ANGIOTENSIN-(1-7): A TARGET FOR STEM CELL MOBILOPATHY AND VASCULAR REPAIR IN DIABETES

A Dissertation
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

Goutham Vasam

In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Major Department:
Pharmaceutical Sciences

March 2017

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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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

Bone marrow stem/progenitor cells (BMPCs) accelerate vascular repair by re-endothelialization and revascularization of ischemic areas. Diabetes causes impairment of BMPC mobilization, a.k.a. stem cell mobilopathy, and reparative functions, which have now been considered as a major contributing factor for the development of macro and microvascular complications and end-organ damage. Therefore, autologous cell therapies for the treatment of diabetic vascular complications are currently not possible. In this study, I tested the effects of Angiotensin (Ang)-(1-7), a heptapeptide member of the protective arm of renin-angiotensin system, on mobilization of BMPCs and their ischemic vascular repair functions that are impaired in diabetes.

Streptozotocin-induced diabetic or db/db mice were used. Circulating and bone marrow Lineage$^-$ Sca1$^+$ c-Kit$^+$ (LSK) cells were decreased in diabetes, which was normalized by Ang-(1-7). Ang-(1-7) specifically increases Rho-kinase (ROCK) activity in diabetic bone marrow (BM) LSK cells, and fasudil, a ROCK inhibitor, prevented the beneficial effects of Ang-(1-7). BM Slit3 levels were increased by Ang-(1-7), which might have activated ROCK in LSK cells and sensitized for stromal-derived factor-1α (SDF)-induced migration. In relation to ischemia, diabetes prevented LSK cell mobilization and blood flow recovery, which were reversed by Ang-(1-7). Ang-(1-7), in combination with G-CSF or plerixafor reversed the stem cell mobilopathy in diabetes. These beneficial effects of Ang-(1-7) were blunted in Mas receptor knockout (MasR-KO) mice. These results suggest that MasR is a promising target for the treatment of diabetic bone marrow mobilopathy and vascular disease. Overall, this study provided strong preclinical evidence, supporting Ang-(1-7) as a promising molecule for the treatment of diabetic stem cell mobilopathy and vascular disease.

ACKNOWLEDGMENTS

The present work is an accomplishment and a result of my efforts during my stay at NDSU. However, this voyage of research was not travelled unaccompanied. Many direct and indirect sources of motivation were behind my every footstep. Among those, the foremost source of inspiration was my mentor Dr. Yagna P.R. Jarajapu. His guidance laid foundation for my dissertation thesis and he is always available to oversee my work and give advice. His critics on my scientific thoughts, presentation and writing styles honed my skills to be a better academic researcher. His nature to encourage in all career related aspects helped me design my future. What kept me stress-free during the tenure as a graduate trainee is he is the beacon for all my scientific endeavors within and outside of NDSU. I feel extremely fortunate to have a research supervisor like him.

The Research Supervisory Committee, which includes Dr. Jagdish Singh, Dr. Stephen T. O'Rourke and Dr. Dale Redmer, improved my logical thinking while critically reviewing my research proposal. Their thoughtful viewpoints made me work around a challenging research issue in all possible angles to find out a solution, thereby created a lasting impression over my dissertation research. They gave me a huge support through their recommendations whenever I am in need which also helped me secure a postdoctoral position before I graduate. I can never forget the moral support and encouragement of Dr. Jagdish Singh as Chair of the Department of Pharmaceutical Sciences while submitting fellowship proposals, competing within and out of the university, and building leadership qualities, which contributed to my overall development.

My thirst for knowledge was persistently provoked by the erudite faculty members at the Department of Pharmaceutical Sciences. They all have provided free access to their laboratory equipment and gave suggestions when needed. I am indebted to each and every one of them as

they made learning a joyful process. I would like to give a special thanks to Janet Krom, Jean Trautmann and Diana Kowalski for making my life easier at NDSU by smooth handling of the administrative processes and streamlining our usage of equipment.

I have gained some excellent friends and fabulous colleagues during my journey at NDSU and I highly appreciate each of their company. Both together, is my lab mate and family friend Shrinidh Joshi. I would like to thank him not only for helping me process some of my samples to obtain data, but also for being with our family and sharing various moments and experiences in life.

I cannot thank enough my parents, Mr. Venkateswara Rao Vasam and Mrs. Rama Rajeshwari Vasam, who have introduced meaning of life to me, and backed me for all important decisions in my life. What I am today is just because of their selfless sacrifice and support. I consider the unconditional love of my younger sister Praveena and my wife Saranya towards me as the most valuable possession. Saranya has been a great support throughout my PhD since she is in my life, taking care of all the household responsibilities while I am busy spending many hours in the lab.

Finally, I would like to thank the funding support. This work is partly supported by American Heart Association (13SDG16960025), and COBRE Pilot Project Grant (Intramural) – Center for Protease Research (P30 GM103332 – 01) at NDSU, who made the flow cytometry experiments possible in my studies. Studies with Mas receptor knockout mice were done by establishing the breeding colonies at our animal facility with the help of generous gift of breeding pairs from Dr. Lisa A. Cassis and Dr. Sean E. Thatcher from University of Kentucky.

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LIST OF ABBREVIATIONS

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

ACE.....Angiotensin Converting Enzyme

AFU.....Arbitrary Fluorescence Units

AGE .....Advanced Glycation End products

a.k.a .....also known as

Ang.....Angiotensin

ANOVA .....Analysis of Variance

APA.....Aminopeptidase A

APN.....Aminopeptidase N

APC.....Allophycocyanin

AT_1R .....Angiotensin II Type 1 Receptor

AT_2R .....Angiotensin II Type 2 Receptor

AUC .....Area Under the Curve

BFU-E .....Burst Forming Unit-Erythroid

b.i.d .....bis in die (twice a day)

BM .....Bone Marrow

BMPC .....Bone Marrow Stem/Progenitor Cell

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

BrDU.....5-Bromo-2ʿ-deoxyuridine

CAC .....Circulating Angiogenic Cell

CAR .....CXCL12-Abundant Reticular

CD .....Cluster of Differentiation

cDNA .....Complimentary Deoxyribonucleic Acid

CFU .....Colony Forming Unit

CFU-GEMM .....Colony Forming Unit-Granulocyte, Erythroid, Macrophage, Megakaryocyte

CFU-GM .....Colony Forming Unit-Granulocyte, Macrophage

CFU-Meg .....Colony Forming Unit-Megakaryocyte

cGMP .....Cyclic Guanosine Monophosphate

CVD .....Cardiovascular Disease

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

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

DAPI .....4',6-Diamidino-2-phenylindole

DC .....Aspartic Acid Decarboxylase

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

DNA .....Deoxyribonucleic Acid

DPP-4 .....Dipeptidyl Peptidase-4

ECFC.....Endothelial Colony Forming Cell

EDTA .....Ethylene Diamine Tetra Acetic Acid

ELISA .....Enzyme Linked Immunosorbent Assay

eNOS.....Endothelial Nitric Oxide Synthase

EOC.....Early Outgrowth Cell

EPC .....Endothelial Progenitor Cell

FBS .....Fetal Bovine Serum

FITC.....Fluorescein Isothyocyanate

FL.....Fluorescence Parameter

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

H&E.....Hematoxylin and Eosin

HbA1c.....Glycated Hemoglobin

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

HIF.....Hypoxia-Inducible Factor

HLI.....Hindlimb Ischemia

HPC.....Hematopoietic Progenitor Cell

HSC.....Hematopoietic Stem Cell

IB-4.....Isolectin B-4

i.p.....Intraperitoneal

IRAP.....Insulin-Regulated Aminopeptidase

IRS.....Insulin Receptor Substrate

JAK.....Janus Kinase

KDR.....Kinase Insert Domain Receptor

kitL.....Kit-Ligand

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

LDI.....Laser Doppler Imaging

LDL.....Low-Density Lipoproteins

Lin^-.....Lineage Negative

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

LPA.....Lysophosphatidic Acid

LS.....Lineage Negative and Stem Cell Antigen-1 Positive

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

MAPK.....Mitogen-Activated Protein Kinase

MasR.....Mas Receptor

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

mKitL.....Membrane Kit-Ligand

MMP-9.....Matrix Metalloproteinase-9

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

NOD/SCID.....Nonobese Diabetic/Severe Combined Immunodeficiency

Ob/Rb.....Leptin Receptor – Long Isoform

PB.....Peripheral Blood

PBMNC.....Peripheral Blood Mononuclear Cell

PBS .....Phosphate Buffer Saline

PCR.....Polymerase Chain Reaction

PE.....Phycoerythrin

PI3K .....Phosphatidylinositol 3-Kinase

qPCR.....Quantitative Polymerase Chain Reaction

RAGE.....Receptor for Advanced Glycation End Products

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

Robo.....Roundabout

ROCK .....Rho-Associated Protein Kinase

ROS.....Reactive Oxygen Species

RPMI.....Roswell Park Memorial Institute

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

s.c. .....Subcutaneous

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

SCF .....Stem Cell Factor

SCT .....Stem Cell Therapy

SDF .....Stromal Cell-Derived Factor

SHP-2.....SH2 Domain Containing Protein Tyrosine Phosphatase

sKitL	Soluble Kit-Ligand
STAT	Signal Transducer and Activator of Transcription
STZ	Streptozotocin
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
TOPCARE-AMI	Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction
VCAM	Vascular Cell Adhesion Molecule
VE-cadherin	Vascular Endothelial Cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR-2	Vascular Endothelial Growth Factor Receptor-2
VLA-4	Very Late Antigen-4
vWF	Von Willebrand Factor
WBCs	White Blood Cells
WT	Wild Type

LIST OF SYMBOLS

bp.....Base Pair

Ca.....Calcium

$CaCl_2$.....Calcium Chloride

cm.....Centimeter

^oC.....Degree Celsius

ΔCt.....Change in Cycle Threshold

dL.....Deciliter

g.....Gram

HCl.....Hydrochloric Acid

kg.....Kilogram

M.....Molar

Mg.....Magnesium

mg.....Milligram

min.....Minute

mL.....Milliliter

mM.....Millimolar

µg.....Microgram

µL.....Mircoliter

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. Diabetes Associated Cardiovascular Complications

1.1.1.1. Diabetes

Diabetes is the seventh leading cause of death in the United States and currently 9.3% of the population has diabetes (American Diabetes Association). Worldwide, one in every 11 adults has diabetes which costs 12% of global health expenditure (International Diabetes Federation). Fasting plasma glucose test and oral glucose tolerance test are the two commonly used predictors of diabetes, where the latter is the best predictor for prediabetes or type 2 diabetes (National Institute of Diabetes and Digestive and Kidney Diseases). HbA1c% estimates the average blood glucose levels over the past 3 months and it acts as a marker to predict diabetes-associated microvascular complications (1). Although tight control of blood glucose levels is optimal to prevent complications, it is not always recommended as it is often associated with hypoglycemia and weight gain leading to other morbidities, especially for children below age 13, people who are elderly, expected to live less than 10 years, or having diagnosed with other complications (American Diabetes Association).

1.1.1.2. Macro and Microvascular Complications of Diabetes

Prolonged hyperglycemia during diabetes affects all major organs of the body due to its cardiovascular complications, making it a strong risk factor for ischemic vascular diseases. The deleterious effects of hyperglycemia are commonly classified as macrovascular complications such as peripheral arterial disease, coronary artery disease, and stroke, and microvascular complications such as diabetic retinopathy, neuropathy and nephropathy (Fig. 1). More than half

of the diabetic individuals had contacted a specialist doctor regarding one of the cardiovascular complications (2). Majority of cardiovascular complications are recurrent, and resistant to conventional treatments and/or surgeries, and are the leading cause of mortality in diabetic individuals. In 2010, in United States alone, the number of emergency department visits related to diabetes-associated kidney, neurological and eye complications were 4.09, 6.75 and 1.86 million respectively (3).

1.1.1.3. Pathophysiology of Diabetes Associated Cardiovascular Complications

The common cause of diabetes associated cardiovascular complications is angiopathy which is precipitated due to chronic hyperglycemia-induced oxidative stress and inflammation (Fig. 1). Increased oxidative stress might be due to increased glucose auto-oxidation, protein glycosylation resulting in advanced glycosylation end (AGE)-product formation, polyol pathway resulting in decreased antioxidant mechanisms, and free radical production through enhanced cytosolic enzyme activity such as that of NADPH oxidase and endothelial nitric oxide synthase (eNOS) uncoupling (4; 5). Enhanced inflammation during diabetes might be due to increased AGE-receptor for AGE (RAGE) interactions and generation of pro-inflammatory cytokines (6).

The central pathological mechanism of diabetic angiopathy is endothelial dysfunction and impaired vascularization which further worsens vascular disease and promotes impaired or delayed recovery from ischemic injury (7-9). Enhanced oxidative stress and diminished availability of nitric oxide (NO) are widely accepted as prominent molecular mechanisms that actuate and accelerate diabetic vascular disease (10). In case of macrovascular disease, increased oxidative stress and inflammation in endothelial cells lead to accumulation of oxidized LDL and infiltration of macrophages forming foam cells ultimately causing atherosclerosis that damages large blood vessels and effects blood supply to the end-organ such as heart, brain or limb (11). In

case of microvascular complications, apart from above-mentioned factors various other pathophysiological mediators are involved. For instance, pericyte depletion, vascular endothelial growth factor (VEGF)-induced permeability, impaired perfusion and formation of fragile capillaries impede vision in diabetic retinopathy (12). Accumulation of extra cellular matrix and mesangial expansion occurs because of profibrotic and proinflammatory signals from macrophages and glomerular cells resulting in apoptosis of endothelial cells which leads to diabetic nephropathy (13).

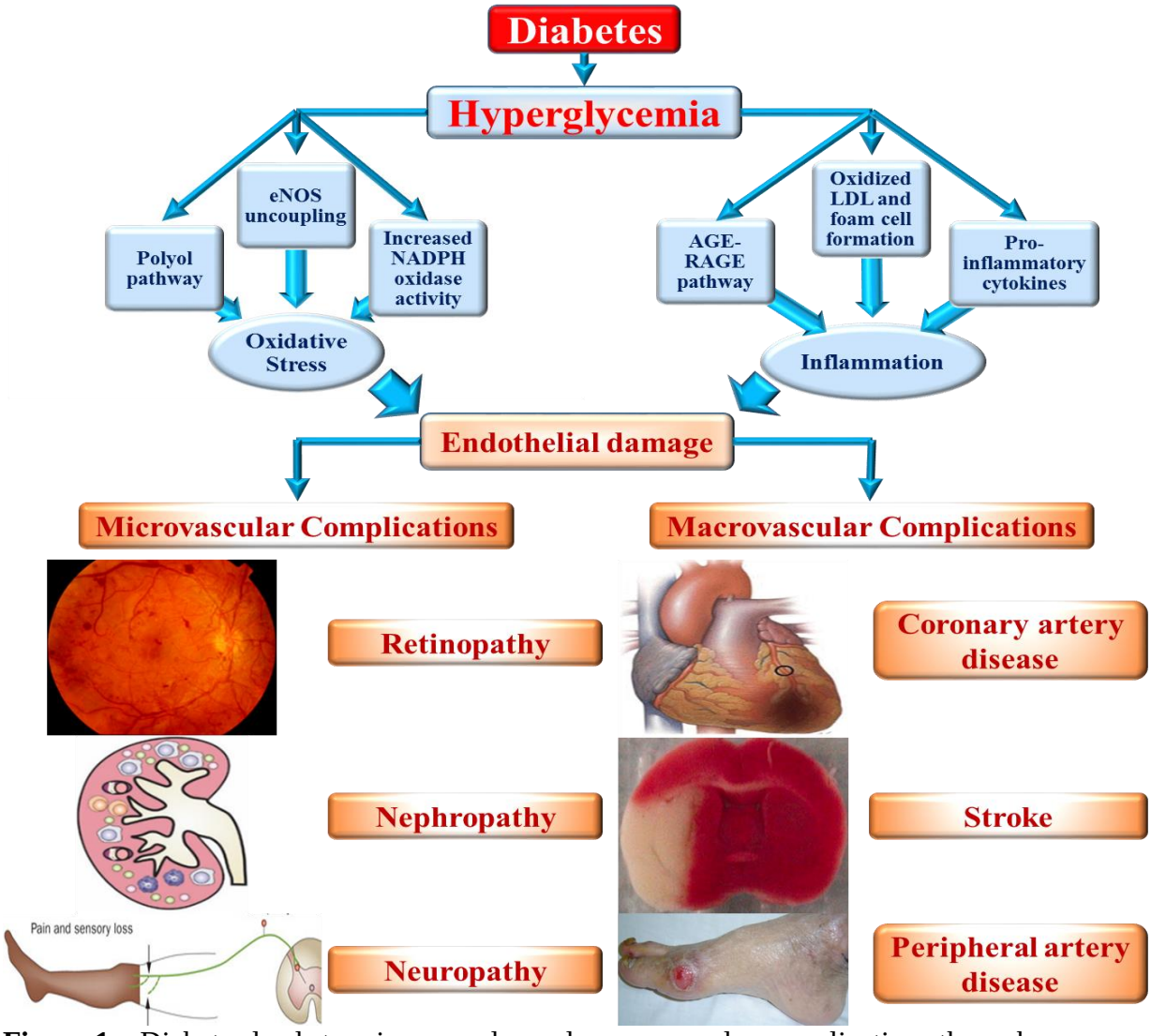


Figure 1 – Diabetes leads to microvascular and macrovascular complications through endothelial damage due to oxidative stress and inflammation. Figure adapted from (14-19).

