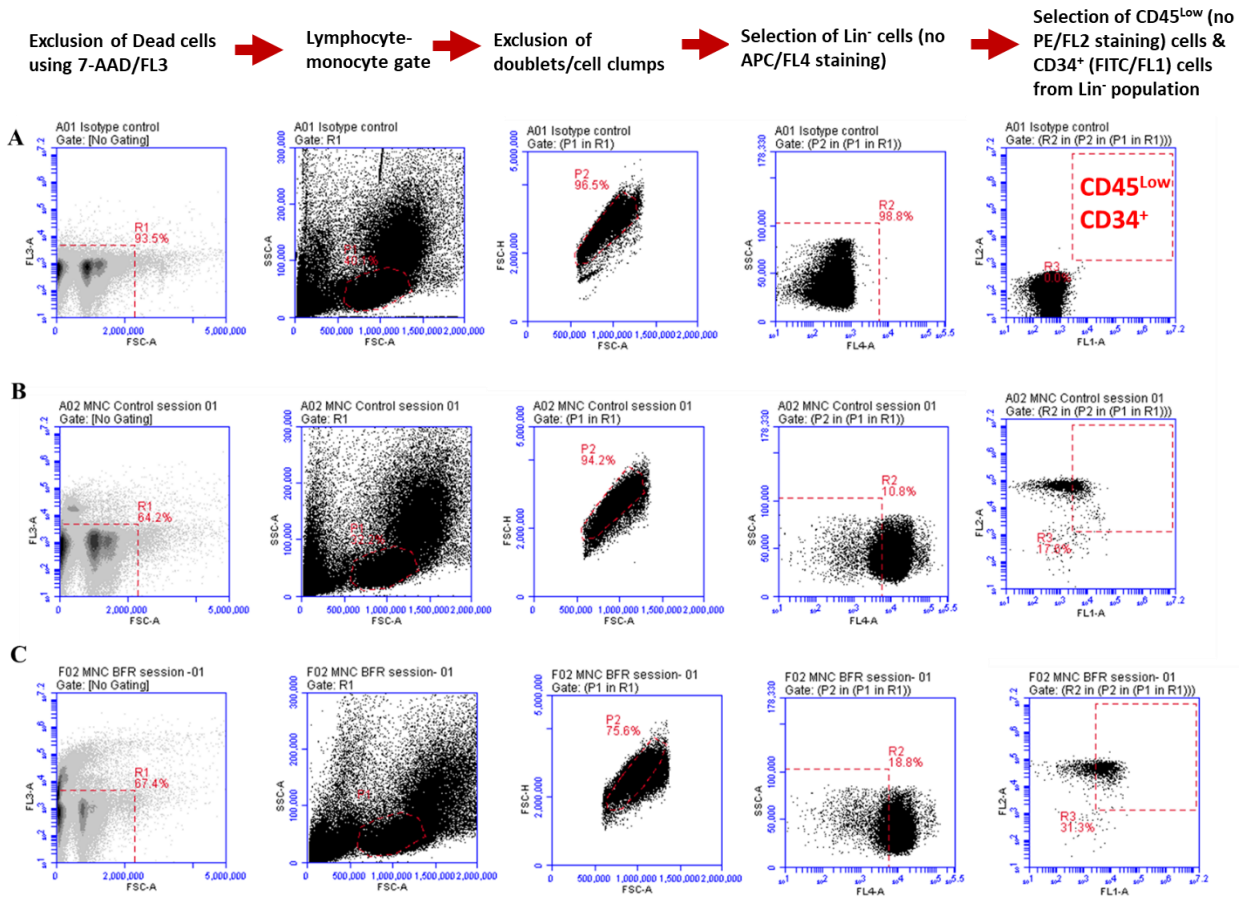

Figure 15. Gating strategy used for flow cytometric enumeration of human Lin$^-$CD45^{low}CD34$^+$ cells. *A* Sample with isotype control, *B* and *C.* Samples from control session and BFR session with fluorescent conjugated antibodies, respectively.

1.3.15. Statistical analysis

Data are presented as mean values ± standard error of mean. Paired t-test was used to analyze results obtained from two treatment groups in a study whereas two-way analysis of variance (two-way ANOVA) was used when paired comparisons were performed over time. Number of experiments 'n' represent the number of donors used in the experiment or number of mice in the experimental group. For the analysis of results obtained from different subjects between two or more different treatment groups unpaired student t-test or one-way analysis of variance (one-way ANOVA) was used. $P<0.05$ was considered as statistically significant and significance values are mentioned in the results. Whenever ANOVA results showed significance in the results, post- hoc analysis was done by using Tukey's test or Bonferroni's correction for one-way and two-way ANOVA, respectively. Statistical analyses were performed using GraphPad InStat 3.0 (GraphPad Software, San Diego, CA). Minitab software (Minitab 17; PA USA) was used to do power analysis to confirm that the sample size of the study was suitable to achieve the statistical significance of 0.05 and analysis was carried out post hoc. All experiments were powered at 80% or higher and effect size was 0.8.

CHAPTER 2. HYPOXIC REGULATION OF ANGIOTENSIN-CONVERTING ENZYME 2 IN HSPCS

2.1. Rationale and preliminary results

We tested if hypoxic preconditioning induced stimulation of reparative potential of HSPCs is regulated by activation of RAS. Previous studies in different cell types have reported increased paracrine protection and proliferation of cells in response to hypoxia. Migration and proliferation are the hallmark signatures of vasoreparative functions of HSPCs. Hypoxia-regulated vasculogenic/angiogenic factors, SDF or VEGF are known to induce migration and proliferation of HSPCs, thus causes recruitment of HSPCs to the areas of ischemia (39,171–173). As mentioned in the previous chapter, activation of ACE2/Ang-(1-7)/MasR axis was shown to stimulate vascular repair-relevant functions of HSPCs. However, convergence of these two distinct strategies, both of which are enhancing the vasoreparative functions of HSPCs was never reported so far. For the first time, we studied the mechanism behind the hypoxic regulation of ACE2 and MasR in HSPCs derived from human and mice. Additionally, shedding of catalytically active form of ACE2 was demonstrated in other cell types. Increased ACE2 in circulation generates vasoprotective Ang-(1-7), which by activating MasR on HSPCs, stimulates vasculogenesis and angiogenesis. This study is the first of its kind to test and identify hypoxia mediated shedding of ACE2 in HSPCs with therapeutic significance. In specific aim 1, we sought to find out the effect of hypoxia on regulation of key members of RAS, ACE and ACE2. We investigated whether hypoxia upregulates the protective ACE2 under *in vitro* conditions. Further, if hypoxic regulation was observed, we investigated the mechanism involved in the gene regulation.

2.1.1. Hypoxia stimulates migration and proliferation functions of HSPCs

Hypoxic exposure (1% O_2) robustly stimulated proliferation of human $CD34^+$ cells as determined by BrdU incorporation assay, in basal conditions ($P<0.05$, n=8) or in response to hypoxia-regulated factors SDF (100 nM, $P<0.01$, n=8) or VEGF (25 nM, $P<0.01$, n=8) (Fig. 16*A*). More profound effects of hypoxia was observed on migratory response of $CD34^+$ cells (Fig. 16*B*). Migration of $CD34^+$ cells was significantly increased compared to normoxic cells in basal, and SDF- or VEGF-stimulated conditions ($P<0.05$, n=8).

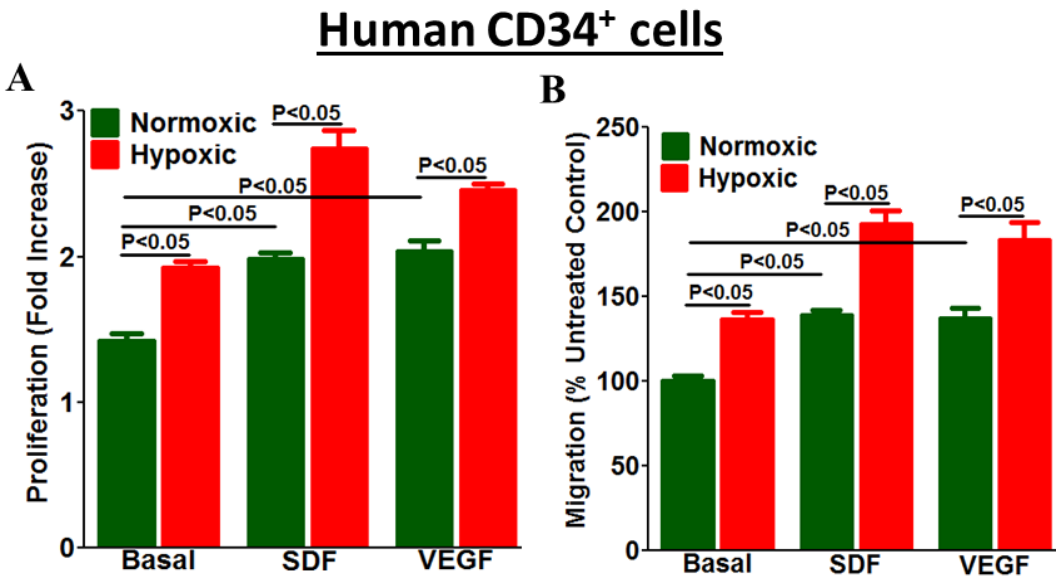

Figure 16. Hypoxic potentiation of proliferation and migration of human $CD34^+$ cells. *A.* Hypoxia increased proliferation in basal, and in SDF- or VEGF-stimulated conditions compared to proliferation in normoxic conditions. ($P<0.05$, n=8). Read out is expressed as fold-increase compared to mitomycin -treated cells. *B.* Exposure to hypoxia increased migration response in both basal conditions, and in response to SDF or VEGF. ($P<0.05$, n=8). Migratory response in cells expressed as percent increase over baseline response in untreated cells in normoxic conditions.

Importantly, hypoxic stimulation of migration and proliferation was not specific for human HSPCs but was also observed in murine LK cells. Hypoxia also robustly increased proliferation and migration of murine LK cells in basal conditions ($P<0.05$ for proliferation, $P<0.01$ for migration, n=8) or in response to SDF or VEGF ($P<0.01$, n=8) compared to cells

which were exposed to normoxic environment (Fig. 17*A* and *B*). These results confirm the role of hypoxic potentiation of key vascular reparative functions of HSPCs.

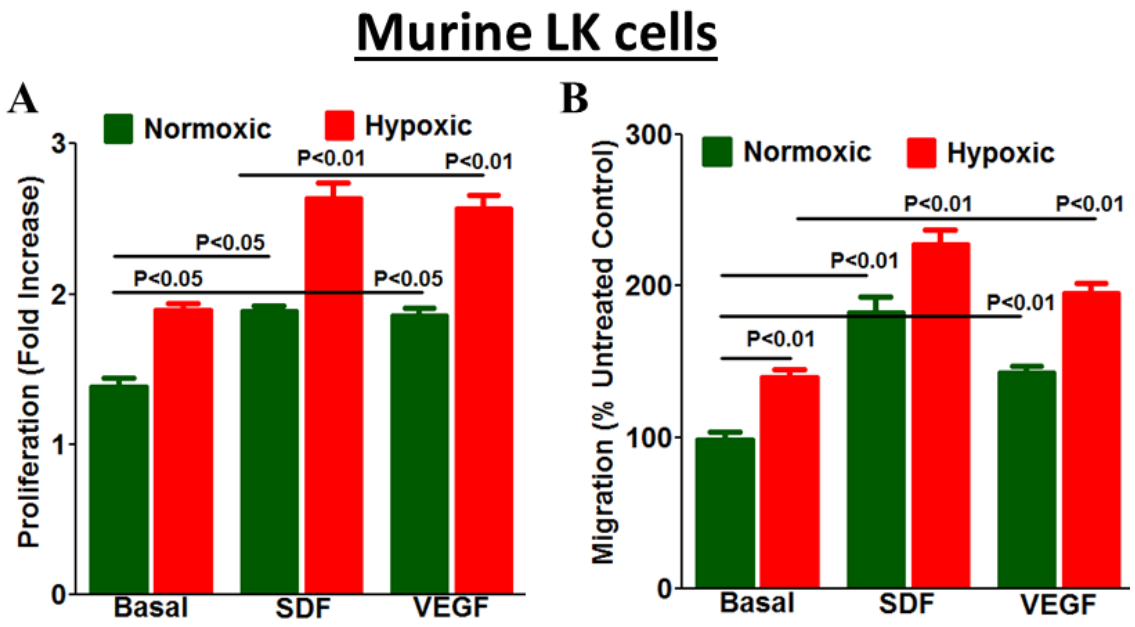

Figure 17. Hypoxic potentiation of proliferation and migration of murine LK cells. *A.* Hypoxia increased proliferation in basal, and in SDF- or VEGF-stimulated conditions compared to proliferation in normoxic conditions. (P<0.05 for basal, P<0.01 for SDF-/VEGF-, n=8). *B.* Exposure to hypoxia increased migration of LK cells in basal conditions, and in response to SDF or VEGF. (P<0.01, n=8).

2.1.2. Hypoxia increases mRNA expression of ACE2 in HSPCs

We measured the effect of hypoxic exposure (12-hour) on ACE2 and ACE mRNA in HSPC lysates. Hypoxia upregulated transcription of ACE2 as indicated by lower Δ*Ct* values for hypoxia treated cells as compared to normoxia (P<0.002, n= 8) (Fig. 18*A*). Expression of ACE messenger RNA transcript was unchanged in either normoxia or hypoxia conditions. Along similar lines, mRNA expression for ACE2 was higher (indicated by lower Δ*Ct* values) in murine LK cells incubated in hypoxia than in normoxia (P<0.01, n=6) (Fig. 18*B*). This indicates the effects of hypoxia in transcriptional stimulation of ACE2.

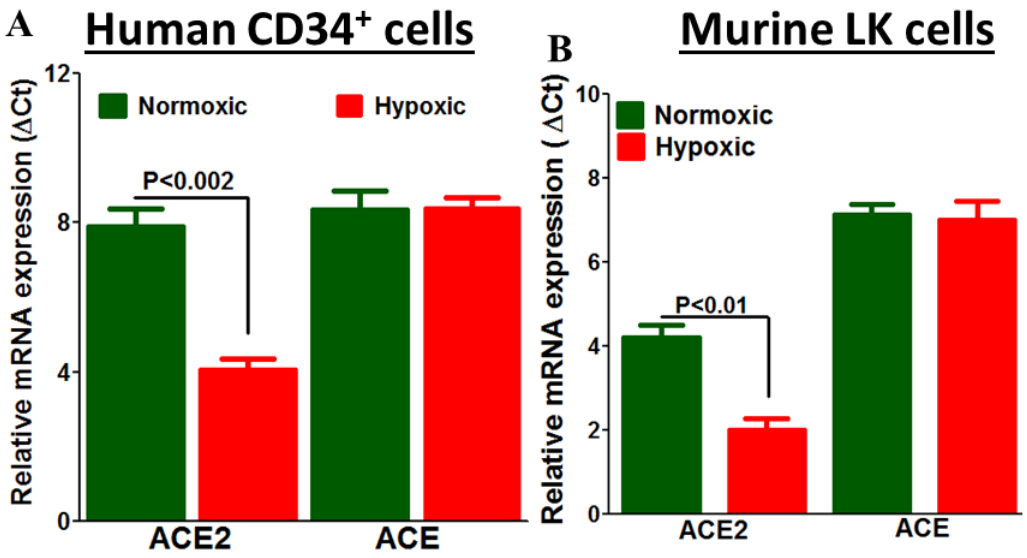

Figure 18. Hypoxia increases ACE2 but not ACE mRNA expression in human $CD34^+$ and murine LK cells**:** *A*. Hypoxia enhanced ACE2 mRNA in $CD34^+$ cell lysate (indicated by low ΔCt values) ($P<0.002$, n=8) and *B*. in murine LK cells ($P<0.01$, n=7).

2.1.3. Hypoxia increases activity and protein expression of ACE2 but not ACE in HSPCs

We measured the effect of hypoxic exposure (12-hour) on the functional activities of ACE2 and ACE in HSPC lysates. Hypoxia enhanced the ACE2 activity in both human $CD34^+$ cells ($P<0.01$, n =8) and murine LK cells ($P<0.05$, n =7) as compared to normoxia (Fig. 19*A* and *B*). However, hypoxia did not the alter vaso-deleterious ACE activity in both the cell types. This indicates the effects of hypoxia in increasing the activity of vascular protective ACE2. Initially, we performed preliminary experiments to determine the optimum level and duration of hypoxia by taking ACE2 activity as a readout signal in human $CD34^+$ cells. We tested different levels of oxygen by keeping the duration of exposure time constant, starting with normoxia (20% O_2), and then decreasing oxygen levels to achieve 10%, 5%, 3%, 1% and 0.1% O2 inside the hypoxia chamber. We collected cells from each treatment conditions. ACE2 activity gradually increased with decreasing levels of oxygen and 1% and 0.1% O_2 levels showed the highest level of ACE2 activity after 12-hour exposure (Fig. 20*A*).

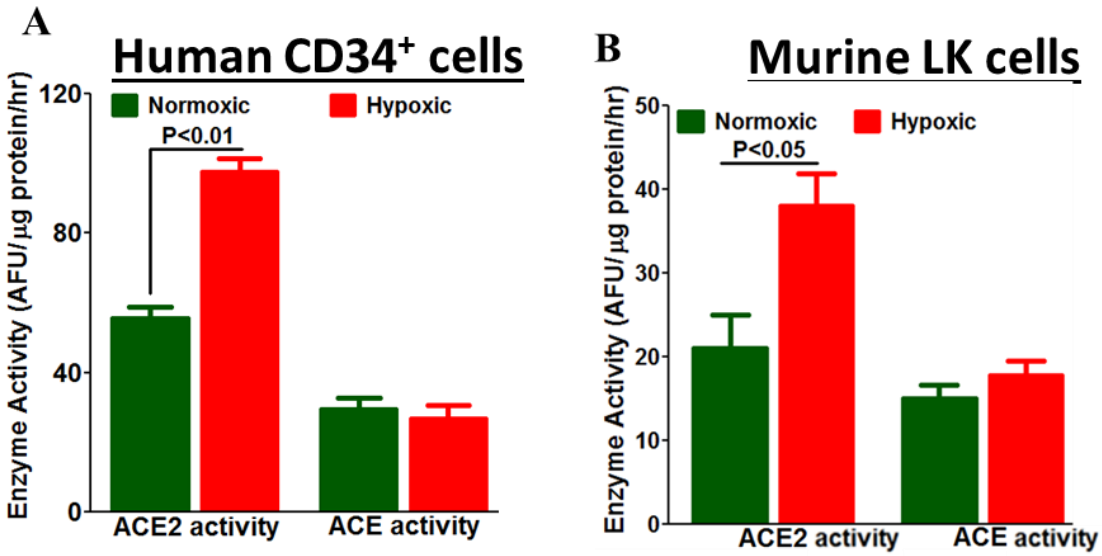

Figure 19. Hypoxia increases ACE2 but not ACE activity in human $CD34^+$ and murine LK cells. *A.* Hypoxia enhanced ACE2 activity in $CD34^+$ cell lysate ($P<0.01$, n=8) and *B.* in murine LK cells ($P<0.05$, n=7). Enzyme activities are defined as captopril- or MLN-4760- inhibitable fluorescence for ACE and ACE2, respectively, in assays with the respective fluorogenic substrates.

Similarly, we tested the effect of different durations of hypoxia (0, 3, 6, 12 and 24 h) on ACE2 activity in $CD34^+$ cell lysates. Among different duration of exposure the effect of hypoxia was similar on ACE2 activity at 12 h and 24 h (Fig. 20*B*). To further confirm the duration of hypoxic exposure, we performed Western blot analysis on $CD34^+$ cell lysates after harvesting them at different time intervals and measuring the ACE2 protein levels. This study revealed that ACE2 protein was enhanced on $CD34^+$ cells after hypoxic exposure and that this up-regulation was highest at 12 h (Fig. 20*C* and *D*). Based on these preliminary studies 1% O_2 for 12 h was considered to be an optimum condition and the rest of the studies of hypoxic exposure were carried out in this condition.

Importantly, hypoxic exposure of HSPCs increased the protein expression of only ACE2 but not ACE. This effect was observed in both human $CD34^+$ (Fig 21*A* and *B*) and murine LK

cells (Fig 21*C* and *D*), where band intensities for ACE2 were significantly higher after hypoxic exposure but were unchanged for ACE.

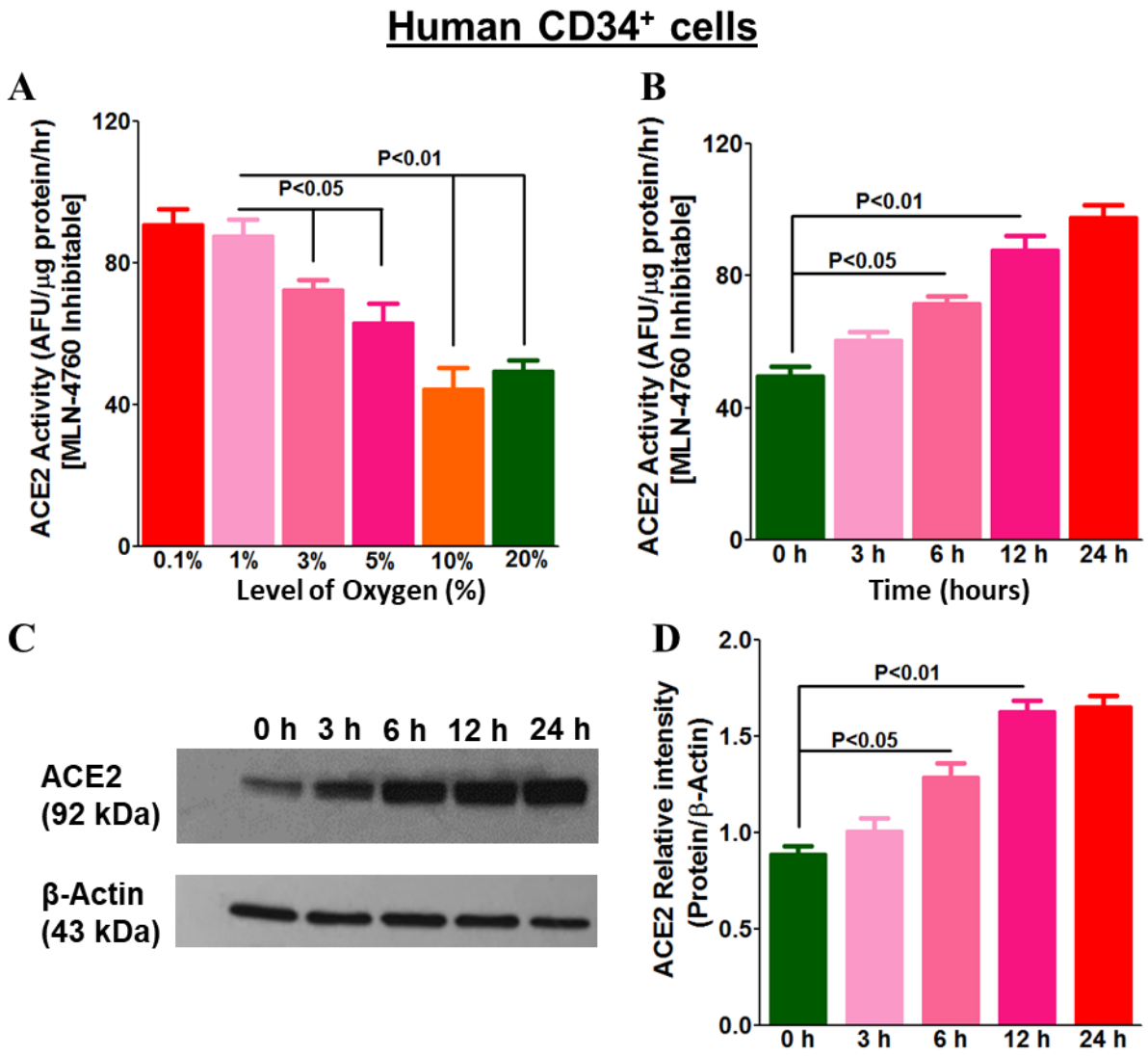

Figure 20. Determination of the optimal level and duration of hypoxia for ACE2 upregulation. *A.* Effects of normoxia (20% O_2) and incremental levels of hypoxia on the ACE2 activity in human $CD34^+$ cells. ACE2 activity is similar at 0.1% and 1% hypoxia, which is higher than that observed at 3-10% hypoxia or normoxia (n=5). *B.* Effects of different durations of hypoxia (1%) on ACE2 activity. Activity is similar at 12 and 24 h exposure and is higher than that observed at 0-6 hrs of exposure (n=5). *C* and *D*. Effects of different durations of hypoxia (1%) on the ACE2 protein levels in human $CD34^+$ cells. Protein expression is similar at 12 and 24 h exposure and is higher than that observed at 0-6 hrs of exposure (n=5).

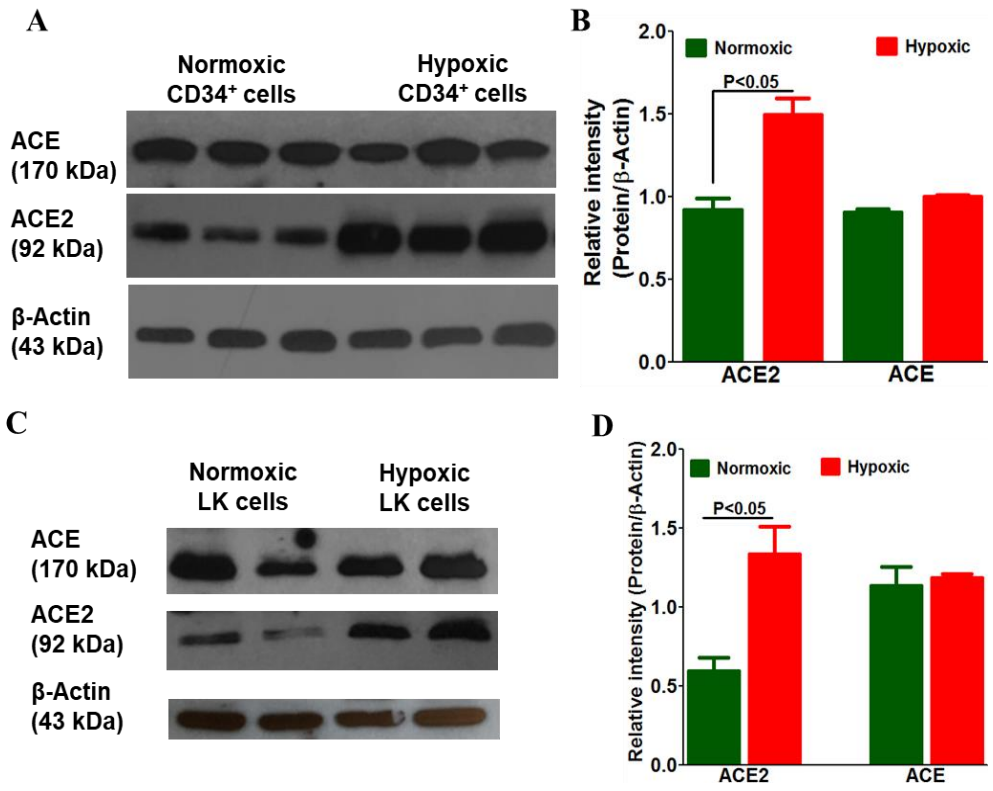

Figure 21. Western blot analysis showing hypoxia increased protein expression of ACE2 in HSPCs but not ACE. *A.* ACE2 protein bands and *B.* bar graph showing densitometric measurement of protein bands relative to β-actin. Band intensity was analyzed using image J. ACE2 band intensity were higher in hypoxia-exposed human $CD34^+$ cells compared to normoxic cells ($P<0.05$, n=6). *C* and *D.* Hypoxic increase in ACE2 protein band and intensity in murine LK cells. ($P<0.05$, n=5)

These studies provided strong evidence that hypoxia stimulates the vascular repair-relevant functions of HSPCs and that this increase is only associated with the increase in vasoprotective member of RAS–ACE2, and not deleterious member of RAS–ACE.

2.1.4. Hypoxia increases ACE2 activity in HSPCs supernatants

In another set of experiments, we analyzed the cell supernatants of HSPCs collected after exposure to normoxia or hypoxia for ACE and ACE2 enzyme activities. Supernatants derived from cells exposed to normoxia showed ACE and ACE2 activities (Fig. 22*A* and *B*) suggesting constitutive shedding of the ectodomains of the enzymes that retained their catalytic activity even in the soluble form. Surprisingly, exposure to hypoxia increased the ACE2 activity by 4-

fold in the supernatants but not that of ACE suggesting that hypoxia increases shedding of catalytically active fragment of ACE2 ectodomain (Fig. 22*A* and *B*).

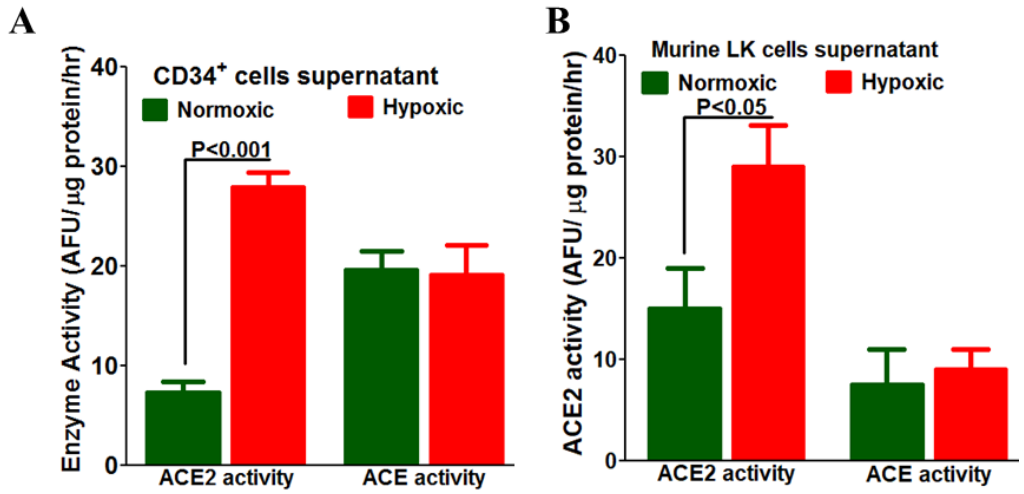

Figure 22. Hypoxia stimulates shedding of ACE2 in HSPCs. *A.* ACE2 activity was increased in the human $CD34^+$ cells supernatants upon exposure to hypoxia in comparison with normoxia. No change was observed in ACE activity in the supernatant ($P<0.001$ for ACE2, n= 8) *B.* ACE2 fluorescence activity was increased in the murine LK cells supernatants upon exposure to hypoxia in comparison with normoxia. No change was observed in ACE activity in the supernatant ($P<0.05$ for ACE2, n= 7)

2.2. Working hypothesis

We hypothesized that the transcription factor hypoxia-inducible factor-1α (HIF1α), which mediates cellular responses to hypoxia, regulates the expression of ACE2 in HSPCs.