1.1.2. Bone Marrow Stem/Progenitor Cells

Bone marrow stem/progenitor cells (BMPCs), often termed endothelial progenitor cells (EPCs), induce endothelialization, promote vascular regeneration and tissue repair (8; 20). Stem cells are the cells with unlimited self-renewal capability and can differentiate into a specific type of cell in the body. Progenitor cells have limited self-renewal capacity, which can form a limited number of differentiated cell types in the body. Whereas, EPCs are the progenitor cells which specifically form endothelial cells upon differentiation. Developmentally, hematopoietic stem cells (HSCs) or hemangioblasts can give rise to hematopoietic progenitor cells (HPCs) which form various blood cells and angioblasts that leads to the formation of endothelium. In 1990, Asahara et al. (21) first discovered the EPCs. Later, various reports demonstrated isolation of three different types of EPCs. Hill et al. (22) have modified the method of Asahara et al. (21) and demonstrated the formation of early outgrowth cells (EOCs) or CFU-Hill population by plating peripheral blood mononuclear cells (PBMNCs) on fibronectin-coated plates, removing non-adherent cells and culturing for 5 days leading to the formation of discrete colonies. Similarly, Dimmeler et al. (23) cultured the PBMNCs in supplemented endothelial growth medium for 4 to 7 days, where the cells exhibit endothelial-like characteristics but does not form colonies, which were named as circulating angiogenic cells (CACs). Late outgrowth cells or endothelial colony forming cells (ECFCs) were later developed by plating PBMNCs in collagen-coated plates, removing non-adherent cells and culturing for 7-21 days in endothelial cultured medium until the occurrence of distinctive cobblestone morphology (24; 25). These cells resemble endothelial cells and can form vessel-like structures both in vitro and in vivo.

Various BMPCs are identified by using different models defined by a specific set of markers for each cell type. To demonstrate angiogenic and vasculogenic activity of BMPCs,

researchers study EPCs or the hemangioblasts and HSCs which can give rise to EPCs and were known to have vasoreparative functions. Over the hematopoietic hierarchy, the cells might lose or gain one or more markers to differentiate. Therefore, selecting a marker which encompasses a wide range of stem and progenitor cells of a particular lineage is a practical approach. For instance, as shown in Weissman et al. (26), human long-term HSCs, multipotent and oligopotent progenitors contains common lineage-negative (Lin^- - devoid of differentiated cell markers) characteristic and $CD34^+$ marker. However, murine hematopoietic hierarchy is widely varying with common $Lin^-Sca1^+c\text{-}Kit^+$ (LSK) markers over similar lineage of cells. As summarized by Urbich et al. (27), $CD34^+CD133^+$ HSCs give rise to $CD34^+CD133^+KDR^+$ EPCs and various other EPCs with endothelial markers – vWF, CD31 and VE-cadherin expression and incorporation of Dil-Ac-LDL. Whereas, in murine studies, LSK cells themselves were used as EPCs (28; 29). Nevertheless, mice cells that expressed CD34 (30) or Sca-1 and VEGFR-2 markers were shown to be functional EPC population (31).

1.1.3. Mechanisms of Bone Marrow Stem/Progenitor Cell Mobilization

Bone marrow (BM) microenvironment plays a key role in BMPC homing and migration. BM is filled with stroma containing mesenchymal stromal cells (MSCs) and CXCL12-abundant reticular (CAR) cells. MSCs, CAR cells and osteoblasts release adhesion factors such as Stromal-derived factor-1 (SDF) or CXCL12, stem cell factor (SCF), vascular cell adhesion molecule (VCAM) and VEGF to which BMPCs express receptors such as CXCR4, c-Kit, very light antigen (VLA)-4 and VEGFR-2 respectively and reside in BM (32). In response to endothelial damage due to ischemia, tissue or vascular injury, the damaged and surrounding cells secrete cytokines such as granulocyte colony stimulating factor (G-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF) (33). These cytokines in turn stimulate BM to

release progenitors by disrupting the SDF, VCAM and/or VEGF interactions with their corresponding receptors. BMPCs enter the blood circulation through a process called 'mobilization', circulate, migrate and home to the damaged regions, and accomplish vascular repair by incorporation, endothelial transdifferentiation and paracrine angiogenic functions (34) (Fig. 2). SDF and VEGF that are generated post ischemia serve as chemoattractants and mediate mobilization of BMPCs and their homing to the ischemic regions (35; 36). Plasma-BM SDF and/or VEGF gradient determines the fate of BMPCs mobilization (37; 38).

The release of BMPCs under steady-state conditions is controlled by circadian rhythm through regulation of nor-adrenaline secretion and activation of sympathetic nervous system at regular intervals operated through core genes of the molecular clock (39). Ischemia is a strong stimulus for BMPC mobilization (40), which is largely mediated by the shift in SDF or VEGF gradient across the blood-bone marrow interface (41). Mobilization can be stimulated by pharmacological agents, called 'mobilizers'. G-CSF (33) and plerixafor (42) are the two extensively used clinical mobilizers in cell-based therapies to collect the BMPCs needed, and have been shown to improve ischemic vascular repair (20; 43-46). G-CSF is a slow mobilizer of BMPCs, binds to homodimer G-CSF receptor (47) and stimulates several pathways. It activates sympathetic nerves which stimulate MSCs and CAR cells to downregulate the release of adhesion factors. It stimulates myeloid cells such as neutrophils to release proteases such as matrix metalloproteinase (MMP)-9 which degrades the adhesion factors. It decreases the number of osteoblasts and can inhibit macrophages which have stimulatory function on MSCs. One or more of these mechanisms mobilizes BMPCs. Unlike G-CSF, plerixafor is a fast acting and potent mobilizer and acts via a single known mechanism; antagonizing CXCR4, a receptor for SDF, thereby releasing BMPCs (32). The process of mobilization of stem/progenitor cells

and the sites of action of G-CSF and plerixafor are depicted in Fig. 2. Cells at various stages of differentiation are mobilized by these pharmacological agents. In addition, G-CSF induces proliferation and differentiation in wide variety of cell types (33). The role of various white blood cells (WBCs) is undeniable in reparative functions, as they release cytokines and other inflammatory mediators at the injured site which will facilitate the recruitment of stem/progenitor cells along with other inflammatory cells (48).

1.1.4. Cell-Based Therapy

Cell-based therapy or stem cell transplantation (SCT) is a novel and promising approach for treating cardiovascular diseases (CVDs). Recent studies and clinical trials indicate that autologous SCT is being actively explored for the treatment of CVDs (49-53). Briefly, the process involves mobilization of BMPCs from an individual using a pharmacological mobilizer, collection of peripheral blood, isolation, purification or enrichment of BMPCs, and their administration back to the individual to treat CVDs. The yield of BMPC collection is an important determinant of the final therapeutic outcome (54). Often, to obtain enough cells and enhance therapeutic outcomes, EPCs are culture expanded ex-vivo, and using this method have shown potent vasoreparative effect (55; 56). As this method alters the phenotypic characteristics and there are no corresponding cells in vivo, enumerating LSK cells in samples obtained from mice would give a better estimate of vasoreparative EPCs.

SCTs are popular choice in treating cancers such as lymphoma and leukemia, several blood and immunological diseases (57; 58). Following radiation or chemotherapy, autologous cells are transplanted to the same individual from whom the cells are collected and allowed for reconstitution. Although their success is preliminary in cardiovascular treatments, there is a huge

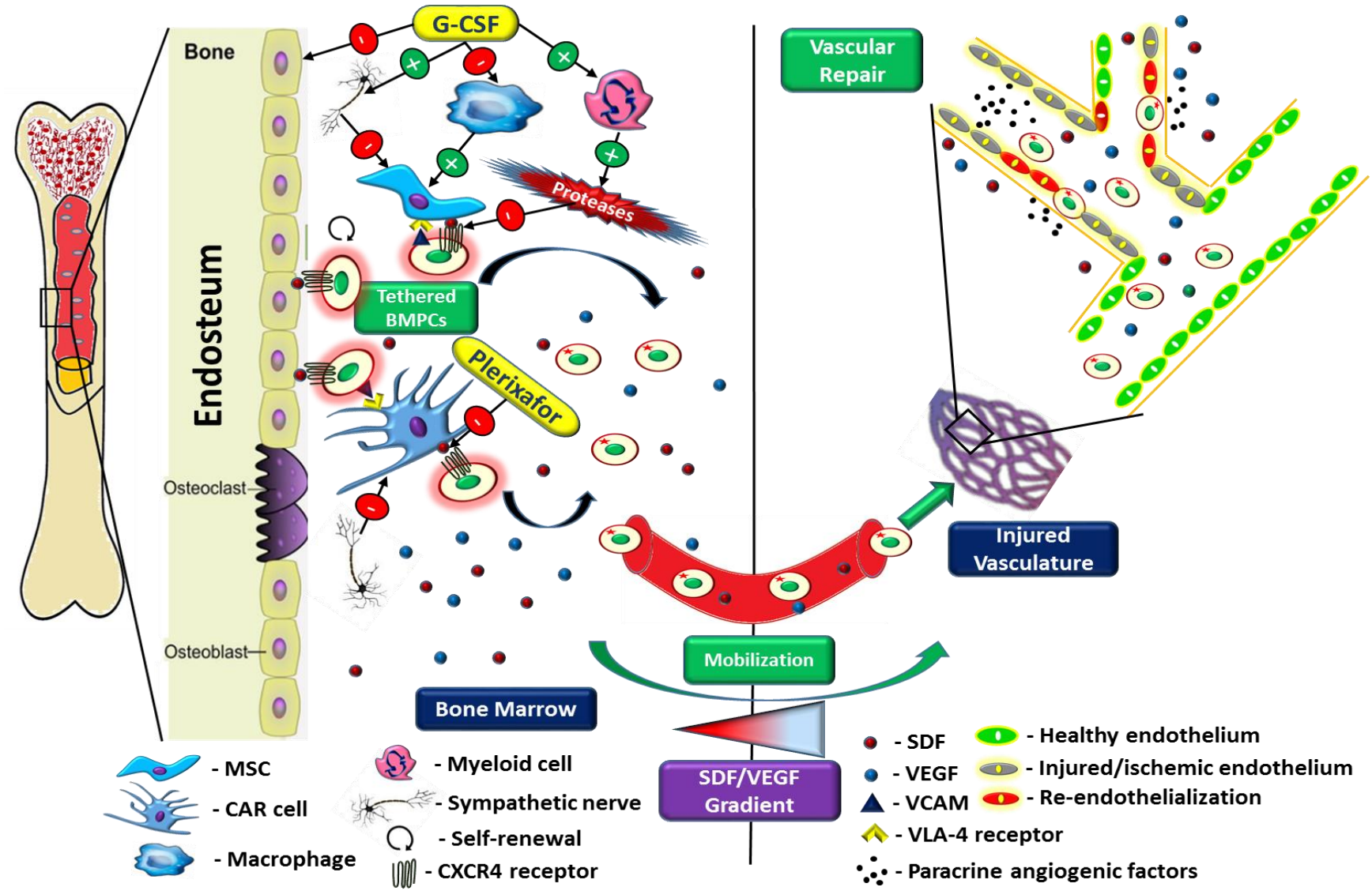


Figure 2 – Stem/Progenitor cell mobilization process depicting the targets for G-CSF and Plerixafor, and vasoreparative functions of BMPCs. Arrows with '-' sign indicates inhibition and '+' sign indicates activation. Figure adapted from (32; 59; 60).

preclinical promise of these therapies and have shown potential in few clinical studies, especially in peripheral arterial disease with positive outcomes in other CVDs. In TOPCARE-AMI trial, intracoronary administration of MNCs or BMPCs showed improvements in left ventricular ejection fraction, reduction in infarct size, and decreased end-systolic diastolic volume in acute myocardial infarction patients (61). Bone marrow derived progenitors were shown to improve left ventricular contractile function in REPAIR-AMI trial (62). Intracoronary administration of MNCs might require administration of cells at regular intervals as demonstrated by BOOST trial (63; 64). According to REGENT trial, the dose of selected $CD34^{+}CXCR4^{+}$ cells is 100 times less than the unselected population of cells for the same positive outcomes in acute myocardial ischemic patients (65) reaffirming the potency of BMPCs. Peripheral arterial disease or critical limb ischemia also strikingly responded to the intramuscular injection of unspecialized bone marrow derived cells or $CD34^{+}$ cells, characterized by improved ankle-brachial index, transcutaneous oxygen tension, collateral vessel formation, blood flow, and pain-free walking time (66; 67). These studies corroborate the inherent capacity of BMPCs to perform vasoreparative functions and treat ischemic diseases.

Currently, several studies are standing at different stages of clinical trials ranging from phase 1 to phase 4 which are aimed at CVDs using whole BM mononuclear cells (MNCs), stem/progenitor cells, or pharmacological interventions that can improve the functions of stem/progenitor cells and have shown positive therapeutic outcomes as described in the Table 1.

G-CSF and/or plerixafor-mobilized BMPCs showed promising results in pre-clinical CVD and wound healing models (46; 68-72). G-CSF was extensively used as mobilizer in clinical CVDs when compared to plerixafor, however, shown to be less beneficial (73-75) which could be attributed to decreased adhesion molecules on mobilized BMPCs that might lead to

Table 1 – Published clinical trials using stem/progenitor cells or improving their functions aimed to treat CVDs and having positive therapeutic outcomes.

Name of the trial	Phase	Condition	Type of stem/progenitor cells	Primary outcomes	References
FOCUS-HF	Phase 1	Ischemic cardiomyopathy	Autologous BMMNCs	Improved quality of life and cardiac perfusion	Perin et al. (76)
FOCUS-Br	Phase 1	Coronary artery disease	HSCs	Improved perfusion and functions of myocardium	Perin et al. (77)
TICAP	Phase 1	Hypoplastic left heart syndrome	Cardiac progenitor cells	Improved right ventricular ejection fraction, and reduced incidence of heart failure	Tarui et al. (78)
TAC-HFT	Phase 2	Left ventricular dysfunction	Autologous BM cells	Improved left ventricular ejection fraction	Trachtenberg et al. (79)
Safety and efficacy of stem cell transplantation to treat dilated cardiomyopathy	Phase 2	Dilated cardiomyopathy	$CD34^+$ cells	Improved survival, ventricular remodeling and exercise tolerance	Vrtovec et al. (80)
Effects of Atorvastatin on EPCs after coronary surgery	Phase 4	Coronary artery bypass surgery	$CD34^+VEGFR\text{-}2^+CD133^+CD45^-$ EPCs	Increased circulating EPCs, preserved sinus rhythm, reduced hsCRP	Baran et al. (81)
Effect of Nebivolol on oxidative stress and EPCs	Phase 4	Hypertension	$CD34^+CD133^+$ EPCs	Increased circulating EPCs	Hayek et al. (82)

reduced homing capacity (83), which might also be true in case of plerixafor (84). Nevertheless, recently approved combinatorial therapy using G-CSF and plerixafor (85), acute administration of these agents (44; 70), or ischemic pre-conditioning of cells (86) after collection might promote

vasoreparative functions of BMPCs. Therefore, for successful SCTs the response of the individuals to these mobilizers is essential.

1.1.5. Bone Marrow Mobilopathy and Failure of Cell-Based Therapies in Diabetic Conditions

The condition in which mobilization of stem/progenitor cells fails or less than adequate in response to a stimulus such as ischemia or clinical mobilizing agent is called 'bone marrow mobilopathy'. The population in which BM mobilopathy occurs is called 'poor mobilizers'. SCTs frequently fail in poor mobilizers as it is difficult to obtain enough number of cells for therapy. There are several risk factors involved in poor mobilization (87-89), where diabetes is one of them. Diabetes associated CVDs are possibly best treated with SCTs, however, severe bone marrow mobilopathy caused by chronic diabetes, affects BMPC yield and subsequently makes cell-based therapies less feasible in diabetic individuals (8; 9). In addition, the vascular repairing functions of BMPCs derived from diabetic individuals are impaired, which mars the process of endothelial regeneration and vascularization by progenitor cells (7; 90-92). A retrospective analysis of SCTs performed in Parma Bone Marrow Transplantation Unit during 2004 to 2008 period has shown a correlation between mobilization failure of $CD34^+$ cells in response to G-CSF treatment and diabetes. This was examined in experimental diabetic mice models which simulated clinical mobilopathy characterized by BM niche dysfunction – derailed sympathetic nervous system, aberrant localization of BMPCs, and non-responsiveness to G-CSF but not plerixafor (93). In a phase 4 clinical trial, the diabetes effect on mobilopathy was further supported, where mobilization of HSCs and EPCs, and angiogenic capacity of PBMNCs in response to human recombinant G-CSF were lowered in diabetic individuals when compared to non-diabetics which might be due to dysregulated dipeptidyl peptidase (DPP)-4 that can degrade

SDF (94). Recently, increased adhesion signaling of α1β4 integrin in EPCs was shown to be a cause of mobilopathy in diabetes (95).

Apparently, the ideal treatment for diabetic vascular disease will be accomplished by pharmacological strategies that can reverse BMPC-mobilopathy and improve vasoreparative functions of BMPCs, which promote therapeutic angiogenesis and/or vasculogenesis – enhancing the blood flow recovery and tissue repair following ischemic insult (96; 97).

1.1.6. Renin Angiotensin System

The role of Renin Angiotensin System (RAS) is well characterized in several cardiovascular disorders. RAS has two 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. RAS involves several enzymes, bioactive peptides and their receptors (Fig.3). Angiotensinogen, a dodecapeptide, is synthesized by liver and is converted to Ang I, a decapeptide by the enzyme renin which is synthesized by juxtaglomerular cells of the kidney. Ang I is further cleaved to from Ang II, an octapeptide and a potent vasoconstrictor. Alternatively, Ang I through the action of Neprilysin (NEP), ACE, and ACE2 can be cleaved to form Ang-(1-7), a heptapeptide. Whereas, Ang-(1-7) is formed predominantly by the action of ACE2 enzyme on Ang II substrate (98; 99). Ang II is further cleaved to form Ang III by the action of aminopeptidase A (APA), which in turn forms Ang IV by the action of aminopeptidase N (APN) enzyme. Ang-(1-7) can further lead to the formation of Ang-(1-5) or Ala^1-Ang-(1-7) by the action of enzyme ACE or aspartic acid decarboxylase (DC) respectively. Ang II and Ang III exerts their actions through AT_1R and AT_2R, where the former is responsible for cardiovascular protective and the later for cardiovascular detrimental effects. Ang-(1-7) acts on MasR and was shown to be cardiovascular

protective. Ala[1]-Ang-(1-7) and Ang IV recognizes Mas-related G-protein coupled receptor member D (mRG-D) and insulin-regulated aminopeptidase (IRAP) respectively (100). mRG-D receptor signaling was shown to have vasorelaxant effects (101).

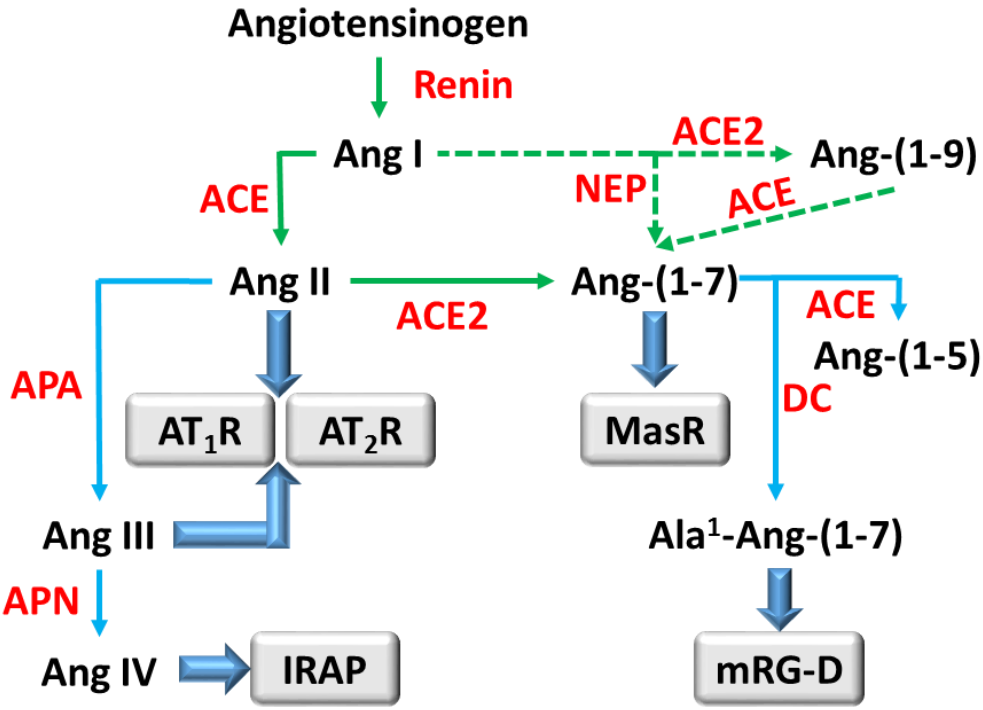


Figure 3 – Biosynthetic pathways of various peptides involved in renin angiotensin system and their receptors. Enzymes and receptors involved are highlighted in red color and grey boxes respectively. Ang-(1-7) biosynthesis is highlighted with green arrows, where the dashed lines indicate minor pathways and solid lines indicate predominant pathways. Adapted from Chappell (100).

ACE/Ang II/AT_1R pathway is aggravated during hypertension, congestive heart failure and other cardiovascular detrimental conditions, which is frequently and successfully targeted to reverse these pathologies (102). Whereas, targeting ACE2/Ang-(1-7)/MasR axis to treat CVDs is still in experimental stage and activating this system has potential therapeutic benefits (103). In fact, beneficial effects of ACE inhibitors in CVDs might also be due to increased circulating levels of Ang-(1-7) through enhanced metabolism of accumulated Ang I by ACE2 enzyme or decreased metabolism of Ang-(1-7) (104). Therefore, activating ACE2 and inhibiting ACE is

clinically beneficial while treating CVDs. Experimental studies have shown that ACE2 overexpression improves cardiac functions and morphology during heart failure (105; 106). Clinically, ACE2 was overexpressed in CVDs, but ACE enzyme is expressed more than ACE2 when compared the fold changes (107), thus nullifying the protective effects. Therefore, higher ACE2 in relation to ACE is cardiovascular protective through enhanced Ang-(1-7)/MasR signaling.

Mas is first discovered in 1986 as a proto-oncogene (108) however the tumorigenic potential was not supported by later studies. It was shown to encode a novel angiotensin receptor in 1988 (109). Until 2001, Mas was thought to modulate intracellular signaling of Ang II signaling through AT_1R (110). After 17 years of its discovery, Mas was identified as putative receptor for Ang-(1-7) and was also shown to activate phospholipase A2/arachidonic acid pathway which can be blocked by A779, a MasR antagonist (111). Another predominant signaling cascade activated by Ang-(1-7)/MasR signaling is phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which induces eNOS phosphorylation, thereby enhancing NO release in endothelial cells (112). These pathways are mainly responsible for vasorelaxant effects of Ang-(1-7). In few cases, Ang-(1-7) was also shown to act through AT_2R (113) but these effects might also need MasR interaction with AT_2R (114). MasR mediated modification of phosphorylation of certain signaling networks such as PI3K/Akt, JAK/STAT, and insulin receptor substrate (IRS) proteins interacts with insulin signaling and improve insulin sensitivity and glucose uptake (115-117). In addition, Ang-(1-7) binds to MasR on fibroblasts and exerts antifibrotic and antihypertrophic effects, thereby antagonizing Ang II-induced detrimental effects (118). This antagonistic effect might involve activation of SHP-2, a protein tyrosine phosphatase that may

inhibit AT_1R-mediated src and mitogen-activated protein kinase (MAPK) activation (119). Ang-(1-7) decreases oxidative stress by inhibiting NADPH oxidase enzyme (120).

1.1.7. Renin Angiotensin System in Bone Marrow

Along with circulating RAS, there is an increased evidence of local RAS that might involve in the homeostasis of various tissues and organs, including bone marrow (121). Proliferation, differentiation, and maturation of various stem/progenitor cells, along with their functions such as mobilization, incorporation into ischemic/injured tissues, and paracrine functions might be affected by local RAS (122; 123). BM RAS is involved in hematopoiesis (121; 124; 125). Both ACE/Ang II/AT_1R and ACE2/Ang-(1-7)/MasR axes promote functions of BMPCs. Ang II and Ang-(1-7) enhance recovery after mild myelosuppression (126; 127). In accordance with this, Ang II was shown to increase BMPCs and long-term HSCs in BM. Moreover, it increased untimely proliferation and differentiation, and decreased engraftment potential of donor HSCs leading to poor reconstitution (128). Unfortunately, Ang II-induced hypertension was shown to be associated with increased proinflammatory cells and decreased EPCs (129). In agreement with this, Ang II chronic infusion was shown to decrease in vitro EOC formation, and deplete in vivo EPCs by enhancing ROS-induced apoptosis (130). In contrast, short-term (< 5 days) administration of Ang II decreased apoptosis, increased NO, adhesion potential, VEGF-induced proliferation and network formation in EPCs (131; 132). Thus, although short-term enhanced ACE/Ang II/AT_1R arm is beneficial, chronic activation will be detrimental.

On the other hand, ACE2/Ang-(1-7)/MasR axis has similar stimulatory effects on BMPCs which in turn promotes cardiovascular regenerative effects without the harmful effects even after chronic activation (133). We and other labs have demonstrated this in various studies

(122; 134-136). Importantly, a recent study from our lab showed that ACE2 is expressed more than ACE in human $CD34^+$ cells and mice Lin^- cells (137). In an experimental stroke model, EPC protective functions were enhanced by ACE2 overexpression via modulation of NADPH/ROS and eNOS/NO signaling pathways, thereby reducing ischemic brain injury and promoting recovery (138). Pharmacological activation of ACE2 by diminazene aceturate showed functional improvement in angiogenic progenitor cells in a rat model of pulmonary hypertension (139). Notably, ACE2 expression was found to be lower in $CD34^+$ cells obtained from diabetic individuals (135), which could be an underlying cause of poor reparative functions of BMPCs. Therefore, diabetic BM mobilopathy and/or vasoreparative functions of BMPCs can be improved by enhancing ACE2/Ang-(1-7)/MasR arm of RAS.

Studies have shown the expression of Mas receptors on bone marrow stromal cells and hematopoietic cells (127; 140). Rodgers et al. (126; 127) have shown that hematopoietic recovery after chemotherapy in mouse model is enhanced by angiotensin peptides in multiple cell lineages. Ellefson et al. (141) have shown that after 5-flurouracil induced myelosuppression in mice, Ang-(1-7) treatment increased the number of early hematopoietic progenitors that bind to it, and increased circulating leukocytes and platelets. Subclinical Neupogen administration has synergistically increased circulating leukocytes with Ang-(1-7). Furthermore, Ang-(1-7) has impact on all hematopoietic lineages confirmed through increase in BFU-E, CFU-Meg, CFU-GM and CFU-GEMM colonies derived from progenitors of myelosuppressed mice bone marrow. Heringer-Walther et al. (142) have shown that Ang-(1-7) enhances proliferation of human mononuclear cells (MNCs) and $CD34^+$ cells. This study also shows the impact of Ang-(1-7) on hematopoiesis, which increases proliferation and differentiation of human cord blood MNCs to form hematopoietic cells in NOD/SCID mice. Rodgers et al. (143) have shown that daily

treatment with Ang-(1-7) after total body irradiation improved survival and reduced bleeding time (reduced thrombocytopenia) in mice. Another study from Rodgers et al. (144) have shown that Ang-(1-7) reduces the dose of Neupogen required for the optimal hematopoietic recovery by 10-fold. Also Epogen act synergistically with Ang-(1-7) to increase erythroid progenitor cells in the bone marrow. Thus, Ang-(1-7) enhances the functional properties of BMPCs to proliferate, differentiate and migrate into the circulation to participate in vasoreparative functions.

Importantly, Ang-(1-7) improves the reparative functions of BMPCs mainly by reversing bone marrow oxidative stress, increasing NO bioavailability in BMPCs derived from experimental diabetes (145) or in $CD34^+$ cells obtained from diabetic individual (135). Wang et al. (146) have shown that circulating Ang-(1-7) enhances cardioprotection after myocardial infarction in rat model, decreased cardiac hypertrophy, and improved cardiac function by increasing c-kit- and VEGF-positive cells in infarcted hearts. Ang-(1-7) stimulated in vitro proliferation of rodent EPCs is blocked by Mas receptor antagonist and this beneficial effect was not observed in the cells obtained from Mas receptor knock out (MasR-KO) mice. Jarajapu et al. (135) have shown that vasoreparative dysfunction of $CD34^+$ cells isolated from peripheral blood of diabetic individuals was improved by Ang-(1-7) treatment. NO bioavailability/cGMP response and migration to stromal cell derived factor (SDF) were restored, and attenuation of NADPH oxidase activity was achieved. Furthermore, the survival and proliferation of these cells was enhanced through Mas/PI3K/Akt-dependent pathway. Overexpression of Ang-(1-7) in these cells also improved in vitro vasoreparative functions and homing efficiency to areas of ischemia, in vivo. To further support the role of Ang-(1-7) in treating cardiovascular disease in diabetes, a recent study by Papinska et al. (147) have shown that short-term Ang-(1-7) treatment improved cardiac function, by reducing cardiac hypertrophy, fibrosis and infiltration of inflammatory cells

into heart tissue, and by increasing angiogenesis in young db/db mice. It also prevented renal glomerular damage and oxidative stress. Along with these beneficial effects, simultaneous increase in circulating EPCs and MSCs was observed. These effects were blocked by Mas receptor antagonist and inhibition of NO formation.

1.2. Objective and Specific Aims

Long-term diabetes is a risk factor for cardiovascular disease. Bone marrow transplantation therapies use mobilized BMPCs to treat various conditions including cardiovascular disease. Recent studies have shown that the defective BMPC mobilization and their function induced by diabetes play a key role in the progression of cardiovascular disease. In response, clinical trials of BMPC transplantation therapies to treat cardiovascular disease in diabetic patients are frequently failed. It has now been documented that BMPC mobilization to clinically used mobilizers or to vascular damage is defective in experimental or clinical diabetes, a condition known as 'diabetic stem cell mobilopathy'. BMPC transplantation therapies being the novel promising treatment option for cardiovascular disease, **development of novel pharmacological approaches to enhance the BMPC mobilization is urgently needed for enhancing reparative functions of cell-based therapies in diabetes**.

1.2.1. Objective

To test the effects of Ang-(1-7) on stem cell mobilopathy and vasoreparative outcomes of BMPCs in diabetic conditions.

1.2.2. Hypothesis

I hypothesized that increasing Ang-(1-7) levels restores mobilization and vasoreparative functions of BMPCs in diabetes, and that the Slit/Robo/ROCK pathway mediates mobilization by Ang-(1-7)/Mas receptor.

1.2.3. Specific Aims

To test the hypothesis, I proposed the following specific aims, which were depicted in the project overview (Fig. 4).

1.2.3.1. Specific Aim 1: To test the hypothesis that Ang-(1-7)-induced mobilization of BMPCs involves Slit/Robo/ROCK pathway

In this aim, I determined whether Ang-(1-7) modulates the expression and/or activity of Slit and Robo proteins in diabetic mouse bone marrow (BM) leading to activation of RhoA/ROCK pathway, thus modifying the interactions between stromal cells and BMPCs.

1.2.3.2. Specific Aim 2: To test the hypothesis that Ang-(1-7) reverses diabetic stem cell mobilopathy in response to G-CSF and plerixafor

In this aim, I tested whether Ang-(1-7) improves the defective mobilization of BMPCs in response to clinically used mobilizers, G-CSF or plerixafor, in experimental diabetes.

1.2.3.3. Specific Aim 3: To test the hypothesis that Ang-(1-7) reverses diabetic impairment in ischemia-induced mobilization and vascular repair

In this aim, I tested whether Ang-(1-7) will improve the ischemia-induced mobilization, neovascularization, and blood flow recovery in diabetic mice with hindlimb ischemia.

These studies, if proven, will provide a novel mechanism-based pharmacological approach to stimulate BMPC mobilization, and to enhance the outcomes of cell-based therapies in diabetes.

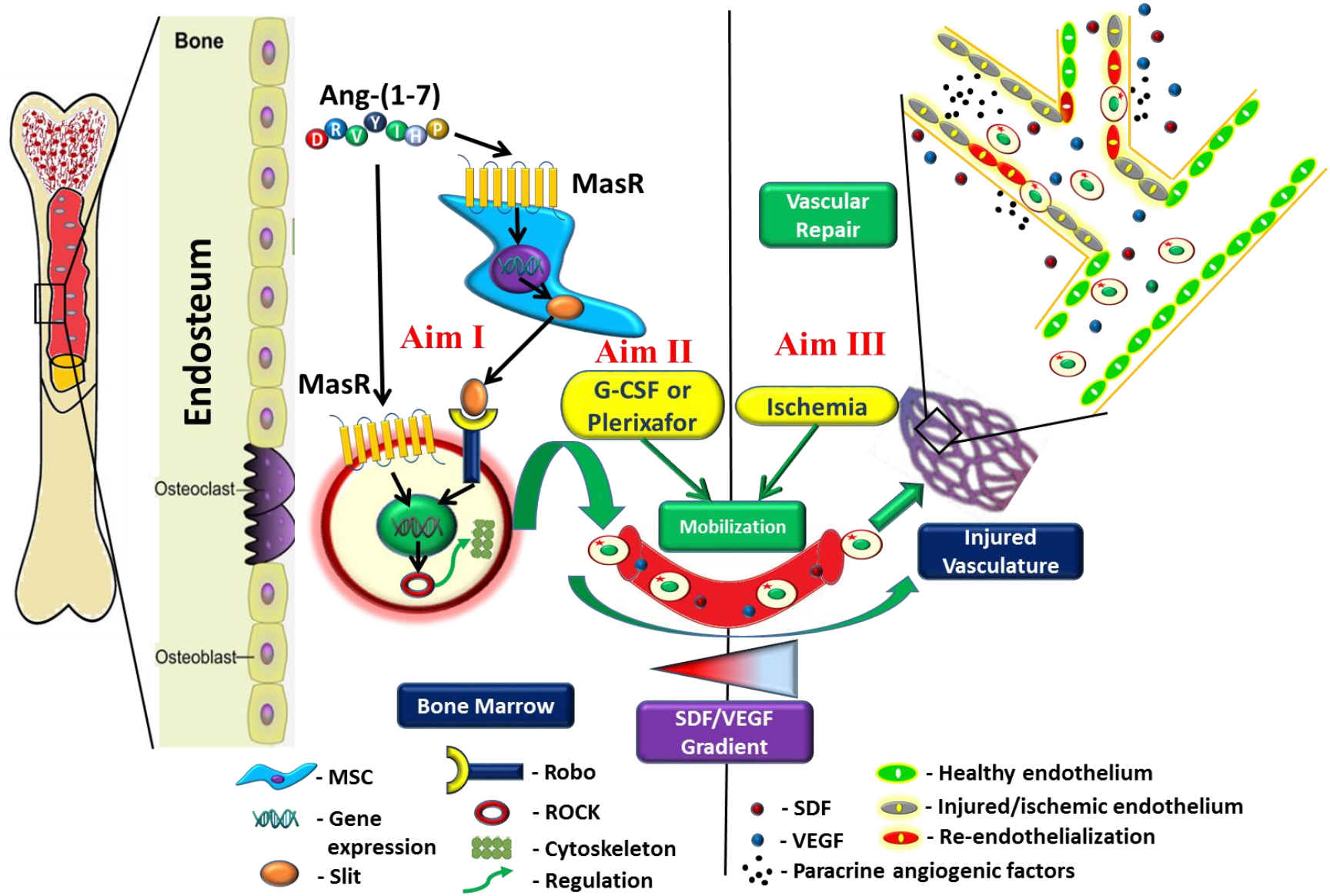


Figure 4 – Project overview. Figure adapted from (59).