2.3. Experimental design

It was critical to determine if the increased expression of ACE2 occurred via HIF1α or its target molecules, SDF or VEGF. To accomplish this working sub-hypothesis, we isolated $CD34^+$ HSPCs from the peripheral blood of healthy individuals and exposed them to normoxia/hypoxia with or without inhibiting HIF1α and measured ACE2 activity and protein expression. To assess the transcriptional regulation of ACE2 via HIF1α, we performed luciferase reporter assays. The HIF1α-regulated factors, SDF and VEGF, are known to mediate pro-angiogenic, vasculogenic

functions, and induce expression of several paracrine factors. Therefore to address the question whether ACE2 expression was directly mediated by SDF or VEGF, we also did reporter assays in human $CD34^+$ cells with SDF or VEGF under normoxic conditions. Additionally, to delineate the mechanism of ACE2 shedding via ADAM17 or by matrix metalloproteases (MMPs), we performed western blotting analysis in the presence of specific inhibitors.

2.4. Results and discussion

2.4.1. Hypoxia's increase of the expression of ACE2 in HSPCs is dependent on HIF-1α

First, we sought to determine if the hypoxic-stimulation of ACE2 expression and activity were mediated by HIF1α and for that we used a pharmacological inhibitor of HIF1α, 2-methyl estradiol (2ME) (0.5μM). This inhibitor was used concurrently while cells where exposed to hypoxic preconditioning. Concurrent treatment of cells with 2ME prevented the upregulation of ACE2 protein ($P<0.05$, n=5) or the enzyme activity ($P<0.05$, n=5) by hypoxia (Fig 23*A* and *B*). This result suggests that the observed effects of hypoxia on ACE2 were indeed HIF1α-dependent.

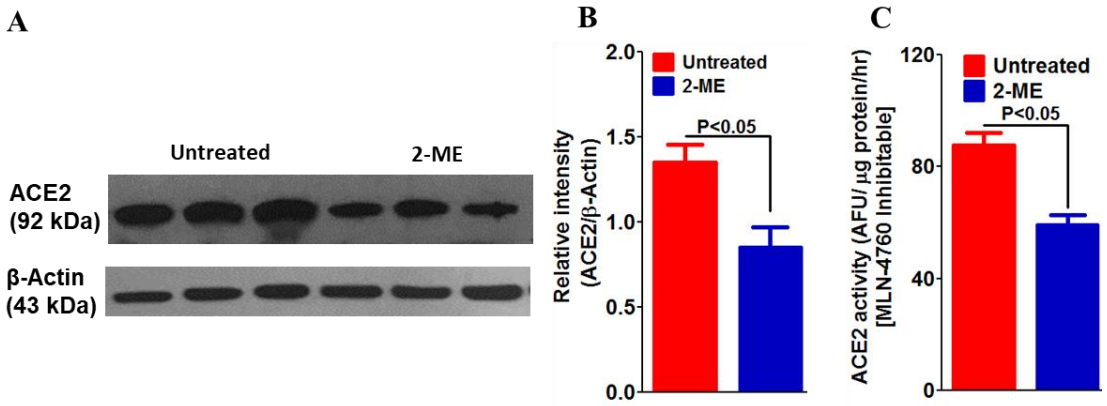

Figure 23. Hypoxic increase in the expression of ACE2 in human $CD34^+$ cells is HIF1α-dependent. *A* and *B.* Protein bands by western blot and densitometry graphs showing treatment with a pharmacological inhibitor of HIF1α -2-methyl estradiol (2-ME) decreased the ACE2 band intensity and prevented the effects of hypoxia on the protein expression of ACE2 ($P<0.05$, n=5). C. ACE 2 activity which was increased by hypoxic stimulation was also decreased in the presence of 2-ME ($P<0.05$, n=5).

2.4.2. Transcriptional regulation of ACE2 expression by HIF1α in HSPCs

We further evaluated the transcriptional regulation of ACE2 by HIF1α by using a luciferase reporter assay. As described in the methods section of the previous chapter, the Lv-luciferase reporter vector was cloned to contain the ACE2 promoter region and the vector was driven by a CMV-SV40 promoter, which contained five putative HIF1α binding sites. Luciferase activity assay showed that neither normoxia nor hypoxia affected the luciferase activity in cells transduced with the control plasmid (blank-luciferase (Luc-Blank)) (Fig. 24). On the other side, ACE2-luciferase activity was significantly increased by exposure to hypoxia compared to normoxia ($P<0.001$, $n=6$) (Fig. 24). Hypoxia-induced ACE2 luciferase expression was abolished by co-expression of ACE2-specific, miR421 in both normoxic ($P<0.05$) and hypoxic ($P<0.001$) conditions ($n=6$) (Fig. 24). Limited information is available related to the regulation of ACE2 by miRs. Target sequence alignment analysis using BLAST confirmed ACE2 as a target for miR421. Evidence for a potential regulation of ACE2 by miR421 has been shown in human cardiac fibroblasts (174). Upregulation of miR421 has been implicated in the development of thrombosis. Elevated miR421 in leucocytes was observed in patients with chronic kidney disease (175). Our study provides a strong support for the hypoxic regulation of ACE2, which was prevented in the presence of miR421.

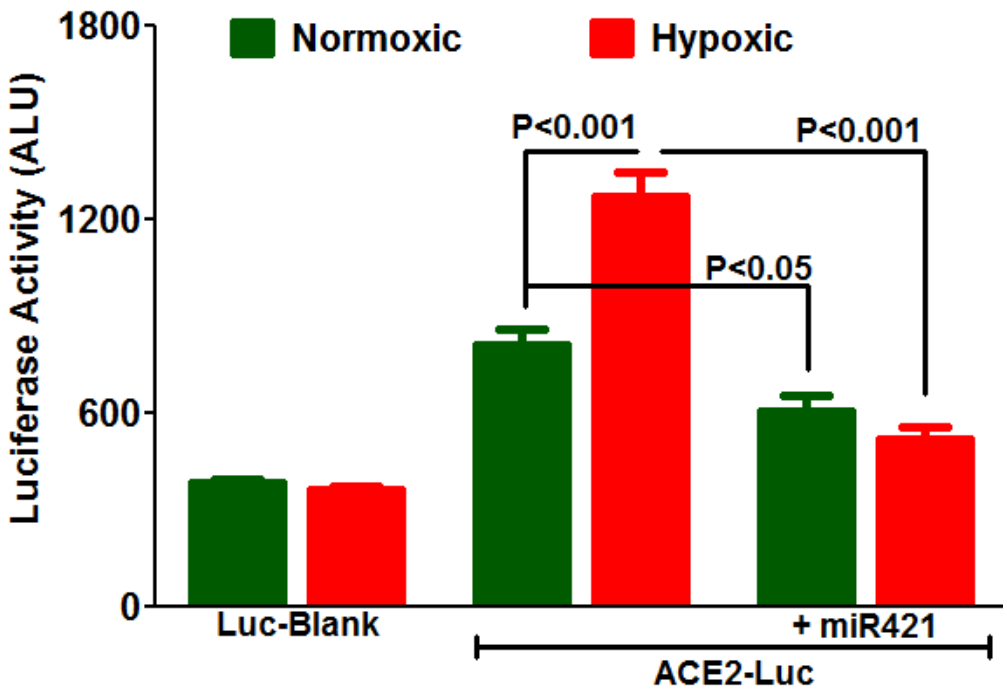

Figure 24. Expression of ACE2 transcription is regulated by HIF1α in human $CD34^+$ cells. Luciferase activity was not changed in cells transduced with the control plasmid (Luc-Blank) upon exposure to hypoxia. Luciferase activity in cells transduced with Luciferase-ACE2 (ACE2-Luc) was higher in hypoxia than in normoxia (P<0.001, n=6). Co-expression of miR421prevented the increase in luciferase activity regardless of the exposure (P<0.05 for normoxia and P<0.001 for hypoxia, n=6).

2.4.3. Transcriptional regulation of ACE2 expression by SDF and VEGF in HSPCs

We investigated if ACE2 transcription was regulated by the hypoxia target molecules SDF and VEGF. It is known that hypoxia is a strong stimulus for upregulation of SDF and VEGF expressions by stabilizing HIF1α. The transcriptional regulation of other angiogenic and vasculogenic factors by SDF and VEGF was shown to have important roles in the recruitment of HSPCs to regions of hypoxia, activation of local endothelium, new vessel formation by angiogenesis and activation of immune cells that play a role in angiogenesis after ischemic injury. Therefore, we investigated if the upregulation of ACE2 transcription by hypoxia was recapitulated by SDF or VEGF under normoxic conditions. I did luciferase reporter assays in

CD34^{+} cells after treating them with either SDF(100 nM) or VEGF(100 nM). Both SDF and VEGF enhanced ACE2 -luciferase activities in normoxic conditions while no activity was observed in cells expressing the control luciferase (Fig. 25).

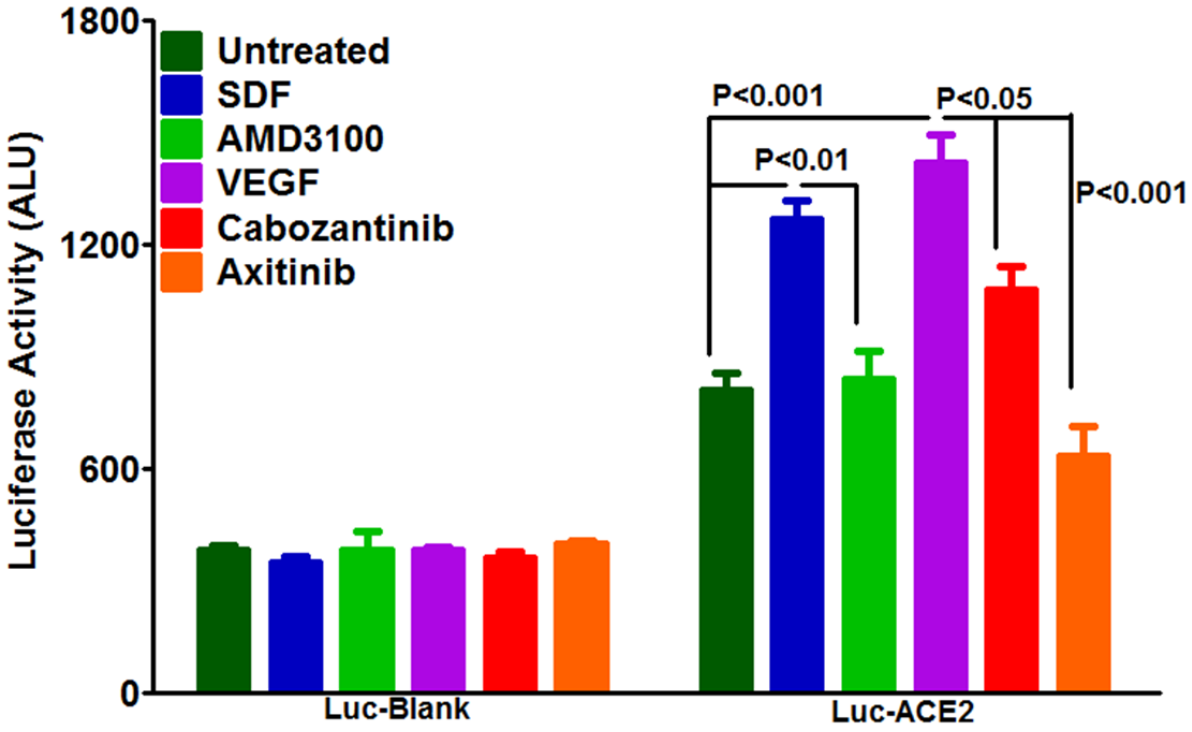

Figure 25. Transcriptional regulation of ACE2 expression by SDF and VEGF in human CD34^{+} cells. No effect of SDF or VEGF in the presence or absence of different receptor inhibitors was observed on luciferase activity in cells transduced with Luc-Blank. Luciferase activity was increased by treatment with either SDF or VEGF in cells transduced with Luc-ACE2 compared to untreated cells (n =6, P<0.001). SDF-induced increase in luciferase activity was prevented by AMD3100 in Luc-ACE2 transduced cells (n=5, P<0.01). Cabozanitib partially reversed VEGF-induced luciferase activity in ACE2-Luc expressing cells (n=5, P<0.05). In contrast, axitinib prevented the effects of VEGF in ACE2-Luc transduced cells (n=5, P<0.001).

SDF and VEGF signal via binding with their specific receptors. We used receptor-selective pharmacological inhibitors to delineate the involvement of specific receptor of SDF or VEGF in mediating ACE2 transcription response. Effect of SDF on ACE2-luciferase activity was abolished by simultaneous treatment of cells with AMD3100 (10 µM) (P<0.01, n=5) confirming the involvement of CXCR4 in mediating this response. Similarly, effects of VEGF

were abolished by a nonselective VEGF receptor antagonist, axitinib (30 μM) ($P<0.001$, n=5). The VEGF receptor family has 3 main subtypes indicated by VEGFR1, 2, and 3. Cabozantinib (100 nM), a selective inhibitor of VEGFR2, partially reversed the effect of VEGF in increasing the ACE2-Luc in $CD34^+$ cells ($P<0.05$, n=5) (Fig. 25).

2.4.4. Hypoxic increase in the ACE2 shedding in HSPCs is dependent on HIF-1α

The preliminary observation of increased soluble ACE2 activity with hypoxia was intriguing. Since, epigenetic regulation of ACE2 and shedding of catalytically active form of ACE2 has been reported in other studies (176–180). To further confirm the stimulation of ACE2 shedding by hypoxia, we performed Western blotting analysis, which detected ACE2 fragments that were observed only in supernatants from hypoxic cells and were absent in the supernatant obtained from normoxic cells (Fig. 26*A* and *B*). ACE2 antibody that can specifically identify N-domain of ACE2 was used and detected a fragment of 90 kDa in the supernatant. A different antibody that is not domain specific detected two distinct fragments, 80 and 90 kDa whereas an antibody which is specific for the C-domain of ACE2 did not detect any fragments in the supernatant (Fig. 26*A* and *B*). Previous studies reported ACE2 shedding in other cell types, mediated largely by ADAM metallopeptidase domain 17 (ADAM17) (178,181,182), also known as TACE (Tumor Necrosis factor-α-Converting Enzyme). ADAM17 is a member of a disintegrins and metalloproteases category and it is localized in the perinuclear compartment with small ADMA17 being present on the cell surface for the shedding activity. Therefore, to determine the role of ADAM17 in ACE2 shedding, we used pharmacological inhibitors of ADAM17 (Tapi-2 (50 μM)), or nonspecific matrix metalloproteinases (MMPs) (GM6001 (25 μM)). Tapi-2, an inhibitor of ADAM17, inhibited ACE2 activity and also prevented ACE2 shedding by hypoxia as indicated by very light bands on x-ray films (Fig. 27*A* and *B*). Similar

inhibition on ACE2 activity and protein expression was also observed with a nonspecific MMP inhibitor GM6001 (Fig. 27*A* and *B*).

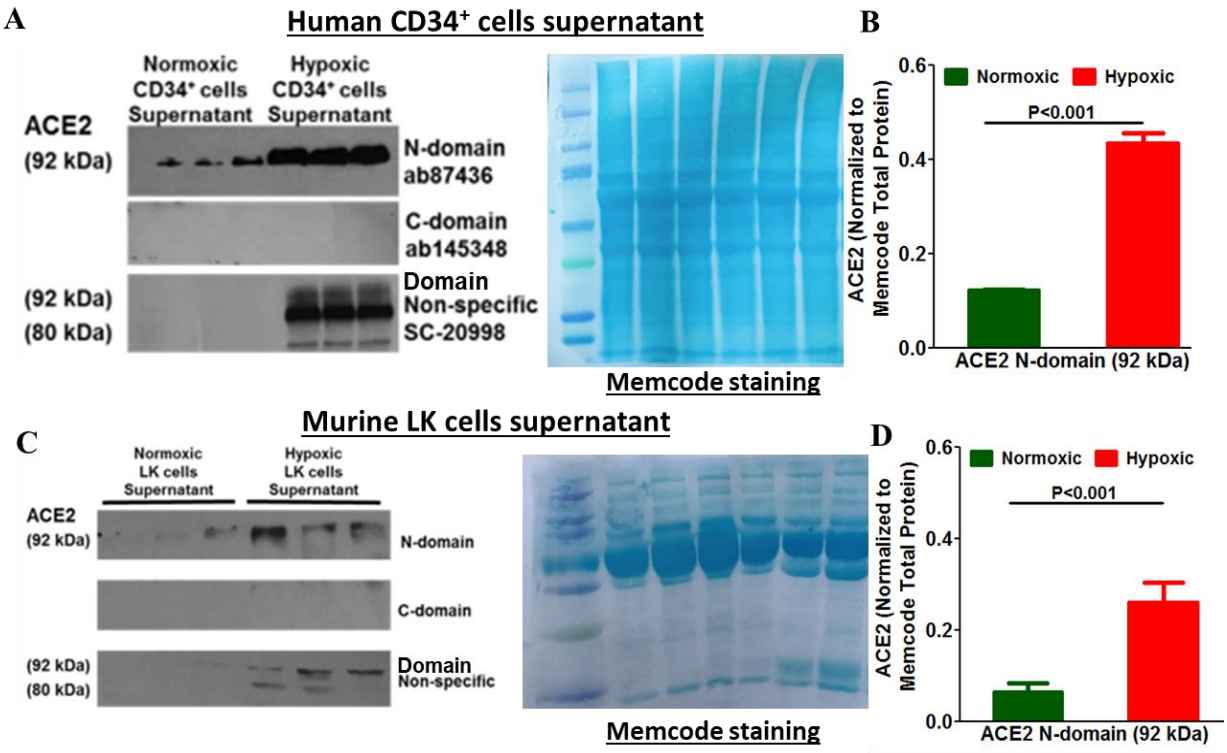

Figure 26. Hypoxia stimulates shedding of ACE2 in HSPCs. *A.* Western blotting of cell supernatants showed that ACE2 fragment as detected with an N-domain specific antibody of ACE2 was higher after hypoxia as compared to normoxia (P<0.001, n=6). ACE2 antibody specific for C-domain did not detect any protein fragments in the supernatants (n=4) while nonspecific antibody detected two distinct ACE2 fragments in supernatants derived from hypoxic cell but not in those derived from normoxic cells (n=5). Memcode staining confirmed equal amount of protein in each lane of the gel. *B.* Protein concentrations were expressed relative to total protein present in the supernatants as detected by memcode (P<0.001, n=5). *C* and *D.* Similar phenotype was observed in the murine LK cell supernatants (P<0.001, n=5).

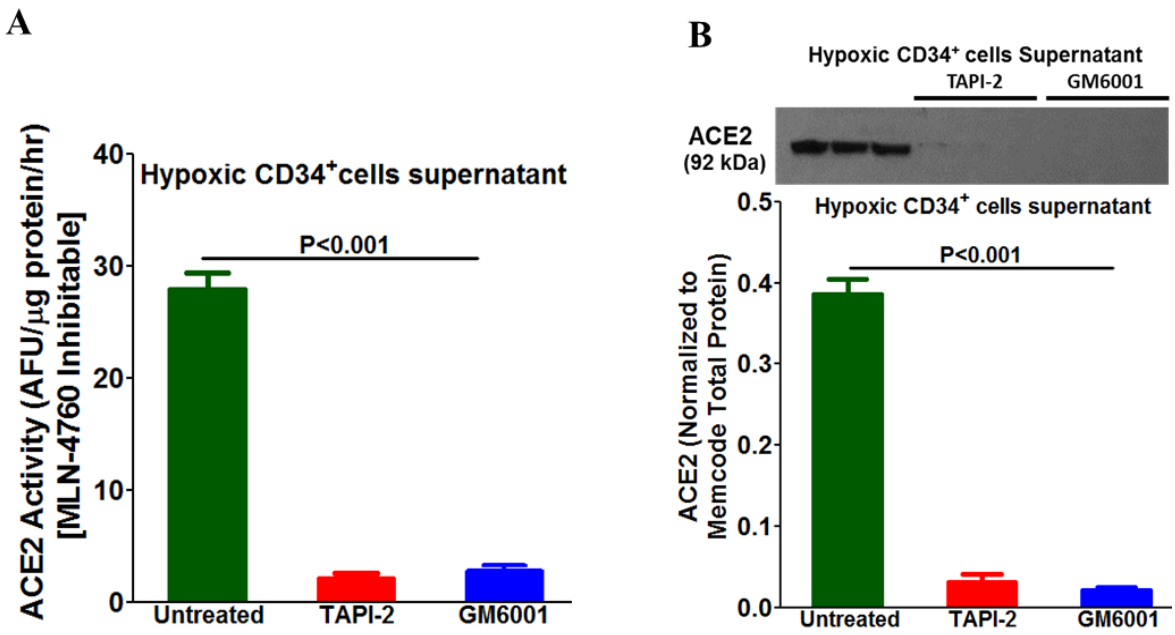

Figure 27. Hypoxia stimulates activity of ACE2 in HSPCs. *A.* ACE2 activity which was increased in the cell supernatants upon exposure to hypoxia in comparison with normoxia, was not observed in the presence of TAPI-2 or GM6001 (P<0.001, n=5). *B.* Western blotting of hypoxic cell supernatants which showed higher ACE2 fragment as compared to normoxia, was not observed in the presence of TAPI-2 or GM6001 (P<0.001, n=5). Densitometry shows ACE2 band intensity normalized against total protein as detected with memcode.

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CHAPTER 3. HYPOXIC REGULATION OF MAS RECEPTOR IN HSPCS

3.1. Rationale and preliminary results

G-Protein coupled MasR is expressed on HSPCs (155,156). As described in the first chapter, there is emerging evidence of MasR activation in HSPCs. Notably, pharmacologic activation of MasR by Ang-(1-7) was shown to stimulate the vasoreparative functions of HSPCs which is comparable to the response produced by hypoxia-regulated factors SDF and VEGF and this response was abolished by MasR antagonist A779. While, we tested the effects of hypoxia on regulation of ACE2 by using pharmacological and molecular tools in the previous chapter, in specific aim 2, we determined the effect of hypoxia on two key receptors of RAS, MasR and AT_1R. We investigated whether hypoxia plays a role in the upregulation of MasR under *in vitro* conditions. Further, if hypoxic regulation was observed, we investigated the mechanism involved in the MasR gene regulation.

3.1.1. Hypoxia increases mRNA expression of MasR in HSPCs

Hypoxia upregulated the transcription of MasR in both human and murine HSPCs as indicated by lower MasR ΔCt values in hypoxia treated cells as compared to normoxia ($P<0.01$, n= 8) (Fig. 28*A* and *B*). Expression of AT_1R messenger transcript was unchanged in either normoxia or hypoxia conditions (Fig. 28*A* and *B*). Likewise, mRNA expression for MasR was higher in murine LK cells as compared to normoxia ($P<0.05$, n=6). This indicates beneficial effects of hypoxia in transcriptional stimulation of MasR but not AT_1R.

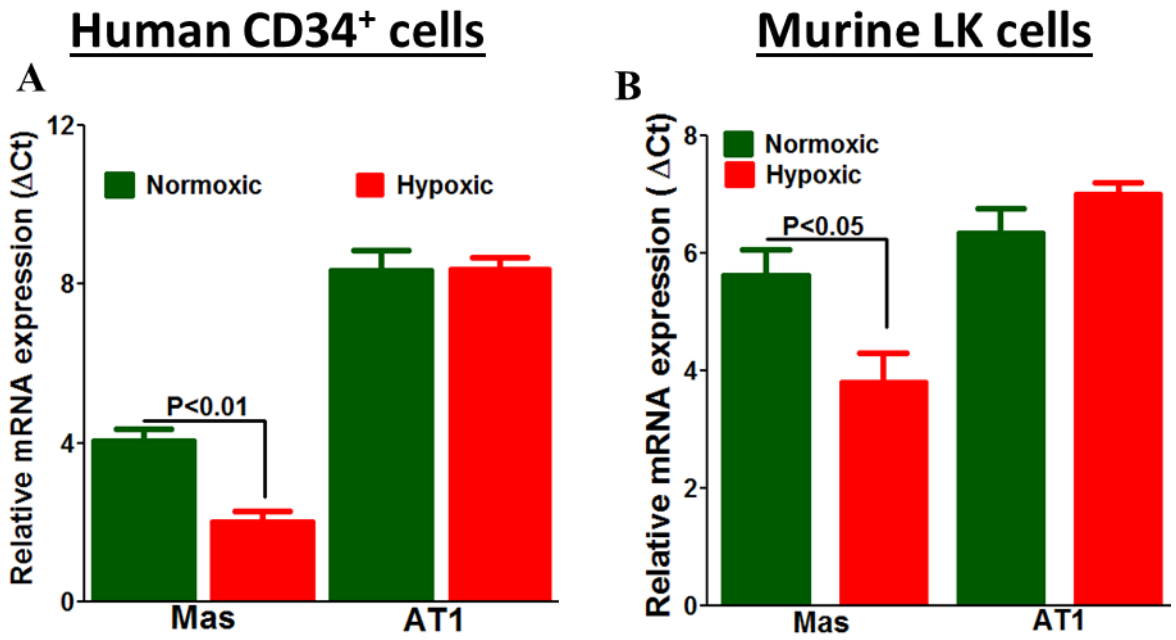

Figure 28. Hypoxia increased the expression of MasR in HSPCs. Exposure to hypoxia increased mRNA expression of MasR (lower ΔCt values) in *A.* human $CD34^+$ cells (n =8) and *B.* Murine LK cells compared to cells in normoxia as observed in the real-time PCR (n=6).

3.1.2. Hypoxia increases protein expression of MasR in HSPCs

We investigated if hypoxic exposure can upregulate the MasR protein expression. In light of recent publication showing non-specificity of MasR antibodies (184), we used flow cytometry to quantify the MasR expression. In this experiment we used an isotype control antibody to cancel the background signal. We observed that hypoxic exposure of HSPCs, increased the protein expression of MasR but not that of AT_1R in human $CD34^+$ cells (Fig 29*A*, *B and C*) where fluorescence intensities for MasR was significantly higher after hypoxic exposure but was unchanged for AT_1R ($P<0.01$, n=8). In case of murine LK cells, we used western blotting to measure the effect of hypoxia on MasR. Here also, we observed the same trend for band intensities of MasR and AT_1R in murine LK cells ($P<0.05$, n=6) (Fig 29*D* and *E*) indicating upregulation of MasR and unaltered AT_1R expression in response to hypoxia.

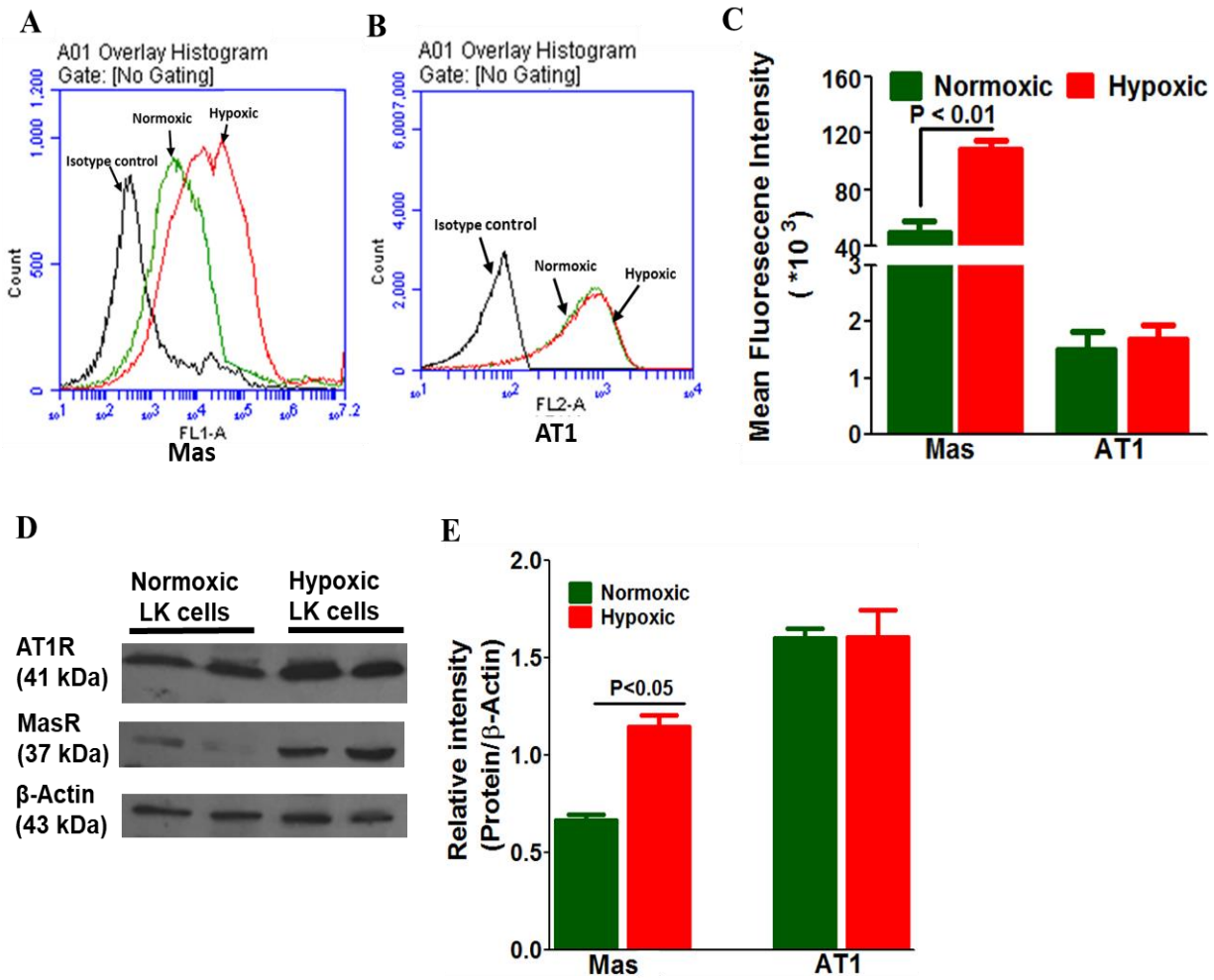

Figure 29. Flow cytometry and western blot analysis showing that hypoxia increased the protein expression of MasR in HSPCs but not AT_1R. *A, B* and *C.* Surface expression of MasR was significantly increased in hypoxia-exposed human $CD34^+$ cells compared to normoxic cells ($P<0.01$, n=8). Intensity corresponding to AT_1R receptor was not affected by hypoxia. *D* and *E* Protein expressions of MasR was also increased in LK cells as indicated by increased band intensity for MasR in hypoxic cells compared to normoxic cells ($P<0.05$, n=6). Expression of AT_1R receptor was not affected by hypoxia (n=6).