1.3. Materials and Methods

1.3.1. Animal Models

All animal studies were approved by the Institutional Animal Care and Use Committee at North Dakota State University. Male C57BL/6NHsd mice were purchased (Harlan Laboratories, USA) and made diabetic by streptozotocin (STZ) injections as described in previous studies (148). Type 1 diabetes was induced using STZ injected at a dose of 50 mg/kg, i.p., dissolved in ice cold 50 mM citrate buffer at pH 4.5, for five consecutive days. Animals were kept on fasting for four hours prior to the injection. Control mice received citrate buffer vehicle. Mice having the blood glucose levels greater than 300 mg/dL were considered diabetic. BKS.Cg-*Dock7*^{m} +/+ *Lepr*^{db}/J diabetic mice (db/db) with homozygous spontaneous mutation to leptin receptor and BKS.Cg-*Dock7*^{m} +/+ *Lepr*^{db}/J non-diabetic litter mate control mice (lean) with heterozygous spontaneous mutation to leptin receptor were purchased (Jackson Laboratory, Bar Harbor, ME, USA). All mice were maintained on a 12-hour light-dark cycle with food and water *ad libitum*. Mice with 8-12 weeks and 20-24 weeks of hyperglycemia were considered as short- and long-term diabetic respectively.

Wild type (WT) and MasR-KO mice of C57BL/6 background, generated by the trans-NIH Knock-Out Mouse Project (KOMP) and obtained from the KOMP repository, were bred in our animal facility.

1.3.2. Glucose Tolerance Test, Blood Glucose and HbA1c Estimation

Glucose was measured in blood samples collected from mice tail veins by using blood glucose test strips (Clarity Plus, Diagnostic Test Group). For glucose tolerance test, mice were fasted overnight, dextrose was injected at a dose of 2 g/kg, i.p., and blood glucose was measured

at 0, 15, 30, 60 and 120 min. after administration. HbA1c% was measured in blood samples collected from mice tail veins by using A1C Now^{+} HbA1c measurement kit (Bayer).

1.3.3. Experimental Protocols

Type 1 or type 2 diabetic mice along with their respective control mice were divided into four groups consisting of two groups of control mice and two groups of diabetics, which were randomly assigned for saline, Ang-(1-7) (Bachem) (149), or A779 (Bachem) (150) treatments, with the dose comparable to previous studies. Ang-(1-7) (1 µg/kg/minute), A779 (0.5 µg/kg/minute), or vehicle, sterile normal saline, was administered by continuous subcutaneous infusion by using osmotic pumps (Alzet; model number 1004) for four weeks, implanted in dorso-cervical area. Peripheral blood samples were collected at the end of the treatment from retro orbital sinus under isoflurane anesthesia and were processed for flow cytometry and/or colony forming unit assay as described below. Mice were euthanized by thoracotomy under anesthesia at the end of the study, bones were isolated from hind-limbs and bone marrow was flushed out and processed as described below.

Diabetic or Ang-(1-7) treated diabetic mice were treated with fasudil or normal saline at a dose of 30 mg/kg; i.p. for 14 days (151). Peripheral blood samples were collected after the end of treatment and processed for flow cytometry to enumerate LSK cells.

Mobilization of BMPCs was induced by using single dose administration of plerixafor (AMD3100, Tocris) at 5 mg/kg, s.c. (152), or 4 days b.i.d. administration of G-CSF (Peprotech) at a dose of 125 µg/kg, s.c. (153). Peripheral blood samples were collected before the treatment, after 1 hour for plerixafor treatment and after 12 hours of last dose administration of G-CSF.

For in vitro studies, wherever applicable, cells were treated with 100 nM of Ang-(1-7) or A779, 30 nM of SDF (Peprotech) or VEGF (R&D Systems), 10 µM of Y-27632 (EMD Millipore), 400 ng/mL of Slit2 or Slit3 (Biomatik), and 3 nM of lysophosphatidic acid (LPA) (Cayman Chemical Company).

1.3.4. Isolation of WBCs from Peripheral Blood or Bone Marrow

Peripheral blood was collected from anesthetized (Isoflurane, Terrell, USA) mice through retro-orbital plexus in EDTA-coated microcentrifuge tubes. Erythrocytes were lysed by mixing the blood with the solution of 0.8% ammonium chloride and 2 mM EDTA, incubated in ice for 10 minutes with occasional mixing. After the lysis of erythrocytes cell suspension was washed thrice by using 1X Phosphate Buffered Saline (PBS) (Corning Cellgro) and centrifuged at 1200 rpm once and 120g twice (Eppendorf Centrifuge 5804R). The supernatant was discarded, resuspended the pellet and counted the total number of WBCs after final wash using Neubauer Chamber. Mice were euthanized after anesthesia (Isoflurane, Terrell, USA) by intra cardiac bleeding. Femurs and tibias were collected from each mouse in ice cold PBS. The bones were flushed with ice cold PBS without Ca and Mg (Corning Cellgro) and single celled suspension was made after pipetting several times. The suspension was filtered through 40 µm filters to remove bone spicules and other contaminants. Erythrocytes were lysed as described above and proceeded for cell count using Neubauer Chamber.

1.3.5. Flow Cytometry

Isolated WBCs from each sample of peripheral blood or bone marrow were suspended in cell staining buffer (Biolegend) and centrifuged (Eppendorf) at 1200 rpm for 10 minutes in 5 mL polystyrene tubes (BD Falcon). Supernatant was removed carefully and added 0.5 µL of Trustain (BioLegend), mixed gently and incubated at 4^0C for 10 minutes to mask non-specific receptors.

Required dilutions of fluorescent conjugated antibodies, Lineage cocktail-FITC, Sca1-APC, c-Kit-PE (BioLegend) were prepared, added to the tubes, mixed gently and incubated for 45 minutes at 4^{o}C in dark. Samples were then washed with 1X PBS (Corning Cellgro) to remove excess antibodies. 7-AAD (BD Pharmingen) was added to detect dead cells. Non-stain sample, with isotype controls was simultaneously prepared. Samples were mixed gently and run using flow cytometer (Accuri C6), and were analyzed for $Lin^{-}Sca1^{+}c\text{-}Kit^{+}$ (LSK) cells, according to the gating strategy shown in the Fig. 5 (154). These cells have vasoreparative functions with highest endothelial cell lineage development potential and have been shown to be vasculogenic in mice (155; 156). This cell population was enumerated in basal, drug- or ischemia-induced mobilization conditions. Other sub populations such as LS or LK cells have comparable reparative functions, and are mobilized in response to ischemic injury (21; 22).

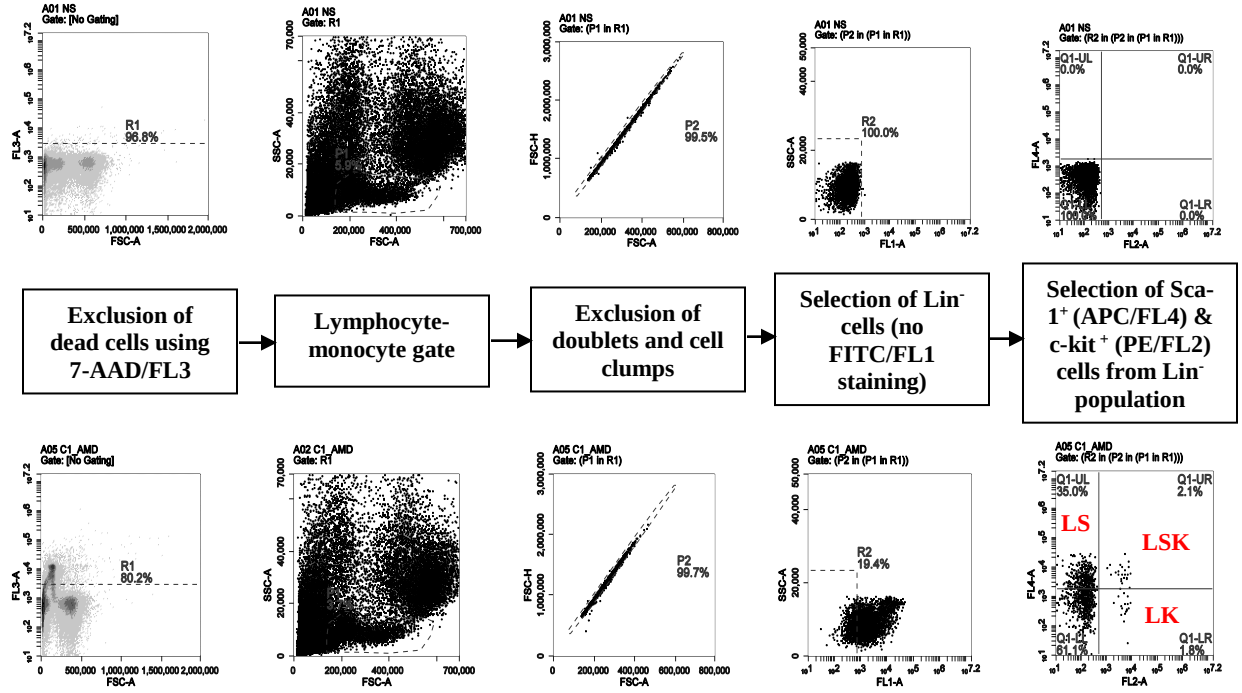
1.3.6. Colony Forming Units (CFU) Assay

WBCs were isolated from peripheral blood or bone marrow. Methocult GF M3434 (StemCell Technologies Inc.) was added to the required number of WBCs (1-3X10^{5} from PB or 2-3X10^{4} from BM), plated and incubated for 10 days at 37^{o}C as per manufacturer's instructions. CFU-GM (Colony-Forming Unit-Granulocyte, Macrophage), BFU-E (Burst-Forming Units-Erythroid) and CFU-GEMM (Colony-Forming Unit-Granulocyte, Erythroid, Macrophage, Megakaryocyte) (Fig. 6) were identified and counted using an inverted microscope.

1.3.7. Isolation of Bone Marrow LSK cells

Lineage-negative (Lin^{-}) cells were isolated by negative selection by using immunomagnetic enrichment kit (Stemcell Technologies Inc.). Briefly, bone marrow monocyte cell suspension was prepared in recommended medium at required dilution, incubated with antibodies for binding CD5, CD11b, CD19, CD45R, 7–4, Ly-6G/C (Gr-1), and TER119-

A. Sample with isotype control



B. Sample with fluorescent-conjugated antibodies

Figure 5 – Gating strategy used for flow cytometric enumeration of mouse LSK cells.

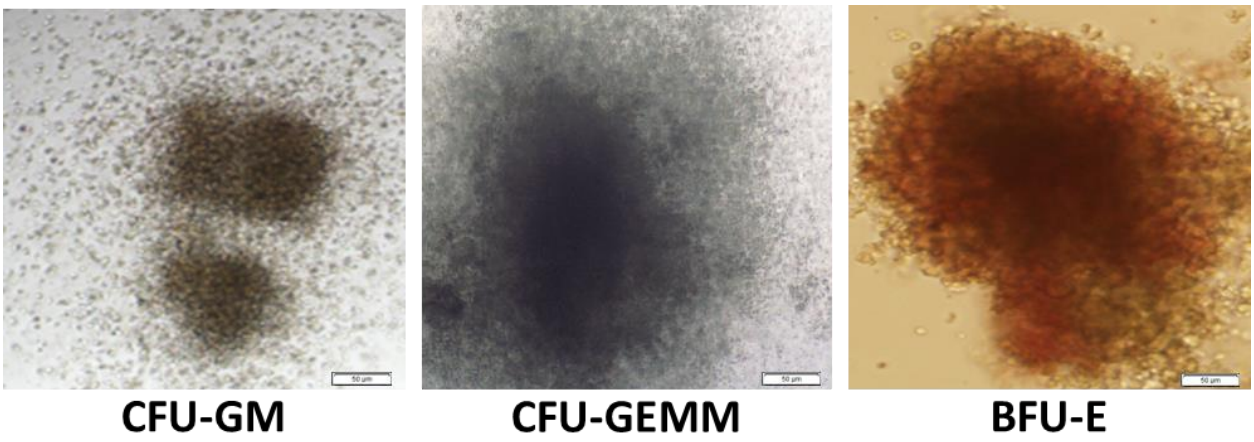


Figure 6 – Representative colonies of blood cells that are observed in Methocult GF M3434 assay. CFU-GM – Colony Forming Unit – Granulocyte, Macrophage; CFU-GEMM - Colony Forming Unit – Granulocyte, Erythroid, Macrophage, Megakaryocyte; and BFU-E – Burst Forming Unit - Erythroid. Scale bar = 50 µm.

expressing cells. These cells are then labeled by Tetrameric Antibody Complexes that recognize biotin and dextran-coated magnetic particles and antibody bound cells were then separated by using EasySep™ magnet, thus obtaining Lin^- cells. Lin^- cells were subsequently enriched for $Sca1^+c\text{-}Kit^+$ cells by using positive immunomagnetic selection kit (Stemcell Technologies). LSK cells were then plated in RPMI1640 (GE Healthcare) in U-bottom, 96-well plate at a low density of $2X10^4$ cells/150 µL per well, until they are used for proliferation or migration assay for less than 24 hours following isolation. This set of experiments was performed by my colleague, Mr. Shrinidh Joshi.

1.3.8. Isolation of Bone Marrow Supernatant Culture of Mouse Bone Marrow Mesenchymal Stromal Cells

Bone marrow of each mouse were flushed using ≤ 1 mL of sterile 1X PBS, on ice cold surface. A single cell suspension was made by pipetting, which was filtered through 40 µm sterile filter to remove debris and bone marrow spicules. The cell suspension was centrifuged at 1200 rpm for 10 min at 4ºC. Supernatant was collected and again centrifuged at maximum speed at 4ºC to remove any remaining cells and the subsequent supernatant was stored at -80ºC.

Following erythrocyte lysis in bone marrow cell suspension, the cell pellet was resuspended in RPMI 1640 media supplemented with 15% FBS and 1X penstrep (100 IU/mL penicillin & 100 µg/mL streptomycin) and were plated in 24 well culture plates at a concentration of $4X10^6$ cells/cm^2 of surface area. Plates were incubated at 37ºC with 5% CO_2. Media containing non-adherent cells was discarded after 24 hours and replaced with fresh media and old media was replaced every week. Adherent cells were cultured at least for two weeks before experimentation.

1.3.9. Proliferation Assay

Proliferation of LSK cells was determined by BrDU incorporation assay as described in Singh et al. (122) by using Cell Proliferation ELISA kit (Roche Bioscience) as per the manufacturer's instructions. 10,000 cells per condition were used for the assay and each sample was tested in duplicate. Proliferation was evaluated 24 hours after plating, in RPMI 1640 with 18 hours of treatment with different drugs or vehicle. Proliferation was expressed as fold increase relative to the effect of mitomycin (1 µM) (Fisher Scientific), an inhibitor of proliferation. This set of experiments was performed by my colleague, Mr. Shrinidh Joshi.

1.3.10. Migration Assay

Migration of LSK cells was evaluated by using QCM™ Chemotaxis cell migration assay kit (EMD Millipore) as per the manufacturer's instructions as described in Jarajapu et al. (157). 20,000 cells were used per treatment in a basal medium, HBSS (Mediatech, Inc.) and were assayed in duplicate. Migration was performed in response to the treatments for five hours, and the response was estimated as Arbitrary Fluorescence Units (AFUs) and analyzed as percentage increase over untreated control of the same treatment group. This set of experiments was performed by my colleague, Mr. Shrinidh Joshi.

1.3.11. Hind-Limb Ischemia

Surgery was performed as described in Niiyama et al. (Fig. 7) (158). Mice were anesthetized (Isoflurane, Terrell, USA), depilated and disinfected the hindlimbs. An incision of 1–1.5 cm was made in the skin of left inguinal region. Femoral artery was isolated 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. Blood-flow in the hind-limbs was measured by imaging the flux (blood $\times$ area^{-1} $\times$ time^{-1}) by using Laser

Doppler imaging system (LDI) (Moor Instruments Inc.) under isoflurane anesthesia, which was expressed relative to the mean blood flux in the contralateral non-ischemic limb. Ischemia-mobilized LSK cells were enumerated before and at different time points over a period of 21 days following HLI by flow cytometry as described above.