3.1.3. MasR deficiency desensitizes HSPCs to hypoxic stimulation of migration and proliferation

Previously we have shown that HSPCs from MasR deficient mice cannot repair the vascular injury after ischemic insult (165). This study also showed that MasR-deficient LK cells have lower basal-migration and proliferation compared to LK cells from wild type mice and

MasR-deficient cells also failed to respond to SDF- or VEGF- induced increase migration or proliferation (165). One of the caveats of that study was that the *in vitro* experiments were done in normoxic conditions. Here, we performed another set of study by obtaining LK cells from wild-type and MasR-knock out (KO) mice and subjected them to hypoxic conditions (1% O_2 for 12 h). We measured their migration and proliferation response in basal and SDF-, VEGF-stimulation. Intriguingly, we observed that hypoxia failed to increase migration or proliferation in MasR-deficient LK cells, either basal or in response to SDF or VEGF (Fig. 30*A* and *B*) (P<0.05, n=4). Our data indicate that MasR is indispensable for the vasoreparative functions of HSPCs and essential for hypoxic stimulation of these functions.

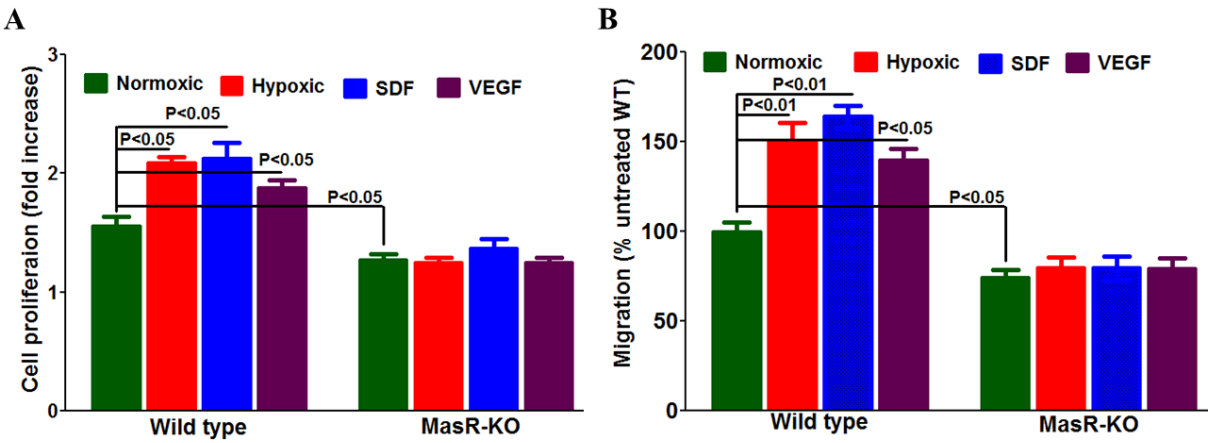

Figure 30. Hypoxic potentiation of proliferation and migration of murine LK cells was absent in MasR deficient mice. *A.* Hypoxia stimulated basal-, SDF- or VEGF-induced proliferation of LSK cells derived from wild type colony mice as compared to normoxic conditions. (P<0.05, n=8). Basal- proliferation was lower (P<0.05, n=4), whereas, SDF- or VEGF-induced proliferation was absent in the LK cells from MasR-KO mice. *B.* Exposure to hypoxia increased migration response in basal conditions, and in response to SDF or VEGF. (P<0.01, n=4). This response was not observed or decreased in the basal conditions (P<0.05, n=4) or was absent in response to SDF and VEGF in the LK cells from MasR-KO mice.

Overall, these preliminary findings corroborate with what we observed in the chapter 2 on hypoxic regulation of ACE2, and now provide evidence that hypoxic stimulation in HSPCs is

specific for the vaso-protective arm of RAS but not for the vaso-deleterious arm. Based on these findings, we sought to further delineate the mechanism for the regulation of MasR by hypoxia.

3.2. Working hypothesis

We hypothesized that regulation of MasR expression in HSPCs occurs via transcription factor hypoxia-inducible factor-1α (HIF1α).

3.3. Experimental design

It was critical to determine if the increased expression of MasR in HSPCs occurs directly via HIF1α or by its target molecules, SDF or VEGF. To accomplish this working sub-hypothesis, We isolated $CD34^+$ HSPCs from the peripheral blood of healthy individuals and exposed them to either normoxia or hypoxia with or without inhibiting HIF1α and measured MasR protein expression. To examine the transcriptional regulation of MasR via HIF1α, we performed luciferase reporter assays. In addition, to answer the question whether MasR expression is directly mediated by HIF1α or hypoxia regulated factors, we performed reporter assays in human $CD34^+$ cells with SDF or VEGF under normoxic conditions.

3.4. Results and discussion

3.4.1. Hypoxic increase in the expression of MasR in human $CD34^+$ cells is dependent on HIF-1α

We investigated if the hypoxic-stimulation of MasR expression is mediated via HIF1α by using a pharmacological inhibitor of HIF1α, 2ME. Concurrent treatment of cells with 2ME prevented the upregulation of MasR protein expression ($P<0.05$, $n=5$) by hypoxia (Fig 31*A* and *B*). This result suggests that the observed effects of hypoxia on MasR are HIF1α-dependent.

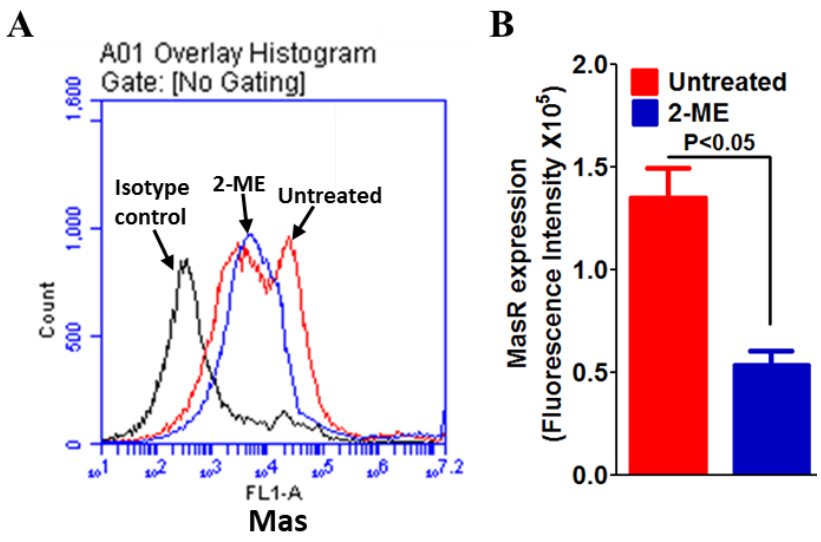

Figure 31. Hypoxic increase in the expression of MasR in human CD34$^+$ cells is HIF1α-dependent. *A.* Flow cytometric overlay histogram representation showing treatment with a pharmacological inhibitor, 2-ME decreased the rightward shift in fluorescence intensity. *B.* MasR fluorescence intensity was decreased by 2-ME (P<0.05, n=5).

3.4.2. Transcriptional regulation of MasR expression by HIF-1α in human CD34$^+$ cells

Transcriptional regulation of MasR expression in human CD34$^+$ cells by HIF1α was tested by using a luciferase reporter assay. Lv-luciferase reporter vector driven by CMV-SV40 promoter was cloned to contain MasR promoter region and it contained 3 putative HIF1α binding sites. As described earlier and shown in Fig. 25, neither normoxia nor hypoxia affected the luciferase activity in cells transduced with Luc-Blank (Fig. 32). On the other side, MasR-luciferase activity was significantly increased by exposure to hypoxia compared to normoxia (P<0.05, n=6) (Fig. 32). Hypoxia-induced MasR luciferase expression was abolished by co-expression of MasR-specific, miR143 in both normoxic (P<0.01) and hypoxic (P<0.001) conditions (n=6) (Fig. 32). While no information was available on the regulation of MasR by miRs, target sequence alignment analysis using BLAST confirmed MasR as a potential target for

miR143. MasR luciferase expression-induced by hypoxia was abolished in the presence of miR143.

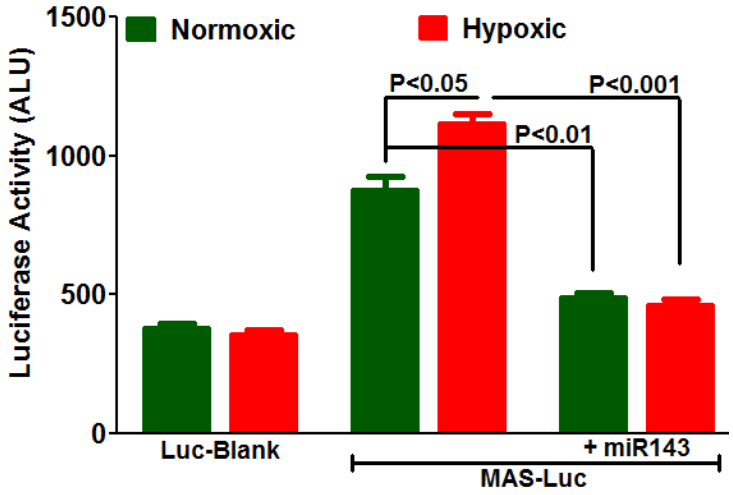

Figure 32. Expression of ACE2 transcription is regulated by HIF1α in human CD34$^+$ cells. Luciferase activity was not changed in cells transduced with control luciferase (Luc-Blank) upon exposure to hypoxia. Luciferase activity in cells transduced with Luciferase-ACE2 (ACE2-Luc) is higher in hypoxia compared to that in the presence of normoxia (P<0.05, n=6). Co-expression of miR421prevented the increase in luciferase activity regardless of the exposure (P<0.01 for normoxia, P<0.001 for hypoxia, n=6)

3.4.3. Transcriptional regulation of MasR expression by SDF and VEGF in human CD34$^+$ cells

We next investigated if MasR transcription was regulated by the hypoxia target molecules, we used a luciferase reporter assays in CD34$^+$ cells after treating them with either SDF(100 nM) or VEGF(100 nM). Both SDF and VEGF enhanced MasR-luciferase activities in normoxic condition, whereas no activity was observed in cells expressing Luc- blank (Fig. 33).

We verified MasR transcription upregulation effect from SDF and VEGF by using receptor-selective pharmacological inhibitors. Effect of SDF on MasR-luciferase activity was abolished by simultaneous treatment of cells with AMD3100 (10 μM) (P<0.01, n=5) confirming the involvement of CXCR4 in mediating this response. Similarly, effects of VEGF were

abolished by a nonselective VEGF receptor antagonist, axitinib (30 µM) (P<0.01, n=5) and unaffected by Cabozantinib (100 nM), a selective inhibitor of VEGFR2 in Luc-MasR transduced $CD34^+$ cells (n=5) (Fig. 33).

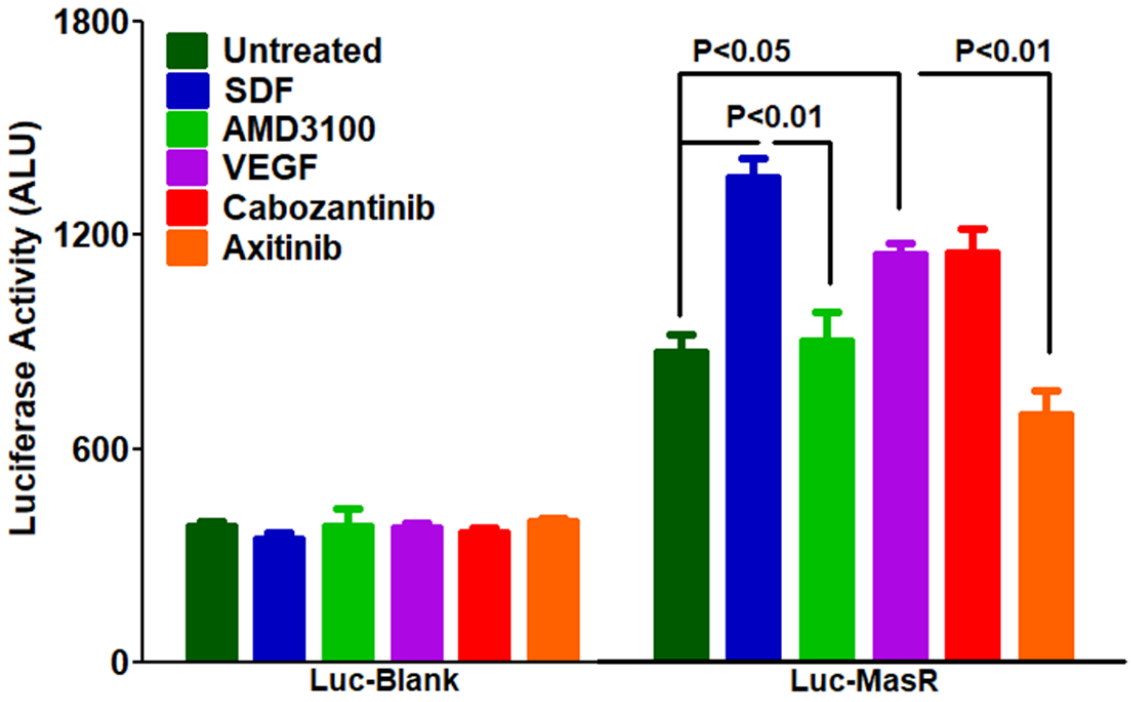

Figure 33. Transcriptional regulation of MasR expression by SDF and VEGF in human $CD34^+$ cells. No effect of SDF or VEGF or inhibitors of their specific receptors was observed on luciferase activity in cells transduced with Luc-Blank. Luciferase activity was increased by treatment with either SDF or VEGF in cells transduced with Luc-MasR compared to untreated cells (P<0.001, n =6). SDF-induced increase in luciferase activity was prevented by AMD3100 in Luc-MasR transduced cells (P<0.01, n=5). Cabozanitib unaffected VEGF-induced luciferase activity in Luc-MasR expressing cells. In contrast, axitinib has prevented the effects of VEGF in Luc-MasR transduced cells (P<0.01, n=5).

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CHAPTER 4. REGIONAL HYPOXIA IN VIVO UPREGULATES ACE2 AND MASR IN VASCULAR PROGENITOR CELLS

4.1. Rationale and preliminary results

Until now, our studies have shown compelling *in vitro* evidence for hypoxic preconditioning as a promising approach for activation of the protective axis of RAS and increasing the reparative potential of HSPCs. Novel findings from this study provided mechanistic insights for the concept of hypoxic preconditioning and also provided very strong evidence for the hypoxic-preconditioning as a promising approach that can be translated to clinical care of individuals needing cell therapies. Now, to reinforce our findings and to provide additional evidence for the biological significance of hypoxia on HSPCs, we extended our studies to *in vivo* hypoxic conditions and for the first time, evaluated the effects of hypoxia on ACE2 and MasR in a mouse model of ischemic injury. Additionally, we performed studies in humans to test the effects of regional hypoxia on ACE2 and MasR expression. Essentially, we aimed to test whether acute non-pathological hypoxia in the peripheral tissues would recapitulate our *in vitro* findings and upregulate the vasoprotective axis of RAS in HSPCs. This goal was accomplished by using low-intensity blood flow restriction (BFR) exercise (185–187). This idea stemmed from the growing body of scientific studies suggesting that physiological stress such as acute resistance exercise can potentiate the mobilization of HSPCs from bone marrow (44,188). Acute exercise was shown to stimulates mobilization by increasing the expression of chemoattractant molecules in the skeletal muscles such as MCP-1, SDF, angiopoietin-1 and VEGF (44). In light of this, as reviewed recently by Emmons et al., exercise can be used in clinical settings as an adjuvant therapy to enhance HSPCs mobilization and improve the outcomes of cell-based therapies (46).

Interestingly, hypoxic exercise also known as BFR exercise, 68atsu training or ischemic exercise, is a novel exercise modality which applies blood flow restriction to the peripheral tissues with exercise cuffs or tourniquet bands applied on limbs and has been shown to occlude all venous blood flow and a portion of arterial blood flow and thereby induces localized hypoxia in the skeletal muscles (189,190). There is strong evidence of support for BFR exercise as a training method which can create transient hypoxia in the muscle and initiate beneficial physiological responses for muscle growth and influences the vascular system (185,186,191). Two widely used forms of BFR are the 68atsu and delphi training cuffs. Kaatsu has been widely used in Japan whereas delphi is showing increasing use in clinical populations.

Low load BFR exercise was shown to initiate beneficial physiological responses for muscle growth, with less strain on bone, ligaments, and joints (189,192,193) and it may be a potential alternative exercise modality for many individuals having difficulty with high intensity exercise training, including those suffering from previous health complications, injury, or inexperience with the methods. Larkin et al (189) showed that BFR exercise not only induced hypoxia and thereby increased the mRNA expression of HIF1α but it also increased the expression of genes related to angiogenesis such as VEGF and its receptor VEGFR2, inducible-NOS (iNOS) and neuronal-NOS (nNOS). This was an important finding since very less is known about the effect of BFR on vascular system despite plethora of evidence suggesting BFR exercise stimulates muscle cross sectional area and strength. Shimizu et al (192) showed four weeks of BFR exercise training increased endothelial health by acutely elevating VEGF and other factors upregulate the vascular growth. Advantages of physical activity and exercise on cardiovascular health are well documented and there has been increasing amount of data showing their beneficial effects on HSPCs. Laufs et al. (44) has showed that exercise training increased the

levels of circulating vascular progenitors via partially NO-dependent effect, inhibited neointima formation and apoptosis and enhanced angiogenesis. This was mainly attributed to increased VEGF and increased local hypoxia in skeletal muscle which triggered the migration of vascular progenitors from bone marrow. However, no information is currently available regarding if BFR exercise can stimulate HSPCs acutely and nonpathological hypoxic conditions by low intensity BFR can upregulate the members of RAS.

4.2. Working hypothesis

We hypothesized that ischemic injury increases the functional ACE2 and expression of ACE2 and MasR on HSPCs. We also hypothesized that localized hypoxia by an acute bout of low intensity BFR exercise will upregulate the chemotactic signals which can stimulate the mobilization of HSPCs from bone marrow into the peripheral circulation. Eventually, this acute hypoxic exposure will increase the expression as well as shedding of ACE2 in HSPCs.

4.3. Experimental design

To test the interesting phenomenon of hypoxic regulation of ACE2 and MasR occurs in *in vivo* settings, circulating HSPCs– LSK cells, were obtained from mice undergoing HLI. On day-2 post-HLI and tested for ACE2 and MasR expression by flow cytometry and for ACE2 activity.

LI-BFR exercise was done in healthy human volunteer as described in the chapter 1 and Fig. 34 shows the flow chart of the study design.

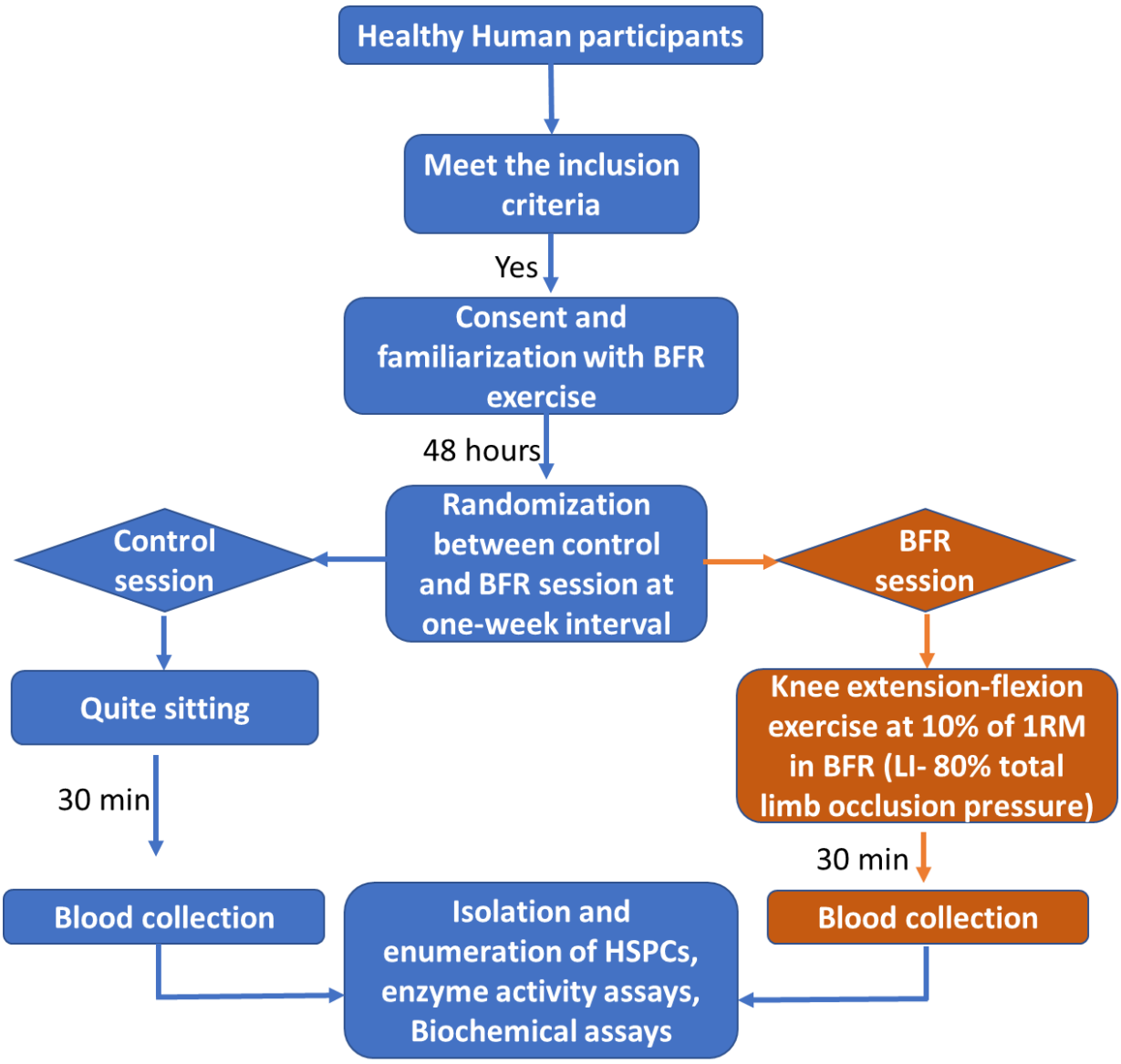

Figure 34. Flow chart depicting the study design for control or LI-BFR exercise.

4.4. Results and discussion

4.4.1. Ischemic injury increases the expression of ACE2 in the circulating HSPCs in mice

Here, we investigated if ACE2 expression is altered in HSPCs that are mobilized from bone marrow in response to ischemic insult (refer to Fig 3 for mobilization of HSPCs after ischemic injury). We performed HLI surgery on wild type mice and collected LSK cells from the peripheral blood of mice which underwent HLI injury (Fig. 13). We have observed that

mobilization of bone marrow LSK cells was maximal on Day-2 post-HLI when the extent of ischemia was still more than 90% as compared to contralateral limb (n =7) (Fig 35*A*). LSK cells were derived on that time point and analyzed by flow cytometry. We compared expression of ACE2 on circulating LSK cells collected after HLI injury with the cells collected from another group which was not subjected to HLI. The assessment of protein expression in cells was done by recording the equal number of events between the ischemic and non-ischemic control mice groups. We tested LSK cells for ACE and ACE2 expression and activities. Cells derived from HLI-mice showed higher mRNA expression of ACE2 compared to the cells obtained from non-ischemic control mice (P<0.01, n=5) (Fig 34*B*). No change was observed in the ACE mRNA expression (Fig 35*B*). Activity of ACE2 was increased in the LSK cell lysates derived from HLI-mice by 2-fold compared to the lysates of control cells derived from non-ischemic mice (P<0.01, n=5), while no change was observed in the ACE activity (Fig 35*C*). Along similar lines, ACE2 protein levels were higher in the LSK cells derived after HLI as compared to cells from non-ischemic mice (P<0.01, n=5) (Fig 36*A* and *B*).

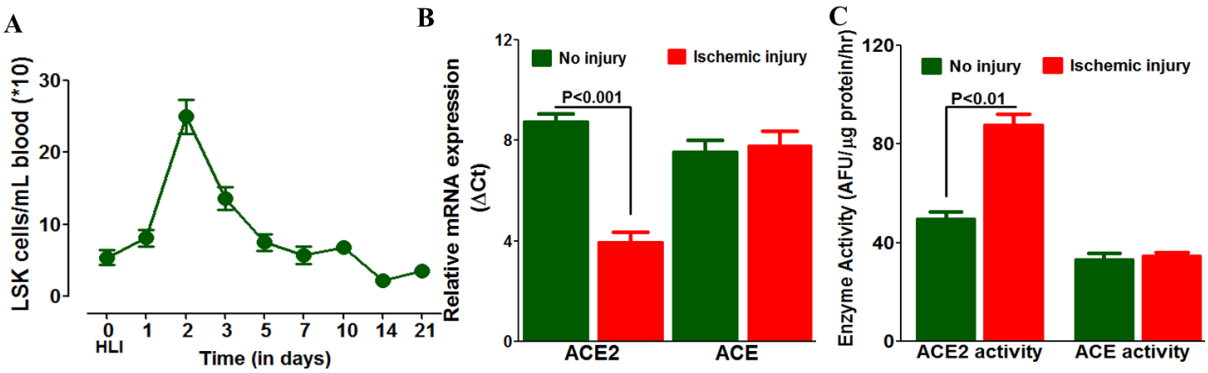

Figure 35. Ischemic injury increases the mRNA expression ACE2 and its activity in the circulating LSK cells in mice. *A.* LSK cells were increased on day 2 following hindlimb ischemic (HLI) injury (n=7). *B.* LSK cells derived from ischemic mice have higher ACE2 mRNA expression (expressed as low Δ*Ct*) compared to LSK cells from non-ischemic mice (P<0.001, n=5). ACE mRNA was unchanged after HLI (n=5). *C.* ACE2 enzyme activity in the cell lysates was higher in cells obtained following HLI compared to cells from non-ischemic mice (P<0.01, n=5).

Observed changes in ACE2 expressions or activity could be attributed to the exposure of circulating LSK cells to hypoxic environment at the areas of vascular injury or due to the direct effects of ischemia-induced factors, SDF or VEGF that are higher in the circulation following vascular injury.

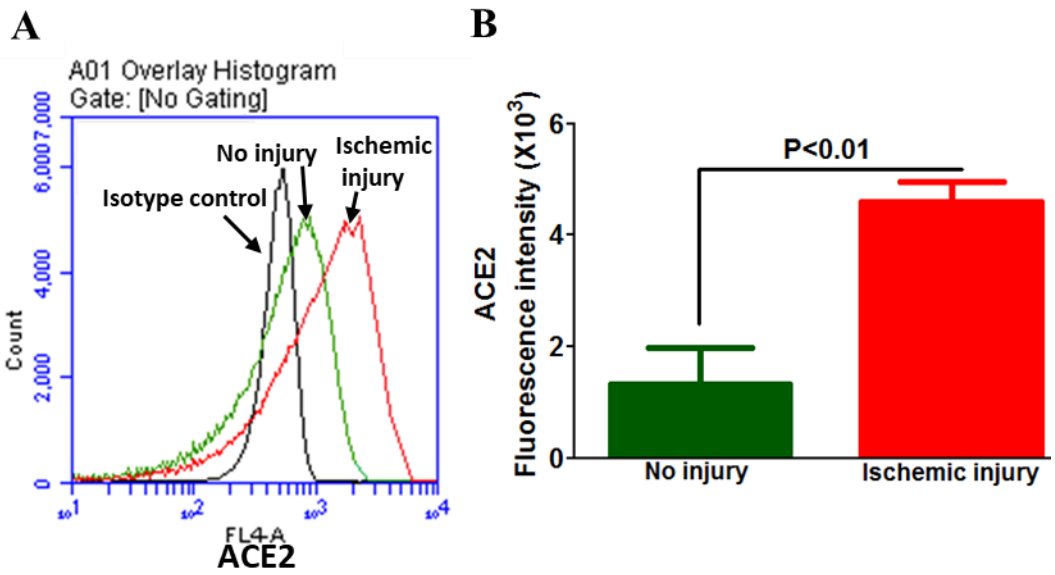

Figure 36. Ischemic injury increases the protein expression of ACE2 in the circulating LSK cells in mice. *A* and *B.* Representative flow cytometric histograms for the determination of surface expression of ACE2. Expression of ACE2 was higher in the LSK cells obtained from the ischemic compared to non-ischemic mice (P<0.01, n=5).

4.4.2. Ischemic injury increases the expression of MasR in the circulating HSPCs in mice

We confirmed the effects of *in vivo* hypoxia on MasR expression by investigating if its expression was altered in HSPCs which are mobilized from bone marrow in response to ischemic vascular injury. We performed HLI surgery on wild type mice and collected LSK cells from the peripheral blood on Day-2 after HLI. We quantified MasR expression on circulating LSK cells by flow cytometry and compared control (non-ischemic) group with the ischemic group. LSK cells derived from HLI-mice showed higher MasR protein levels compared to cells from non-ischemic mice (P<0.01, n=5) levels (Fig 37*A* and *B*).

Again, the observed changes in MasR expression could be due to exposure of the mobilized LSK cells to hypoxic environment at the areas of vascular injury or due to the direct effects of ischemia-induced factors, SDF or VEGF, that are higher in the circulation following vascular injury.