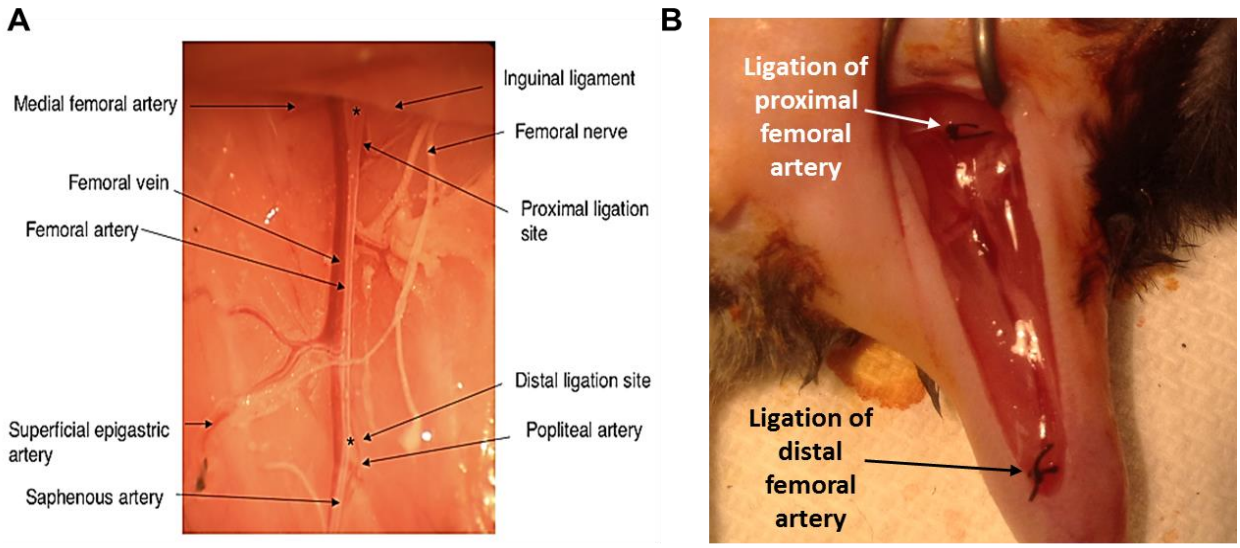


Figure 7 – Femoral artery location in hind limb and the sites for ligation and excision. *A*: Location of femoral artery in the hind limb in relation to other anatomical parts (158). *B*: Ligated sites on the femoral artery after the surgery.

1.3.12. Immunohistochemistry for Estimation of Capillary Density in Skeletal Muscles

Ischemic gastrocnemius skeletal muscle samples were processed for embedding in paraffin blocks. Sections were made at a thickness of 8 µm. Then sections were deparaffinzed in histoclear and hydrated. Antigen retrieval was performed in citrate buffer of pH 6.0. Blocking of non-specific receptors were performed using normal horse serum (Vector Labs), then stained using CD31 primary antibody (Santa Cruz Biotechnology), subsequently with secondary antibody (Vector Labs) and isolectin-B4 (Enzo Life Sciences Inc.), and mounted in DAPI-containing mounting media (Vector Labs). Stained sections were imaged under Olympus fluorescence microscope. Sections were also stained with hematoxyline and eosin (H&E) to observe the amount of necrosis in ischemic skeletal muscle.

1.3.13. Biochemical Analysis

Plasma or BM supernatant SDF-1 was measured by using Quantikine ELISA kit (R&D Systems). MMP-9 was estimated in BM plasma with zymography and analyzed through densitometry by using ImageJ. BM supernatant Slit1, Slit2, or Slit3 protein were estimated by using ELISA kits (Aviva Systems Biology, Abbexa and Biomatik respectively). Rho-kinase activity was estimated through activity response units using Y-27632, a ROCK inhibitor, by using Rho-kinase assay kit (Cyclex).

1.3.14. RNA Isolation and Real-Time PCR

Total RNA was isolated from progenitor cells using the RNeasy Plus Mini Kit according to the manufacturer's protocol (Qiagen). The concentration and purity of RNA was determined by NanoDrop (ND-1000 UV-Vis spectrophotometer; NanoDrop Technologies). cDNA was synthesized from 50 ng of RNA using iScript cDNA Synthesis kit (Biorad, USA). Real-time PCR was performed by SYBR Green gene expression assays for the gene expression of Slit1, Slit2, Slit3, Robo1, Robo2, Robo3, and Robo4 by using SYBR-green real-time RT-PCR with All-in-One™ qPCR Mix (GeneCopoeia, USA) using primers as listed in the Table 2 (Invitrogen). Quantitative PCR system, Applied Biosystems 7500, was used to carry out the PCR. The relative expression was calculated according to the ratio of the copy numbers of the target genes to the housekeeping gene β-actin in each sample by ΔCt method.

1.3.15. MMP-9 Zymography

Zymography was performed to estimate MMP-9 activity in BM supernatants as described in Kupai et al. (159) . Equal amounts of protein were loaded in gelatin containing 8% gels. Electrophoresis was done at 90 V for 30 min after the dye front disappears. Gels were washed in 2.5% Triton X-100 solution followed by incubation in a buffer containing 50

mM Tris HCl, 0.15 M NaCl, and 10 mM $CaCl_2$ of pH 7.8-8.0 at 37ºC for 20 hours. Then the gels were stained with 0.05% Coomassie Brilliant Blue G-250 for 1 hour and washed in destaining solution for 2 hours. Gelatinolytic activity was detected as transparent bands on blue background and quantified by using densitometry.

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

	Primer Sequence	
Gene	Forward	Reverse
Slit1	5' -TGA CAA CTG CAG CGA AAA TC -3'	5'- ACA GCT CAA CTG CAA TGT GG -3'
Slit2	5'- CTG CCT GAG ACC ATC ACA GA -3'	5'- AAT CCG CTA GCC ACT TGA GA -3'
Slit3	5'- CTC AAG GAG ATT CCC ATC CA -3'	5'- CAC AGT GGA GAG GGT TGG TT -3'
Robo1	5'- TCC GAT ACA GTG TGG AGG TG-3'	5'- GCA GTC ATT GTG GTT GTT GC -3'
Robo2	5'- TGA TGT CAT CGT CTC CAA GG -3'	5'- CGG ACT TTG TCC TTT TTC CA -3'
Robo3	5'- CAG GGG GAT GAC TCA CAG TT -3'	5'- TGG CCT AGA TAG GCT GGT GT -3'
Robo4	5'- CCA GAC ACG AGC ACG TTT TA -3'	5'- TTC TCC ACA CAC TGG CTC TG -3'
MasR	5'- GTC CTC TAC TTG CTG TAC TAC GAG -3'	5'- GTT GGC GCT GCT GTT GAT G -3'
LacZ	5'- GGT AAA CTG GCT CGG ATT AGG G -3'	5'- TTG ACT GTA GCG GCT GAT GTT G -3'
β-actin	5'- CCA TCA TGA AGT GTG ACG TTG -3'	5'- CAA TGA TCT TGA TCT TCA TGG TG -3'

1.3.16. Mouse Genotyping

Genotyping of MasR-KO mice was carried out in ear punch samples. PCR reactions were carried as described above by using the primers listed in Table 2. PCR amplification products

were resolved by gel electrophoresis on 1% agarose at 100V for 30 min. MasR, LacZ and β-actin were detected at 93, 210 and 150 bp, respectively, by using Quick-Load 100 bp DNA Ladder (New England BioLabs, Inc.). Representative images of DNA gel electrophoresis were shown in the Fig. 8. This set of experiments was performed with the help of Ms. Su Yamin Myat, a student lab intern.

Often, it is difficult to distinguish wild types and homozygotes from heterozygote mice based on Ct values alone. PCR amplified samples need to be assessed using DNA gels in those cases. Freshly amplified DNA products need to be used to accurately determine the genotype of mice. Resolution of the DNA gel image varies based on the purity of DNA and storage of the samples.

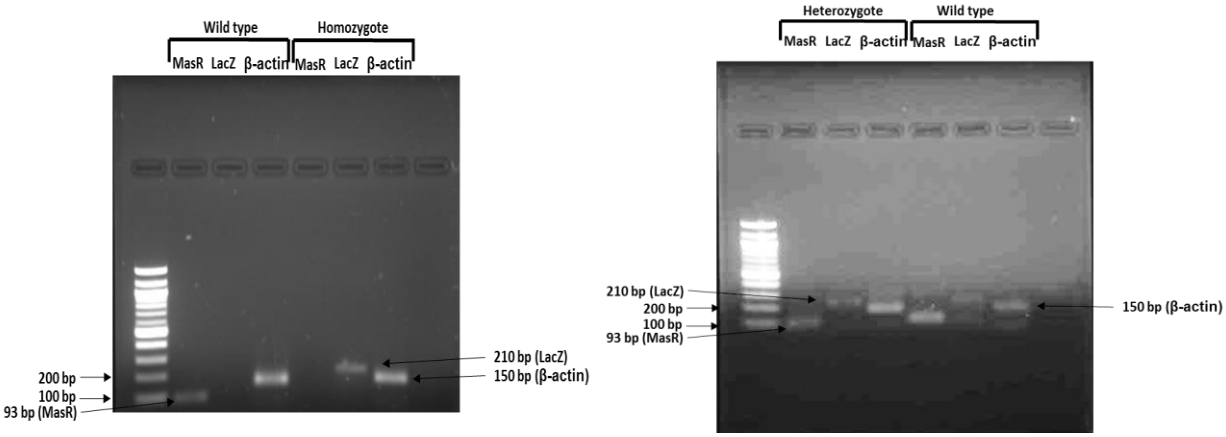


Figure 8 – Genotyping of MasR-KO mice. Shown were representative DNA gels of relative gene expression of MasR (wild type band), LacZ (knockout band), or β-actin (housekeeping gene) in mice which are wild type MasR, heterozygous, or homozygous for MasR knockout.

1.3.17. Statistical Analysis

Data were represented as mean ± standard error of mean. Results obtained from two treatment groups in a study were analyzed by using paired t-test. Paired comparisons done over time were analyzed by using two-way analysis of variance-paired comparisons. Analysis of results obtained from different subjects between two or more different treatment groups were

done by using unpaired student t-test or one-way analysis of variance (one-way ANOVA) respectively. $P<0.05$ was considered as statistically significant. If one-way ANOVA results show significant results, post hoc analysis was done by using Tukey's test. If two-way ANOVA paired comparison results show significant results, post hoc analysis was done by using Bonferroni's correction. Statistical analysis was performed by using GraphPad Prism version 5.0 software (GraphPad Software, Inc.). Sample sizes for all experiments were selected to achieve minimum power of 80% based on effect size of 0.8 and statistical significance of 0.05 estimated after preliminary experiments by using Minitab statistical software.

CHAPTER 2. ANG-(1-7)-INDUCED MOBILIZATION OF BMPCs INVOLVE SLIT3/ROBO/ROCK PATHWAY

2.1. Rationale and Preliminary Results

I tested if diabetes-induced stem cell mobilopathy can be reproduced in type 1 and type 2 mice models. Previous studies in mice have reported progenitor cell dysfunctions in early (duration of less than 8 weeks) diabetes (93; 160; 161). For the first time, I studied various aspects of mobilopathy in experimental diabetes including decrease in circulating progenitor cells, drug-induced, and ischemia-induced mobilization of BMPCs. These aspects were tested over three specific aims in this study in both short-term (8-12 weeks) and long-term (20-24 weeks) diabetes. In specific aim 1, first, I sought to find out the duration of diabetes that causes mobilopathy aspect of decreased circulating progenitor cells. As the expression of MasR in BMPCs and enhanced proliferation of progenitor cells with Ang-(1-7) were reported, using mice with appropriate duration for diabetic mobilopathy I investigated whether Ang-(1-7) reverses this condition. Further, if beneficial effects are observed, I investigated the mechanism involved, which were described in the preliminary results.

2.1.1. Diabetes Decreases Circulating BMPCs

Circulating WBCs ($P<0.05$, n=8), LSK cells ($P<0.001$, n=8) and CFUs ($P<0.01$, n=6) were significantly decreased in short-term STZ-diabetic mice when compared to the respective age-matched controls (Fig. 9*A*). This was also observed in short-term type 2 diabetic mice, where circulating WBCs ($P<0.01$, n=8), LSK cells ($P<0.05$, n=8) and CFUs ($P<0.001$, n=6) were significantly decreased in db/db mice when compared to the age-matched lean controls (Fig. 9*C*).

More profound effects of diabetes were observed on circulating WBCs ($P<0.05$, n=8), LSK cells ($P<0.001$, n=8) and CFUs ($P<0.001$, n=8) in long-term STZ-diabetic mice (Fig. 9*B*).

Comparably, long-term db/db mice have decreased circulating WBCs (P<0.001, n=8), LSK cells (P<0.05, n=8) and CFUs (P<0.05, n=8), where the absence of circulating LSK cells and CFUs in these aged mice makes the data seem less significant compared to the short-term diabetic mice (Fig. 9*D*).

These results indicate that mobilopathy aspect of decreased circulating BMPCs is prominent even in short-term duration, but more pronounced in long-term diabetic mice. Therefore, long-term diabetic mice were used throughout this study.

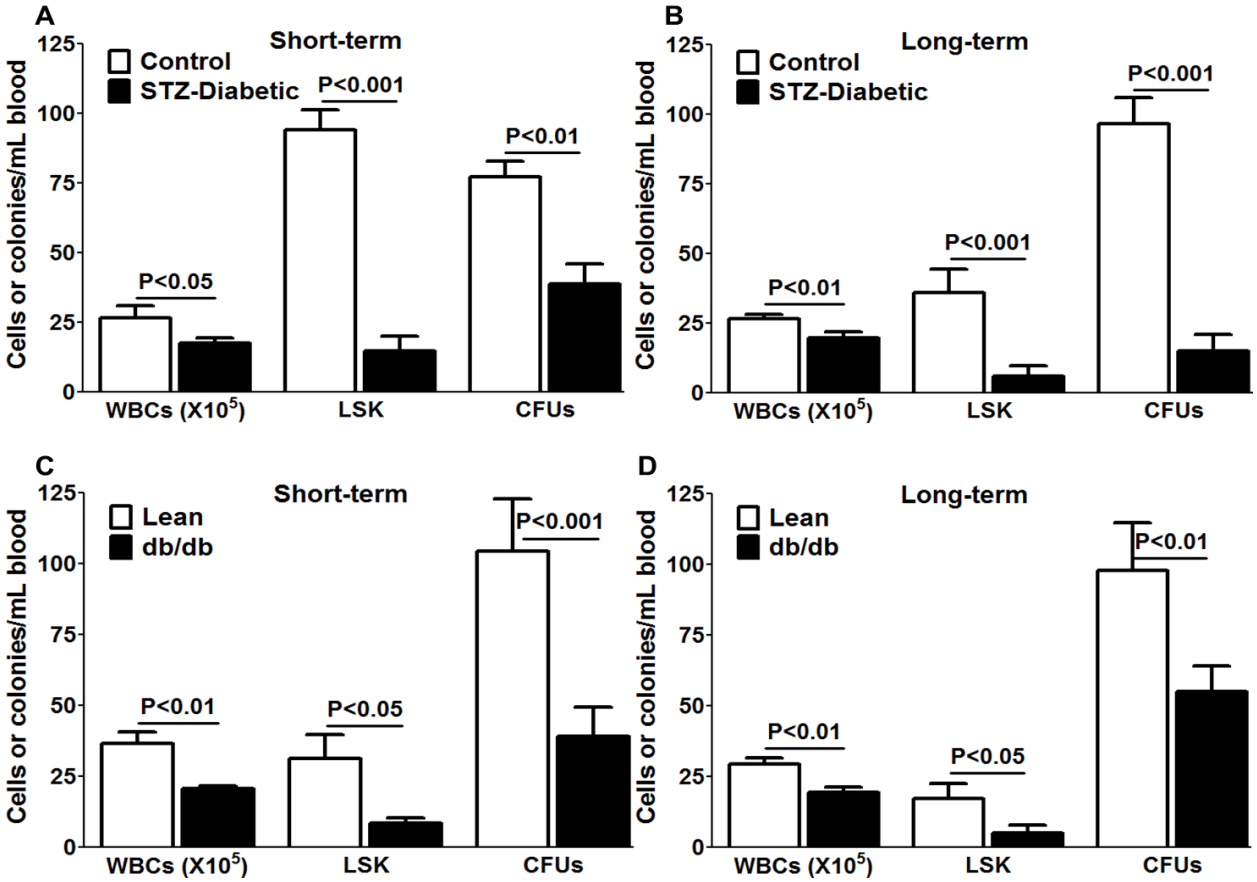


Figure 9 – Experimental diabetes is associated with decreased number of circulating BMPCs. *A* and *B*: WBCs, LSK (n=8) and CFUs (n=6-8) of short-term and long-term STZ-Diabetic mice; *C* and *D*: WBCs, LSK (n=8) and CFUs (n=6-8) of short-term or long-term db/db mice respectively.

2.1.2. Ang-(1-7) Restores Depleted Circulating BMPCs in Diabetes

Circulating WBCs ($P<0.05$, n=6-8), LSK cells ($P<0.05$, n=6-8) and CFUs ($P<0.05$, n=6-8) were restored to normal in Ang-(1-7) (1 µg/kg/min, s.c. for 28 days) treated STZ-diabetic mice when compared to the untreated (Fig. 10). Similarly, Ang-(1-7) restored circulating WBCs ($P<0.05$, n=8) and CFUs ($P<0.05$, n=8) in db/db mice when compared to the lean controls (Fig. 11*B* and 11*D*). Although LSK cell increase (data not shown) seems to be non-significant in Ang-(1-7) treated db/db mice due to the absence of those in limited blood sample size, the immediate progenitors, LK cells ($P<0.05$, n=8) were restored to normal in Ang-(1-7) treated mice compared to the untreated (Fig. 11*C*).

A lower dose of Ang-(1-7) (0.5 µg/kg/min, s.c. for 28 days) was tested in STZ-diabetic mice, but it has partially reversed mobilopathy (Fig. 12). Therefore, 1 µg/kg/min, s.c. for 28 days was used for the rest of the study. This dose was comparable to that of previous studies in mice (82 µg/kg/hour, s.c. for 6 weeks) (149; 162). Even lower dose of Ang-(1-7) (300 µg/kg/day, s.c. for 21 days) was shown to potentiate the effects of neupogen on reconstitution of blood cells in mice subjected to chemotherapy. Whereas, in the absence of neupogen Ang-(1-7) is able to stimulate the recovery of all progenitor cell lineages, but at a higher dose of 1200 µg/kg/day, s.c. for 21 days (144) . Furthermore, the dose I selected was in compliance with toxicokinetic studies (163), where up to 10 mg/kg/day, s.c. for 28 days was deemed to be safe with no toxic effects.

To confirm that Ang-(1-7) is mediating its actions through MasR, A779 at a dose of 0.5 µg/kg/day, s.c, for 28 days, was administered concurrently in STZ-diabetic mice, which prevented the restoration of mobilopathy by Ang-(1-7) (Fig. 13). Moreover, I observed that Ang-(1-7) has no effect on hyperglycemia, HbA1c% and glucose tolerance in both diabetic models

(Fig. 14). This indicates that the beneficial effects of Ang-(1-7) in restoring circulating BMPCs in diabetes is independent of hyperglycemia.

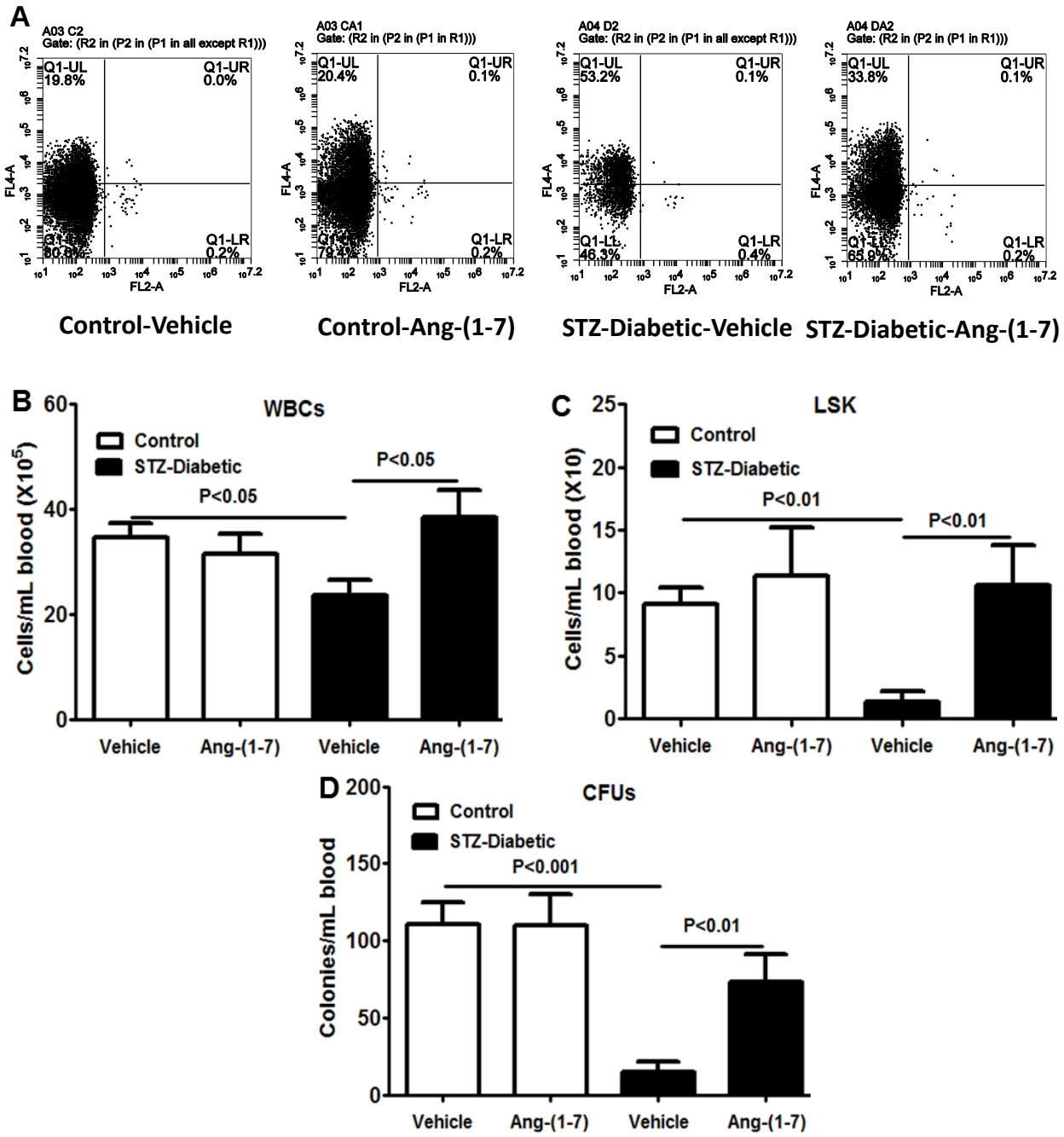


Figure 10 – Ang-(1-7) restored circulating BMPCs in experimental type 1 diabetes. *A*: Representative dot plots of flow cytometric enumeration of LSK cells. Circulating *B*: WBCs, *C*: LSK cells, and *D*: CFUs in different treatment groups (n=6-8).

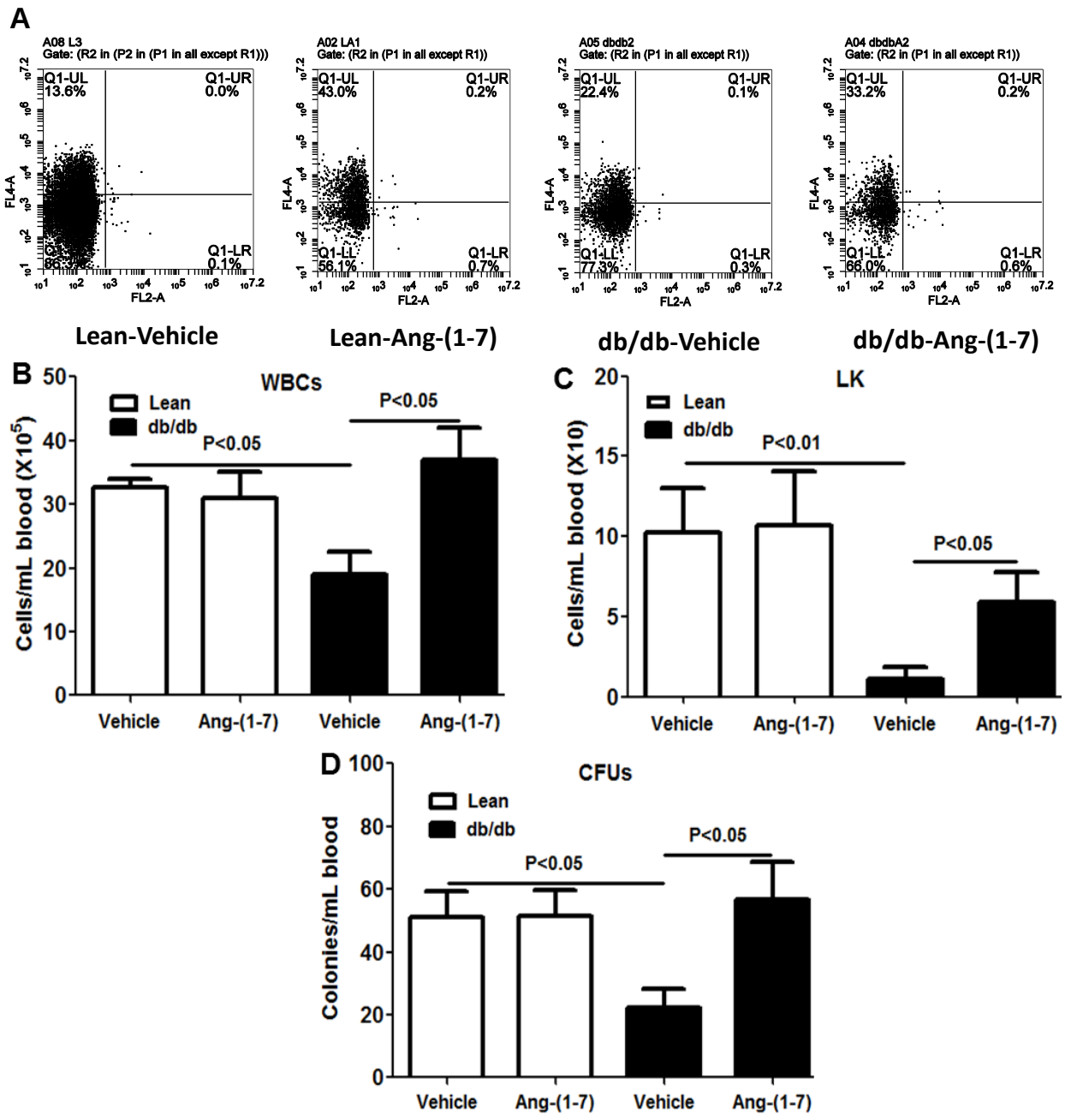


Figure 11 – Ang-(1-7) restored circulating BMPCs in experimental type 2 diabetes.
A: Representative dot plots of flow cytometric enumeration of LSK cells. Circulating *B*: WBCs, *C*: LK cells, and *D*: CFUs in different treatment groups (n=6-8).

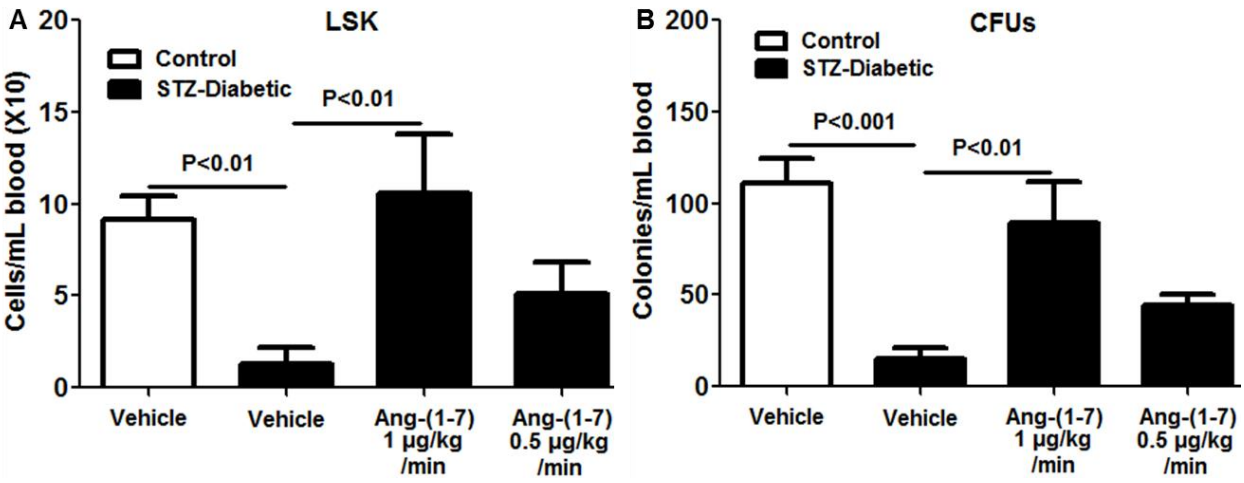


Figure 12 – Ang-(1-7) at lower dose partially reversed diabetic decrease in the circulating BMPCs in type 1 model. Circulating *A*: LSK cells and *B*: CFUs in different treatment groups (n=3).

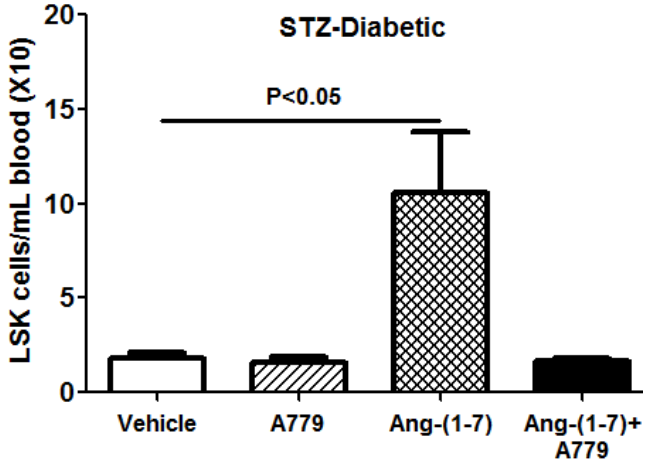


Figure 13 – Concurrent administration of A779 prevented the normalization of circulating LSK cells in experimental type 1 diabetes by Ang-(1-7) (n=3).

2.1.3. Reversal of Mobilopathy by Ang-(1-7) in Diabetes Is Mediated by ROCK

Rho-kinase (ROCK) is a secondary messenger belonging to the family of serine-threonine kinases which mediates several important cellular functions including migration and proliferation through modification of the actin cytoskeleton (164; 165). In a previous study from our lab we have reported that activation of ROCK is essential for in vitro migration of human

$CD34^+$ cells irrespective of the stimulus involved (134). I tested if Ang-(1-7) modulates ROCK activity in progenitor cells of diabetic mice.

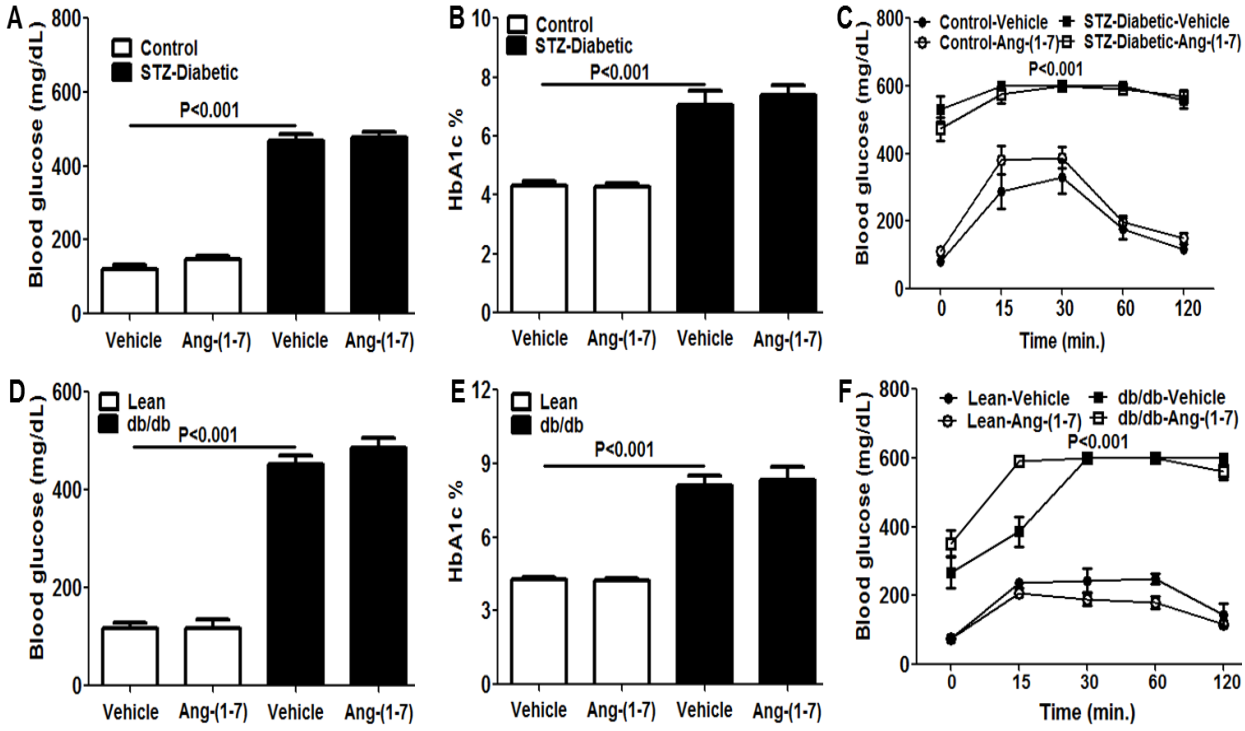


Figure 14. Ang-(1-7) has no effect on glucose condition in experimental type 1 and type 2 diabetes. Blood glucose (*A* and *D*), HbA1c% levels (*B* and *E*), and glucose tolerance curves (*C* and *F*) in different treatment groups (n=6). *C* and *F*: P<0.001 indicates significant difference in the time-glucose curves in STZ-Diabetic-Vehicle or db/db-Vehicle mice compared to their respective controls, as analyzed by two-way ANOVA.

ROCK activity in BM LSK cells was unchanged in both models of diabetes. Whereas, ROCK activity was enhanced in these cells by Ang-(1-7) treatment (P<0.05 vs untreated) (Fig. 15). Then I tested whether increase in ROCK activity is mediating mobilization of BMPCs in Ang-(1-7) treated diabetic mice. Fasudil, a ROCK inhibitor, was administered concomitantly in the last two weeks of Ang-(1-7) administration in STZ-diabetic mice. Diabetes effect on impaired LSK cell mobilization was reversed by Ang-(1-7) as observed before, and the beneficial effect of Ang-(1-7) was blunted by pharmacological inhibition of ROCK by fasudil. Whereas, fasudil itself has no effect on circulating LSK cells (Fig. 16*B*). Moreover, enhanced in

vitro migration of LSK cells in response to SDF or VEGF was attenuated by Y-27632, a ROCK inhibitor, when compared to the SDF or VEGF treated cells alone ($P<0.05$). Similar to fasudil, Y-27632 itself has no effect on migration (Fig. 16*C*). These results indicate that Ang-(1-7) might reverse diabetic stem cell mobilopathy by increasing the ROCK activity in stem/progenitor cells.