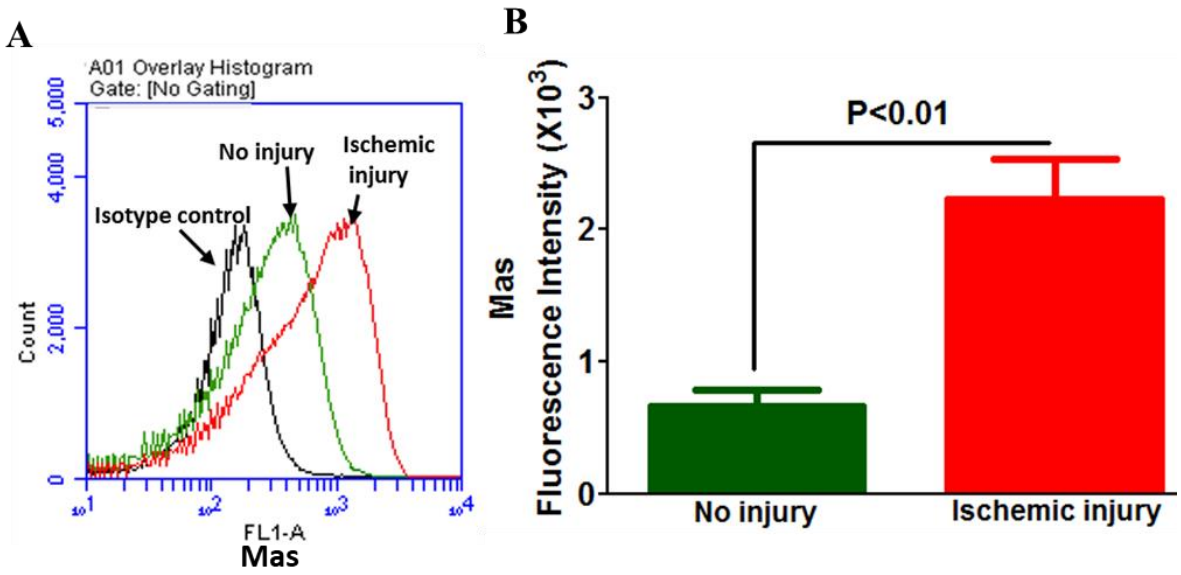

Figure 37. Ischemic injury increases the protein expression of MasR in the circulating LSK cells in mice. *A* and *B.* Representative flow cytometric histograms for the determination of surface expression of MasR. Expression of MasR was higher in the LSK cells obtained from the ischemic compared to non-ischemic mice (P<0.01, n=5).

4.4.3. Low intensity-blood flow restriction exercise induces hypoxia

BFR exercise at low intensity induces severe hypoxia as observed by 60% reduction in the muscle oxyhemoglobin (Oxy-Hb) and increased deoxyhemoglobin (Deoxy-Hb) (Fig. 38*A*). After each bout of BFR exercise, deoxy-Hb was increased from baseline levels and oxy-Hb was decreased. Maximum heart rate observed during BFR was not higher than 160 bpm and this returned to baseline levels after exercise session (Fig. 38*B*). Muscle oxygenation (SmO_2) during control session as measured using near IR sensor was 50.97 ± 3.58 which was dropped after each

set of exercise and average SmO_2 during resting condition was 14.43 ± 0.33 after exercise session, indicating 71.69% drop in muscle oxygenation (Fig. 38*C*).

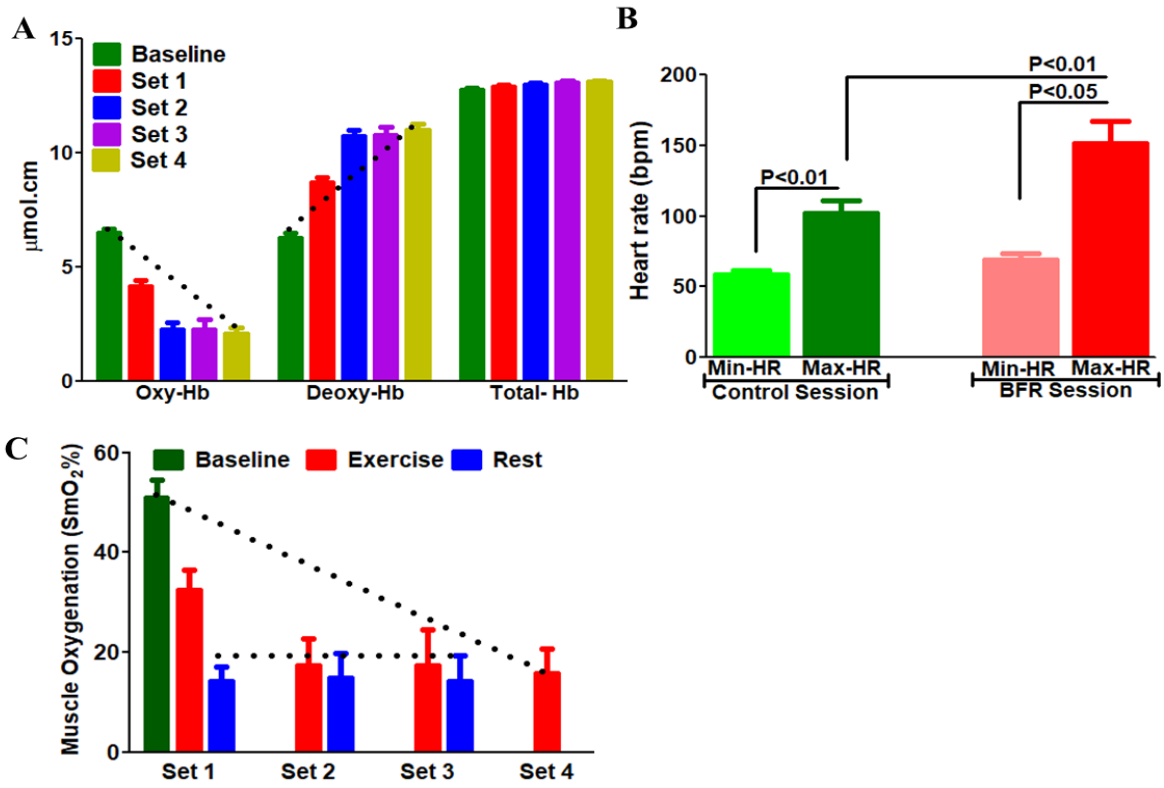

Figure 38. Induction of hypoxia by Low Intensity-Blood Flow Restriction exercise. *A* Reduction in the Oxy-Hb and increased Deoxy-Hb, *B.* Maximum heart rate observed during control and BFR sessions *C.* Smo_2 measured across different sets using MOXY.

4.4.4. Low intensity-blood flow restriction increases plasma SDF and VEGF

Induction of hypoxia by BFR exercise was further confirmed by analyzing plasma samples collected after BFR exercise for hypoxia regulated factors. We observed increased plasma SDF and VEGF levels by 6- and 4- fold respectively post-exercise ($P<0.001$, $n=5$) as measured by ELISA assay (Fig. 39*A* and *B).*

Together these results suggest that BFR even at a low intensity exercise provides severe hypoxic environment in the skeletal muscles and increases the circulating SDF and VEGF levels.

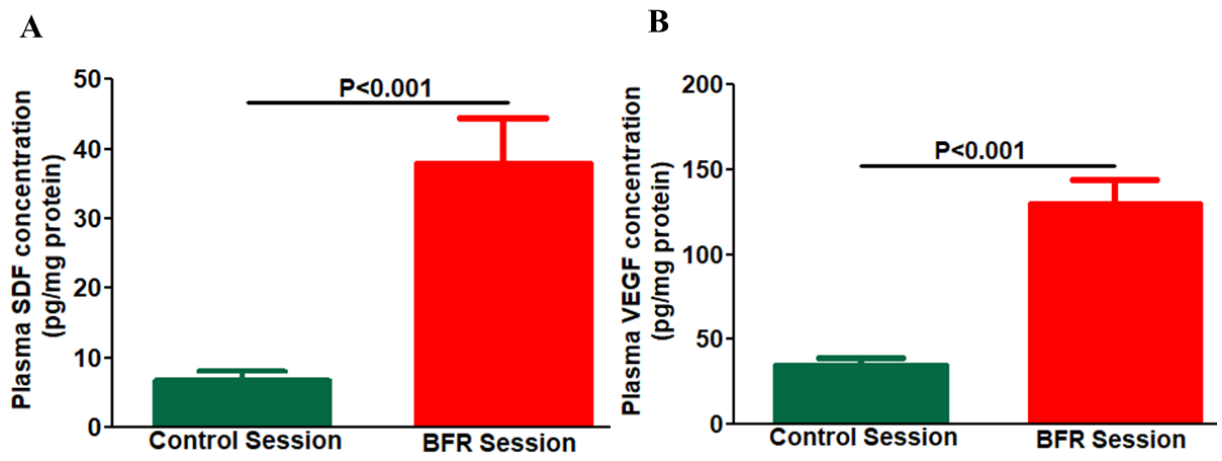

Figure 39. Induction of hypoxia-regulated factors by BFR. *A.* Plasma SDF and *B.* were increased by LI-BFR exercise as compared to control session (n=5, P<0.001).

4.4.5. BFR exercise increases circulating HSPCs in healthy individuals

Number of circulating Lin^- cells were significantly increased following BFR exercise session as compared to control session (P<0.0001, n=5) (Fig. 40*A*). Similarly, circulating $CD45^{Low}CD34^+$ HSPCs were increased after hypoxic exercise session as compared to control sitting session (P<0.0001, n=5) (Fig. 40*B*). Thus, LI-BFR stimulated mobilization of HSPCs, Lin- or $CD45^{low}CD34^+$ cells. To this end, several studies have investigated the effects of exercise on mobilization of HSPCs (47,194–199). High-intensity exercise intervention such as 1000-m sprint on rowing ergometer (197), 4 h cycling (199), 1500 m run (195), incremental cycling test (196) or high-intensity exercise training such as half/full marathon (194) or 8-week cycling training (200) were shown to increase circulating $CD45^{Low}CD34^+$ HSPCs post-exercise. As reviewed recently by Emmons et al. (46), magnitude of HSPCs mobilization is directly related to exercise intensity but high-intensity training is not a viable option for all individuals specially for patients with ischemic vascular diseases. To our knowledge, this is the first study to show that low-intensity BFR training can also mobilize HSPCs and effect of transient hypoxia created by

BFR exercise on HSPCs mobilization was similar to those observed after standard high-intensity exercise.

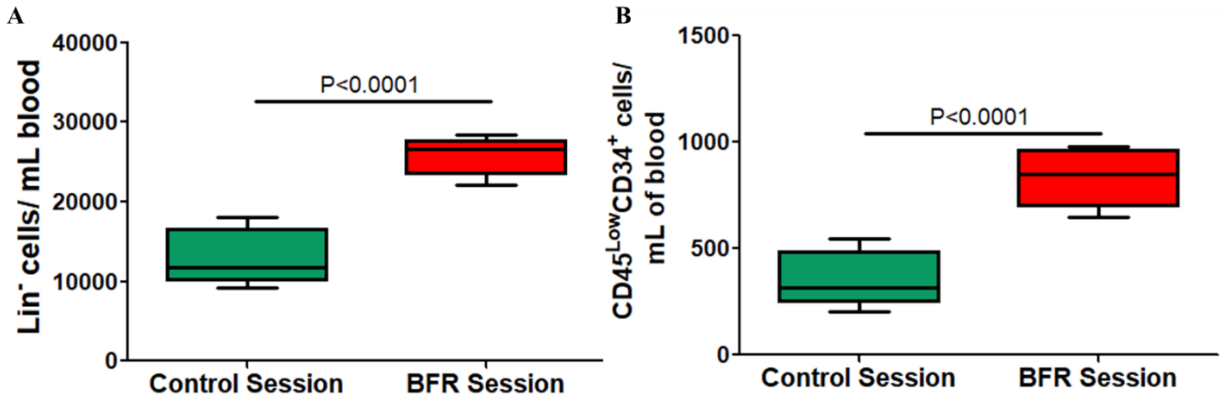

Figure 40. Mobilization of HSPCs by low intensity-BFR. *A.* LI-BFR rapidly increased Lin^- or *B.* $CD45^{low}CD34^+$ cells in the circulation as compared to control session (n=5, P<0.001).

4.4.6. BFR exercise increases ACE2 shedding and protein expression in the circulation and increases ACE2 mRNA and activity in HSPCs

We next measured ACE2 and ACE activity in the plasma. The most notable response to LI-BFR was on the ACE2 activity. We observed an increase of ACE2 activity and unaltered ACE activity in the plasma following LI-BFR exercise session which resulted in the increased ACE2/ACE ratio (Fig. 41*A* and *B*). Similarly, ACE2 activity was also increased in Lin^- cell lysates (Fig. 41*C* and *D*) and cell supernatants (Fig. 41*E* and *F*), derived from individuals performing BFR compared to control group. In agreement with the ACE2 enzyme activity, we observed ACE2 shedding following LI-BFR and ACE2 fragments were detected by western blotting in the plasma (Fig 42*A* and *B*). Then we pooled Lin^- cell lysates of 5 participants and quantified the mRNA expression of four key members of RAS, ACE2, ACE, MasR and AT1R. We observed increased ACE2 and MasR messenger expression whereas ACE and AT1R where not changed after LI-BFR exercise session (Fig. 43).

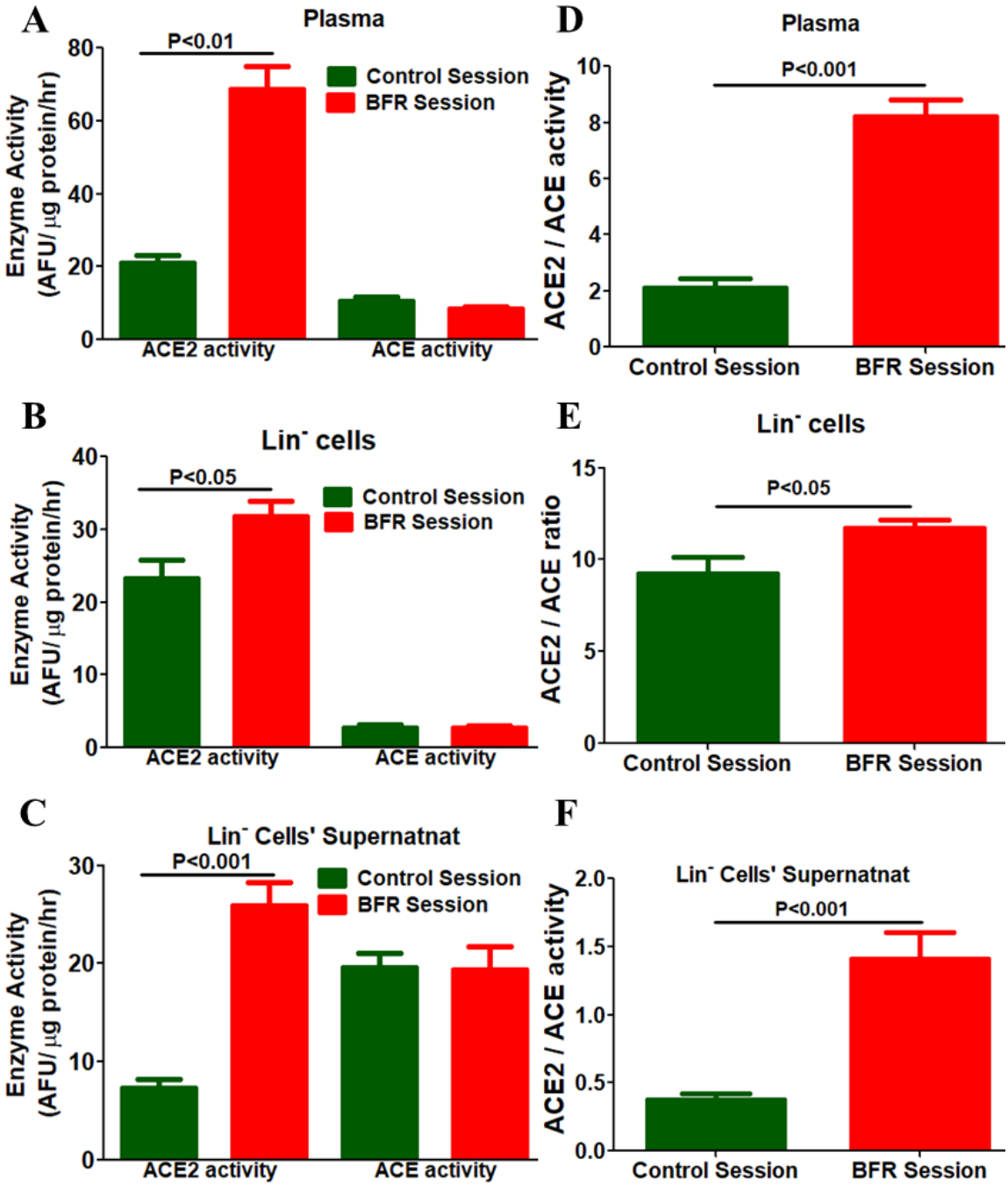

Figure 41. Increased ACE2 activity and unchanged ACE activity after LI-BFR exercise. Increased ACE2 activity and ACE2/ ACE ratio was observed in *A.* and *B.* plasma (P<0.01, n=5), *C.* and *D.* Lin- cells (P<0.05, n=5) and *E.* and *F.* Lin- cells supernatant after LI-BFR exercise session as compared to control session (P<0.001, n=5).

Collectively, these findings provide compelling evidence for the stimulation of vascular regenerative capacity and activation of vasoprotective axis of RAS by LI-BFR, which would enhance vasculogenesis and muscle growth. Hypoxic, LI-BFR exercise regimens are promising means of enhancing vascular and muscle health or restoring cardiovascular health in aging individuals or those with cardiovascular complications or astronauts.

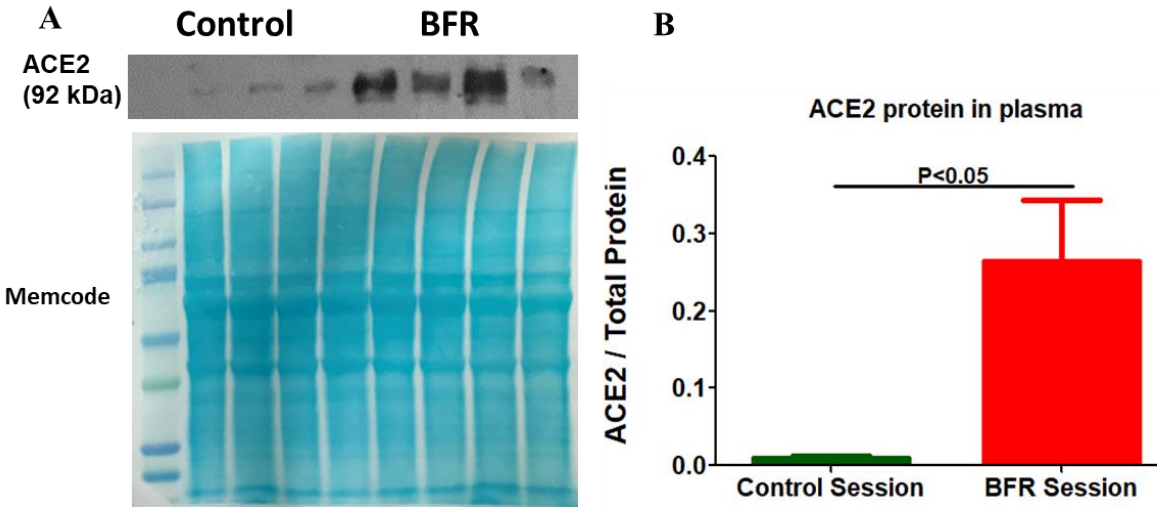

Figure 42. ACE2 shedding was observed in plasma. *A* and *B*. In agreement with the enzyme activity, ACE2 fragments were detected by western blotting and expressed relative to the total protein in the samples by using MemCode.

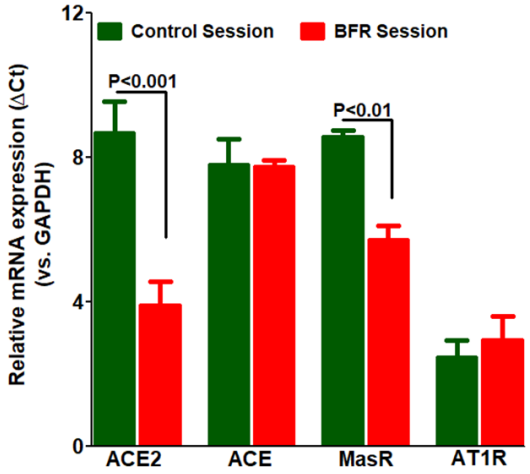

Figure 43. Li-BFR altered the expression of members of RAS in Lin$^-$HSPCs. Li-BFR session increased mRNA expression of ACE2 and MasR ($P<0.001$ and $P<0.01$, respectively, n=5) as compared to control session.

CHAPTER 5. SUMMARY AND CONCLUSIONS; CLINICAL RELEVANCE; LIMITATIONS; AND FUTURE DIRECTIONS

5.1. Summary and conclusions

Successful outcomes of cell-based therapies require i) sensitization of HSPCs to ischemic, pharmacological or priming stimulus for mobilization of optimum number of HSPCs for collection, ii) vascular engraftment of implanted HSPCs into peri-ischemic areas, and iii) paracrine functions of HSPCs to release pro-angiogenic factors and enhance angiogenesis. Genetic approaches to increase the level of pro-survival or angiogenic genes have shown proof-of-concept in the experimental studies (109–112,115,116,122,201,202), however genetically modified HSPCs are still not available for therapeutics. Alternatively, methods that can be easily employed with minimal cell manipulation but could stimulate their reparative functions would be ideal for therapeutics.

Hypoxic preconditioning has been tested in different cell types and was shown to play a central role in the regulation of different genes via its master nuclear factor HIF1α. This study tested whether hypoxia could increase the vasoreparative functions of HSPCs and increase the circulating number of HSPCs that can initiate, participate and accomplish the vascular repair. A scientific novelty of this study is that hypoxic exposure increases the expression as well as shedding of ACE2 in human HSPCs, which are destined to the areas of ischemia. In this context, ACE2 shedding would increase the local functional ACE2 activity in the peri-ischemic environment, which indeed opposes the detrimental effects of local Ang II. These provocative findings imply that hypoxic environment is a strong stimulus for HSPCs to accomplish vascular repair by producing vasoprotective molecule, Ang-(1-7), which by activating MasR on HSPCs, stimulates vasculogenesis/ angiogenesis. Thus, hypoxic upregulation of ACE2 and MasR

expression, and ACE2 shedding would contribute to the ischemic vascular repair. This beneficial effect would be further amplified by concurrent exposure of cells to hypoxia-regulated factors, SDF and VEGF, which also stimulate the expression of ACE2 and MasR.

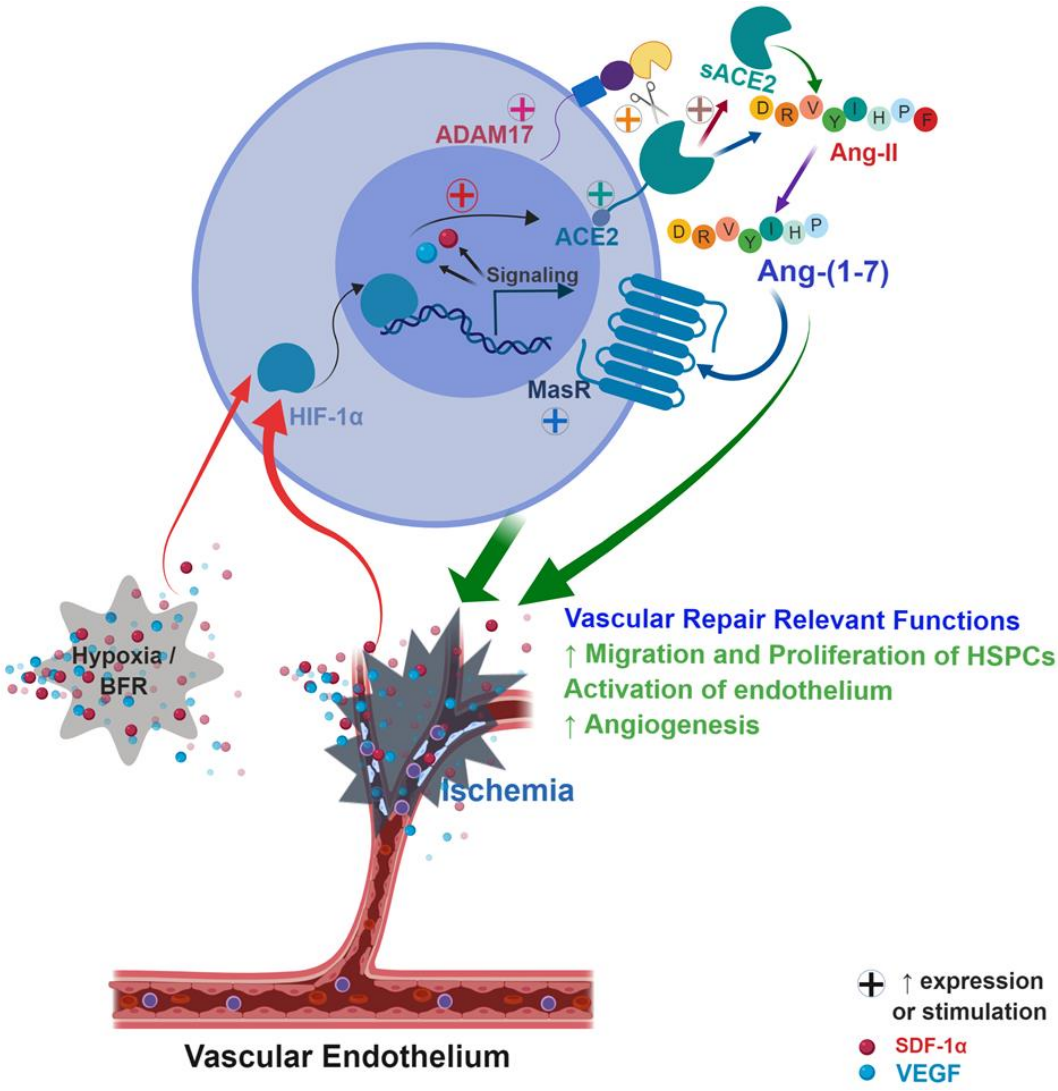

Figure 44. Summary of Research.

This study reports several novel findings. Hypoxia stimulated key vascular repair-relevant functions, migration and proliferation of human and murine HSPCs, which was associated with increased expression of both ACE2 and MasR. Hypoxic upregulation of the expression of ACE2 and MasR was dependent on HIF1α, evidently due to increased gene

transcription. Hypoxia-induced SDF and VEGF increase transcription of ACE2 and MasR in hypoxia-independent manner. Hypoxic exposure increased shedding of ACE2 ectodomain fragments in HSPCs with detectable ACE2 activity in the supernatants. In a mouse model of ischemic injury, HSPCs that were mobilized from bone marrow in response to ischemia were shown to have increased expression of ACE2 and MasR.

Importantly, for the first time this study reports significance of non-pathologic transient hypoxia, which recapitulated the *in vitro* observations on vascular protective axis of RAS in mice and humans. Low intensity hypoxic exercise training BFR, stimulated vascular regenerative capacity partly by activating vasoprotective axis of RAS, which would enhance skeletal muscle angiogenesis thereby muscle growth.

This multidimensional and integrative study has both conceptual and technical novelties. Conceptually, this study provides evidence for a novel role of hypoxia in regulating ACE2/Ang-(1-7)/Mas receptor axis of RAS in HSPCs and stimulating reparative functions of HSPCs for participating in vascular repair. Use of LI-BFR exercise is recently gaining popularity for its clinical utility in elderly individuals or those recovering from the long cardiovascular illness or astronauts who experiences microgravity. This study used that novel technique to induce transient non- pathologic hypoxia in the peripheral tissues and thereby stimulated the reparative functions of HSPCs.

In conclusion, collectively, these results demonstrated for the first time that hypoxic-preconditioning would be a cost-effective as it requires minimal cell manipulation and a better approach that could be translated to clinical care of individuals needing cell therapies.

5.2. Clinical relevance

Clinical trials have already demonstrated the safety and efficacy of cell-based therapies for ischemic vascular diseases and current findings also support the use of genetically modified HSPCs to increase their therapeutic potency. Translation of hypoxic preconditioning into clinics is a better and more feasible approach. The hypoxic preconditioning approach can be easily included in the current treatment strategy which is generally a threes step process i.) 4-day protocol to mobilize the cells, ii.) leukapheresis on day 5-10 and iii.) transplantation on day 6-11. This study has shown that at least 12 h of hypoxia can produce beneficial effects on HSPCs. This clearly shows that hypoxic priming can be easily performed before reinfusion of HSPCs in to patients as therapeutics. Effect of hypoxic preconditioning does not require long protocols for expansion of cells, therefore, this approach could have tremendous clinical applications in cell-based therapies as it could be used within the existing treatment protocol.

Further, studies confirmed the beneficial effects of hypoxia in increasing circulating HSPCs. Low load BFR is already used by athletes and clinically in musculoskeletal rehabilitation post ACL injury, knee osteoarthritis (OA) and now being considered for cardiovascular applications. In this study, low load BFR technique has recapitulated beneficial effects of hypoxic preconditioning on vasoreparative functions of HSPCs. Use of this technique makes this study more relevant to translate into clinics. Therefore, intervention with low load BFR can be easily employed as an adjuvant therapy in older age individuals and those with vascular disease who cannot tolerate high intensity resistance training, to simultaneously enhance the outcomes of cell-based therapies.

5.3. Limitations

This study resulted in several novel findings but is not free from limitations.

We examined the effects of hypoxia only on the ACE2/Ang-(1-7)/MasR pathway but it is known that Ang-(1-7) can also be generated from ACE2- independent pathways. Ang-(1-7) can also act via AT_2R or MrgD receptors. In case of ACE2 shedding, previous studies have shown two distinct bands at 70 kDa and 90 kDa. However, in the current study the 70 kDa fragment was not observed in the supernatant but instead an 80 kDa fragment was observed only by N-domain-specific antibody but not by the domain nonspecific antibody. ACE2 is post-transcriptionally glycosylated in most cell types, however in my studies I have seen ACE2 protein bands related to non-glycosylated form of ACE2.

This strategy may not be beneficial in diabetic individuals, prevalence of which is more in adults aged 65 or older in US and interventions targeting hyperglycemia have limited or no success in reducing risk of vascular diseases. This is due to previous reports showing hypoxic desensitization of diabetic HSPCs partly due to decreased HIF1 signaling.

BFR study is not sufficiently powered to give more conclusive insights and needs to be done in larger groups of participants. We did not include the control group who performed the exercise without low intensity blood flow restriction. Another limitation is the gender bias as we have employed only male participants in the BFR study.

5.4. Future directions

Though hypoxic preconditioning is found to regulate ACE2 and MasR and stimulate the reparative functions of HSPCs, the regulation of other protective and Ang-(1-7) receptors yet to be answered. Reviewing the literature, it is clear that MasR is not only a cognate receptor for Ang-(1-7), however, confirmation studies need to be performed on effects of hypoxia on AT_2R and MrgD receptors. BFR studies need to repeated in large data set to increase the power of

study and probably in both the genders. Secretome of hypoxic cell supernatants need to be analyzed for upregulation of other proangiogenic, pro-survival and anti-inflammatory genes.

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