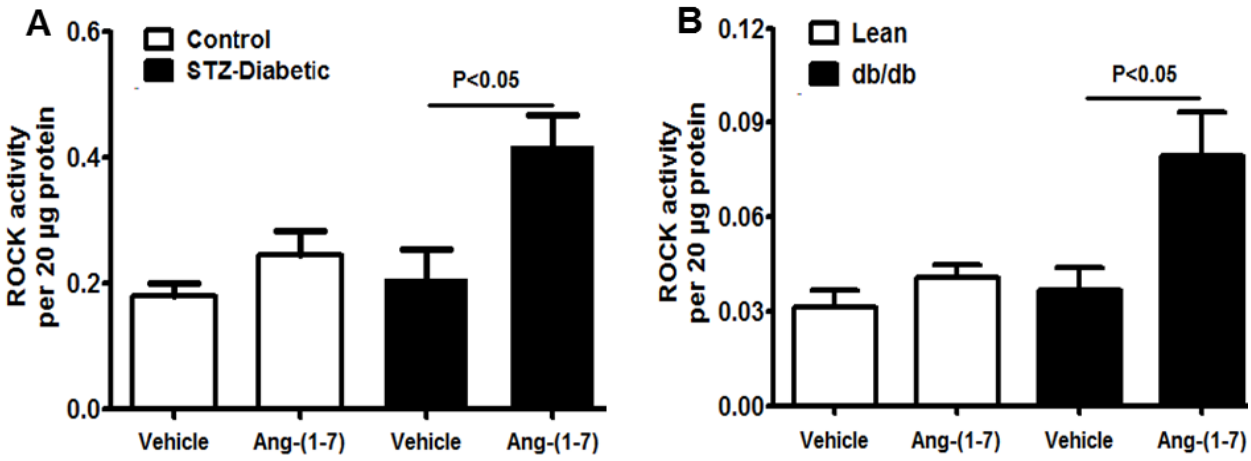


Figure 15 – Ang-(1-7) induces ROCK activity in diabetic BMPCs. ROCK activity is enhanced in *A*: STZ-diabetic and *B*: db/db mice bone marrow-derived progenitor cells after in vivo Ang-(1-7) treatment (n=5-7).

2.1.4. Ang-(1-7) Did Not Induce ROCK Activity in Peripheral Cardiovascular Tissues

ROCK enzyme also plays an important role in producing cardiovascular detrimental effects such as inflammation, hypertrophy and fibrosis (166-168). Simultaneous increase in ROCK activity in cardiovascular tissues as in LSK cells could be detrimental. Surprisingly, I observed that ROCK activity was unaffected in cardiovascular tissues, heart and aorta (Fig. 17). This suggests that the enhancement of ROCK activity by Ang-(1-7) is specific to BMPCs in diabetic conditions.

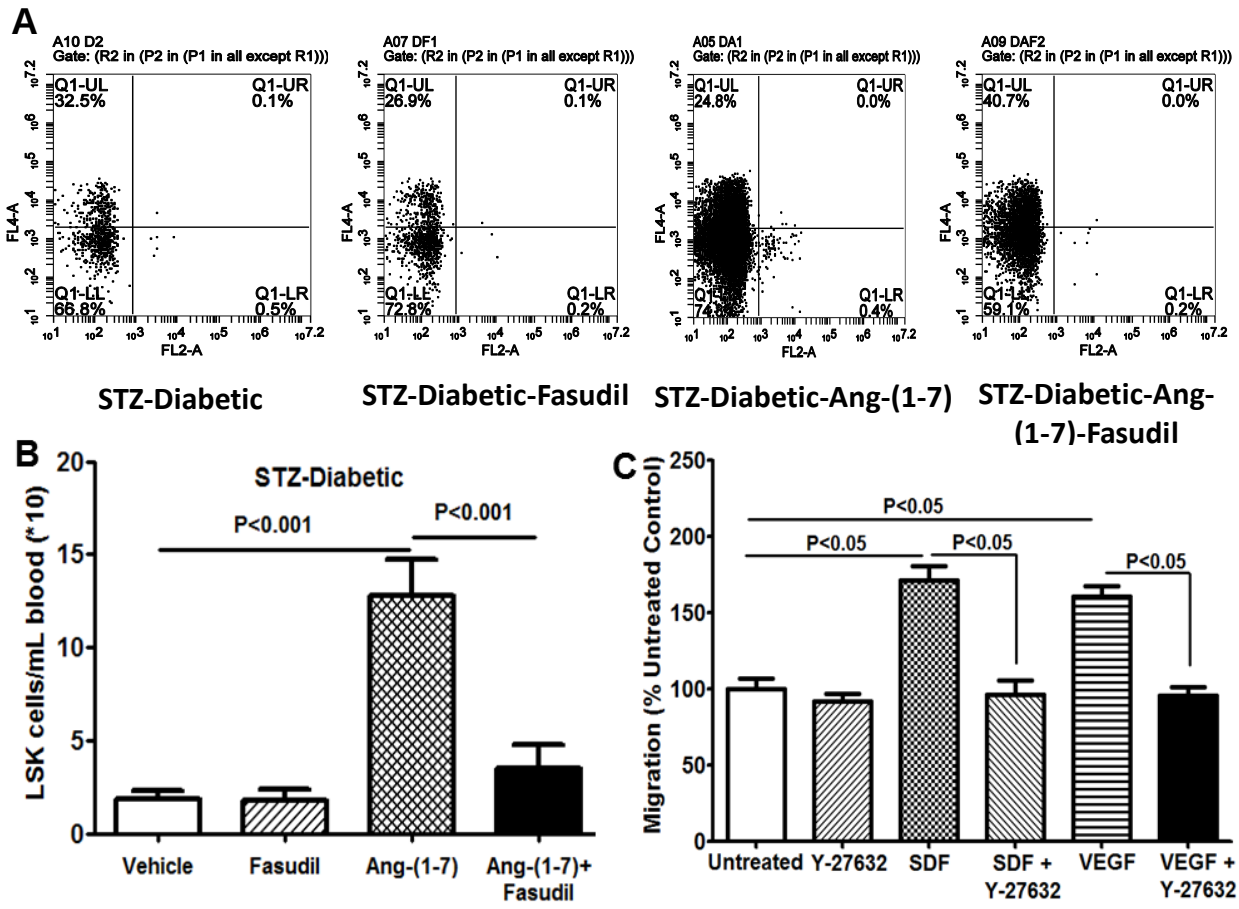


Figure 16 – Ang-(1-7)-induced mobilization of BMPCs is mediated by ROCK in experimental type 1 diabetes. *A*: Representative flow cytometric dot plots of LSK cell enumeration in different treatment groups. *B*: Fasudil blunted Ang-(1-7)-induced mobilization of BMPCs (n=6). *C*: SDF or VEGF induced migration was blunted by Y-27632 (n=5).

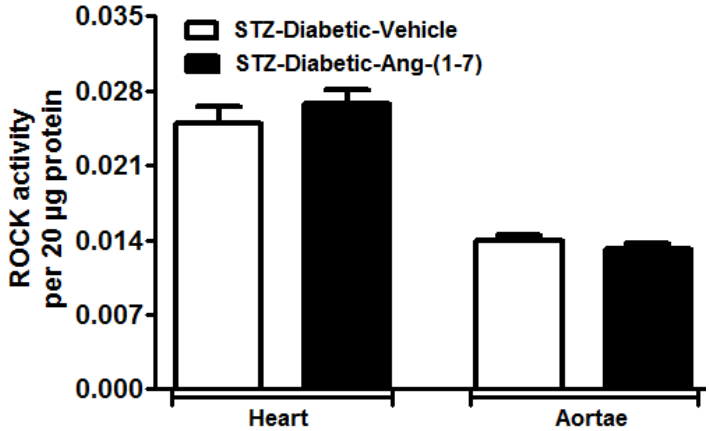


Figure 17 – ROCK activity in heart and aortae of experimental type 1 diabetic mice was unchanged after Ang-(1-7) treatment (n=4).

2.1.5. Slit and Robo Genes Are Expressed in MSCs and BMPCs

I investigated the locus of action of Ang-(1-7). It might act either directly on BMPCs or indirectly through MSCs which can then influence BMPCs. I found that direct in vitro treatment of LSK cells with Ang-(1-7) did not enhance ROCK activity (Fig. 18). This shows that Ang-(1-7) is not acting directly on BMPCs but through MSCs. Then, I sought to find out the mediators of enhanced ROCK activity by Ang-(1-7), where I focused on Slit/Robo pathway. Slit proteins belong to a family of secreted extracellular matrix glycoproteins that includes Slit1, Slit2 and Slit3 which are highly homologous. They play an important signaling role by acting through roundabout (Robo) receptors and mediate diverse cellular functions in various cell types including BMPCs (169; 170).

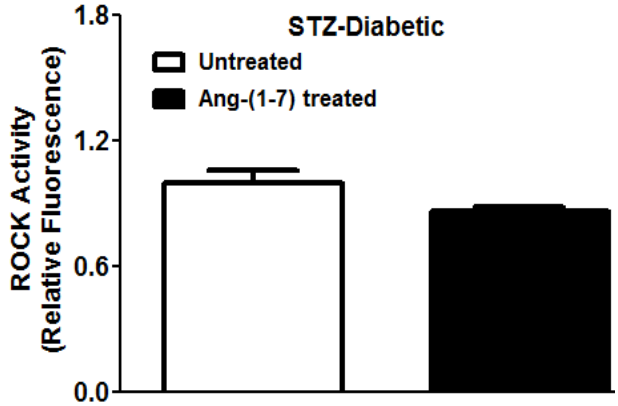


Figure 18 – In vitro Ang-(1-7) treatment has no effect on ROCK activity of BMPCs derived from experimental type 1 diabetes (n=3).

I examined whether mRNA transcripts were expressed in BM cells. Although not conclusive, I observed that mRNA transcripts of Slit1, Slit2, Slit3, Robo1 and Robo3 were predominately expressed in BM-MSCs, whereas Robo1, Robo3 and Robo4 were in LSK cells (Fig. 19). This indicates that Slit proteins secreted by MSCs might act through Robo receptors on BMPCs to exert its effects.

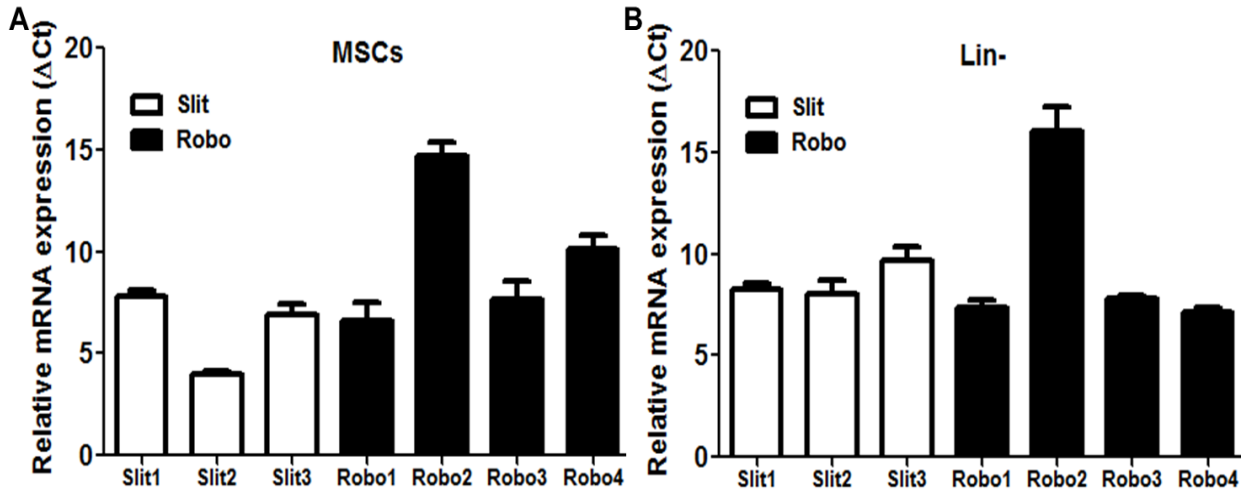


Figure 19 – Relative mRNA expression of Slit homologs and Robo receptors in bone marrow cells. Shown were ΔCt values relative to β-actin in *A*: MSCs and *B*: Lin$^-$ cells (n=5-7).

2.2. Working Hypothesis

I hypothesized that increased Ang-(1-7) levels mediates mobilization of BMPCs in diabetic conditions through MasR activated Slit/Robo/ROCK pathway.

2.3. Experimental Design

Experimental design for the specific aim 1 is depicted in Fig. 20*A* and 20*B*. The effects of Ang-(1-7) if any were further confirmed by using MasR-KO mice. This also helped us determine whether Ang-(1-7) is acting via MasR or not.

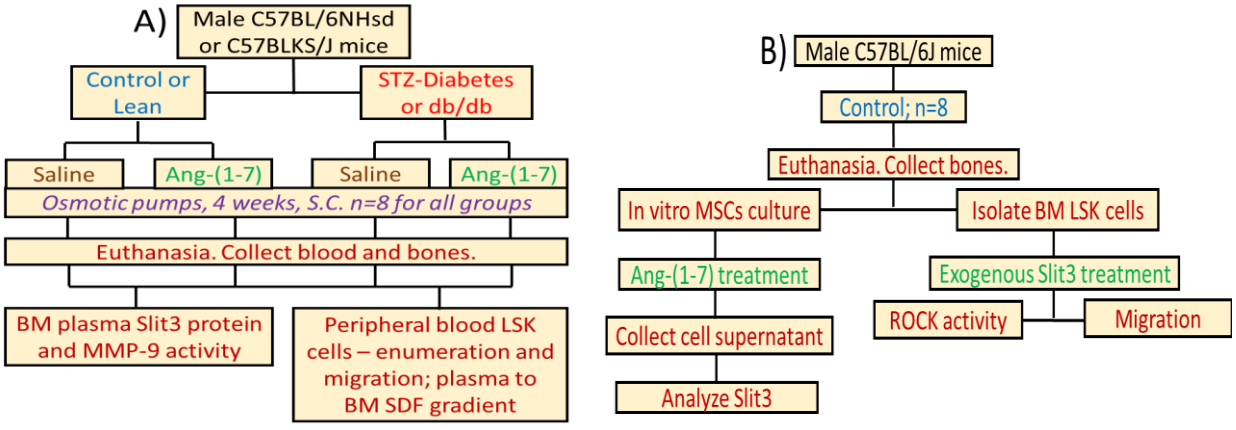


Figure 20. Flow charts depicting experimental design for specific aim 1 – *A*: in vivo and *B*: in vitro experiments.

2.4. Results and Discussion

2.4.1. Ang-(1-7) Reversed Depleted BM Reserves of Stem/Progenitor in Diabetes

I examined if diabetic stem cell mobilopathy is a result of depleted bone marrow resident progenitor cells, a.k.a. BMPC-reserve. The number of LSK cells were significantly decreased in diabetic bone marrow in both models, which was reversed by Ang-(1-7) (Fig. 21*A* and 21*B*). Similarly, BM CFUs were found to be decreased in STZ-diabetes and was reversed by Ang-(1-7) (Fig. 21*C*). Surprisingly, db/db mice BM CFUs are comparable to lean mice and were unaffected by Ang-(1-7) (Fig. 21*D*). This could be because of increased proliferation of BMPCs in the complete absence of leptin receptor, ObRb, which is nullifying the effect of diabetes on in vitro functions of progenitor cells. Although there is no direct evidence for this, leptin is known to maintain self-renewal capacity of breast cancer stem cells (171) and promote quiescence and self-renewal in BMPCs (172). If the same phenomenon applies here, reduced quiescence might have resulted in lower number of BM LSK cells and simultaneously enhanced proliferation resulting in increased in vitro functional BMPCs, which can be derived from successors of LSK cells (such as LK and LS cells). The involvement of leptin in hematopoiesis is well known (173; 174) but its actual mechanism in bone marrow and impact on hematopoiesis is yet to be discovered. Recently, we have shown that stem/progenitor cell population from db/db mice mobilize differently when compared to STZ-diabetes in response to clinical mobilizers, though the effect of diabetes on their vasoreparative functions is comparable (154).

In accordance with these observations, LSK cells from diabetic bone marrow showed decreased basal, SDF- or VEGF-induced proliferation in both models, which was restored to normal in LSK cells derived from Ang-(1-7) treated diabetic mice (Fig. 22). This indicates

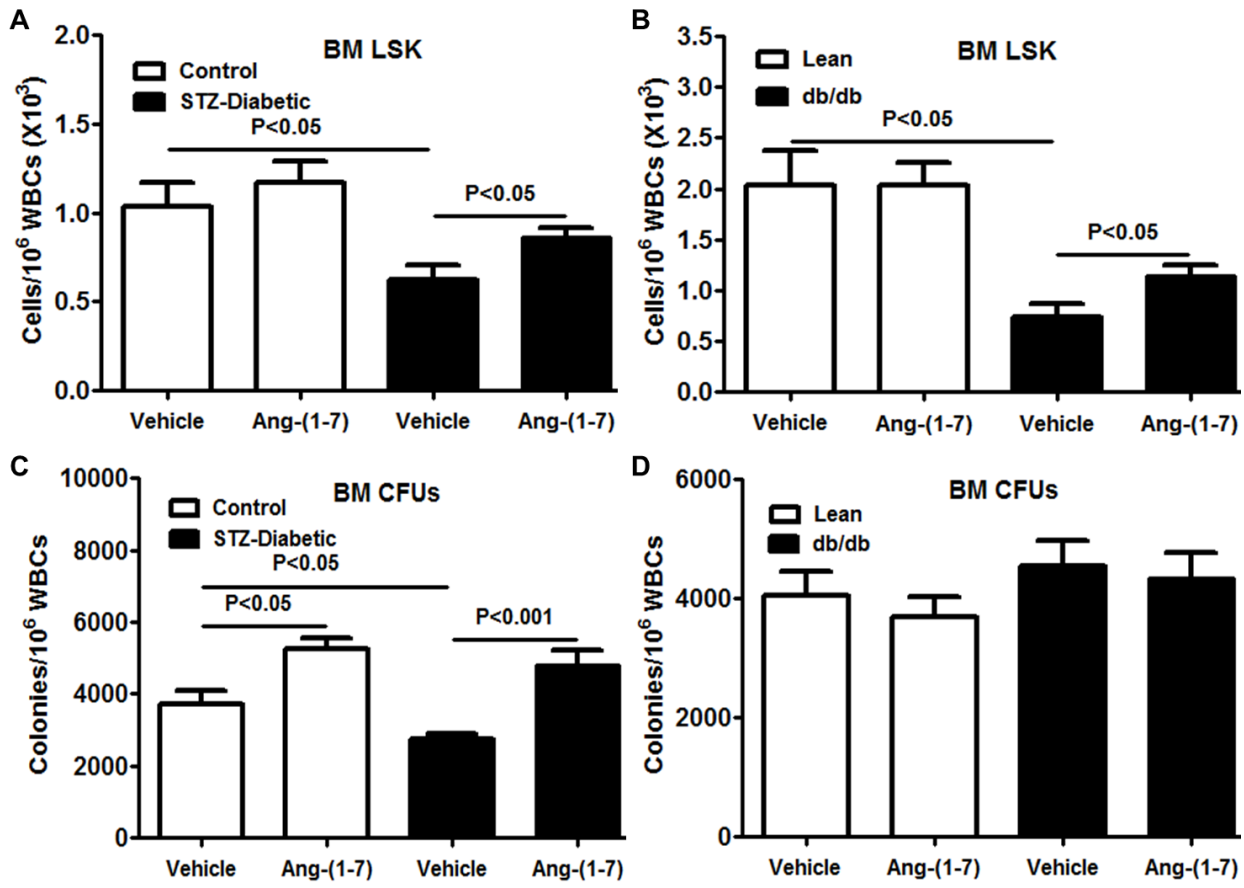


Figure 21 – Reversal of depleted bone marrow reserve of BMPCs in type 1 or type 2 diabetes by Ang-(1-7). *A*: Bone marrow LSK cells and *C*: CFUs from different treatment groups involving STZ-diabetic mice (n=7-8). *B*: Bone marrow LSK cells and *D*: CFUs from different treatment groups involving db/db mice (n=5-6).

Ang-(1-7) reversal of diabetic stem cell mobilopathy might involve reversing the depletion of BMPC reserve. The decrease in proliferation of diabetic BMPCs and the reversal by Ang-(1-7) is in compliance with the previous studies where Ang-(1-7) was shown to increase proliferation and accelerate hematopoietic recovery subsequent to chemoradiation therapy (7; 126; 135). Thus, mobilization of diabetic BMPCs might likely be increased by enhanced proliferation by Ang-(1-7).

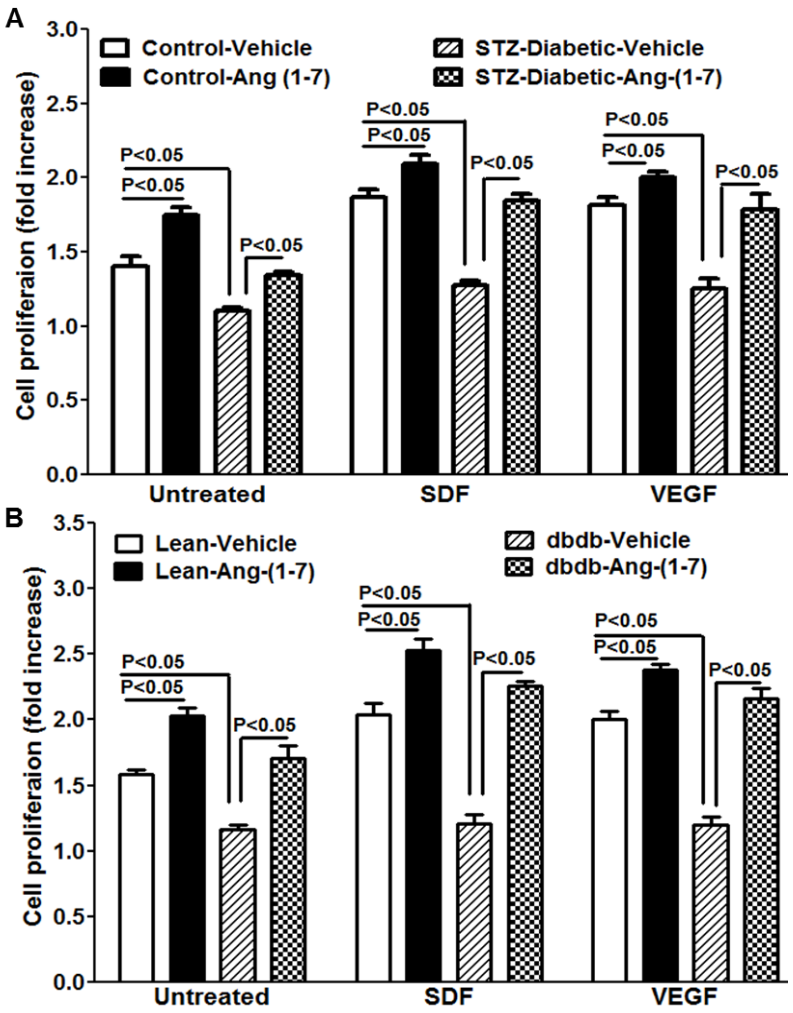


Figure 22 – Reversal of decreased in vitro proliferation of LSK cells derived from experimental type 1 and type 2 diabetes in basal conditions, or in response to SDF or VEGF by Ang-(1-7). Proliferation of LSK cells in different treatment groups involving *A*: STZ-diabetic and *B*: db/db mice (n=6).

2.4.2. Ang-(1-7) Reverses Mobilopathy by Shifting SDF Gradient in Favor of BMPC Mobilization in STZ-Diabetes but Not in db/db Mice

SDF gradient drives BMPC mobilization and largely mediates mobilization in response to ischemia (35; 36). I tested whether SDF gradient plays a role in the reversal of mobilopathy by Ang-(1-7). I estimated the levels of SDF in plasma and corresponding BM supernatants of mice from all the treatment groups. Plasma SDF levels were decreased in STZ-diabetic mice (P<0.05, n=5-7) (Fig. 23*A*) but were unchanged in BM supernatants when compared to the age-matched

controls (Fig. 23*B*). Plasma-BM SDF gradient showed a decreased trend which could deter the mobilization of BMPCs (Fig. 23*C*). Plasma SDF levels were normalized (Fig. 23*A*) and BM supernatant SDF was decreased (Fig. 23*B*) by Ang-(1-7) treatment, therefore, increasing the SDF gradient in favor of mobilization (Fig. 23*C*). The reduction in plasma SDF levels in diabetes might be due to increased oxidative stress which leads to decreased hypoxia-inducible factor (HIF)-1α (175), and the restoration of SDF might be because of reduced oxidative stress after Ang-(1-7) treatment (176), which can normalize HIF-1α activity.

In contrast, plasma levels of SDF were unchanged in db/db mice when compared to the lean mice with or without the treatment of Ang-(1-7) (Fig. 24). The reasons for this are unknown, but bone marrow leptin signaling might play a role in this altered phenomenon as well. These results indicate that the reversal of diabetic stem cell mobilopathy by Ang-(1-7) might involve shifting of SDF gradient in favor of mobilization of BMPCs in STZ-diabetes but not in db/db mice.

2.4.3. Ang-(1-7) Restores Migratory Function in BMPCs

Irrespective of SDF levels, I observed that in vitro migration sensitivity of BM LSK cells in response to SDF was reduced in both models of diabetes ($P<0.05$, n=6 vs respective controls). Ang-(1-7) treatment restored the lost sensitivity of LSK cells to SDF in both diabetic models when compared to the untreated ($P<0.05$, n=6). Similar impairment of in vitro migration in LSK cells was observed where VEGF was used as a reference for comparison, which was reversed by Ang-(1-7) (Fig. 25). These results indicate that hypoxia-regulated factors such as SDF or VEGF that might play a key role in reversal of mobilopathy by Ang-(1-7), which increases the sensitivity of LSK cells to them. Thus, increased circulating BMPCs in STZ- diabetic mice

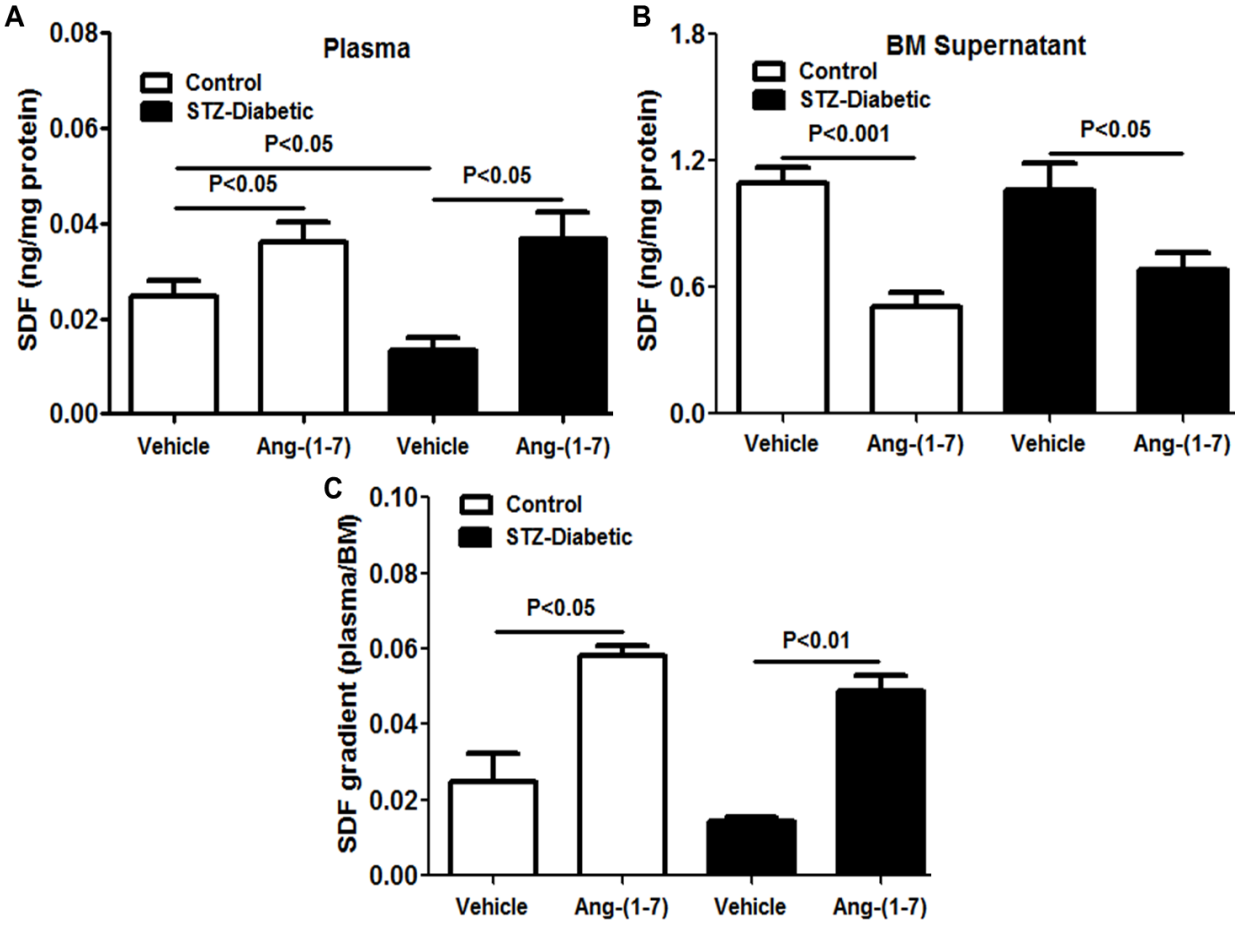


Figure 23 – Ang-(1-7) shifts SDF gradient across blood-bone marrow interface in favor of BMPC mobilization in experimental type 1 diabetes. SDF levels in *A*: Plasma and *B*: BM supernatant in different groups (n=5-7). *C*: SDF gradient, which is the ratio of plasma SDF to BM supernatant SDF normalized to unit protein, in different treatment groups (n=3).

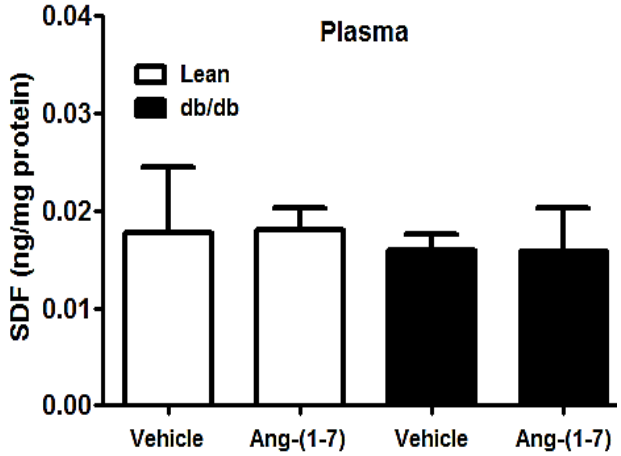


Figure 24 – Plasma SDF levels were unchanged in db/db mice before and after Ang-(1-7) treatment (n=4).

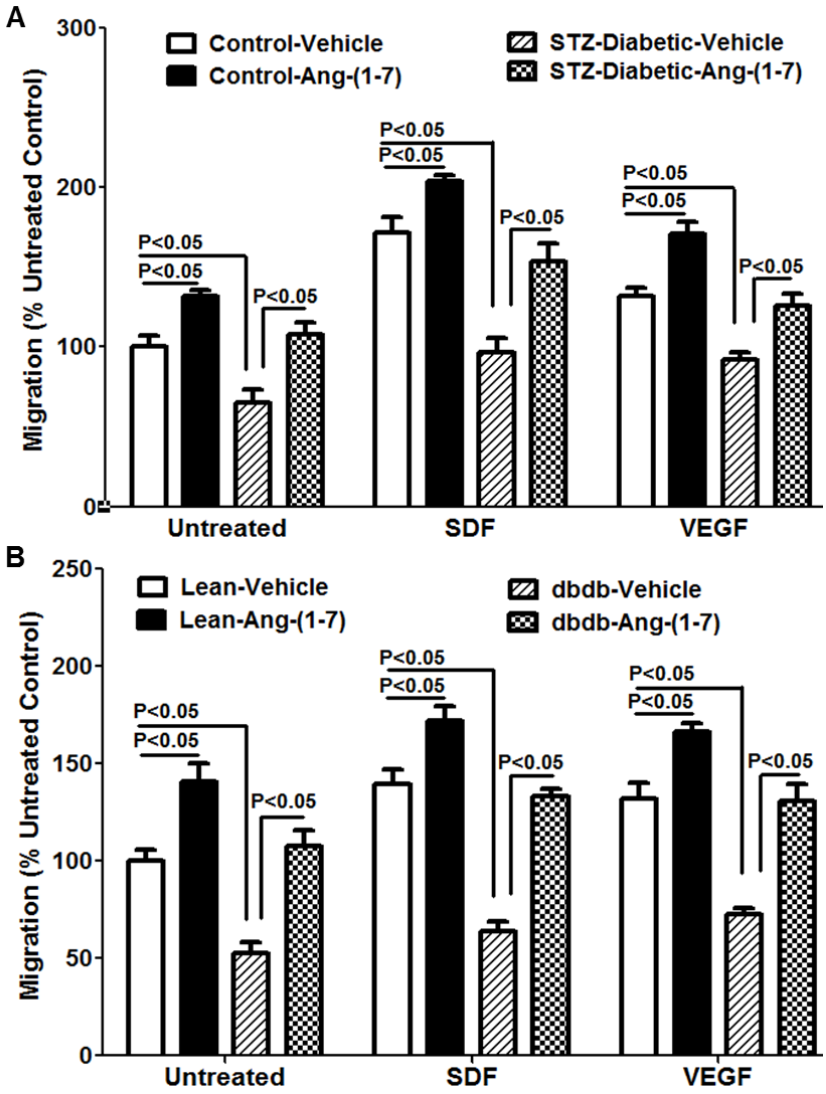


Figure 25 – Reversal of decreased in vitro migration of LSK cells derived from experimental type 1 and type 2 diabetes in basal conditions, or in response to SDF or VEGF by Ang-(1-7). Migration of LSK cells in different treatment groups involving *A*: STZ-diabetic and *B*: db/db mice (n=6).

after Ang-(1-7) treatment might be as a result of restored plasma SDF levels and sensitization of cells to hypoxia-regulated factors, whereas only later is true in case of Ang-(1-7) treated db/db mice.

2.4.4. MMP-9 Activity in Diabetes Is Unaffected by Ang-(1-7)

BMPCs express c-Kit, a tyrosine kinase receptor, which plays a key role in tethering of these cells in BM by binding to the stem cell factor or Kit ligand (KitL) (177). The membrane Kit ligand (mKitL) will be cleaved to soluble Kit ligand (sKitL) by MMP-9 enzyme (178; 179), which releases BMPCs to enter the blood circulation, thereby enhancing mobilization. I tested if MMP-9 plays any role in reversal of mobilopathy by Ang-(1-7). The estimation of MMP-9 levels in BM supernatants in STZ-diabetic mice showed that they were unaffected by diabetes and were unchanged even after Ang-(1-7) treatment (Fig. 26). This suggests that MMP-9 might not have a role in the effect of Ang-(1-7) on diabetic stem cell mobilopathy.

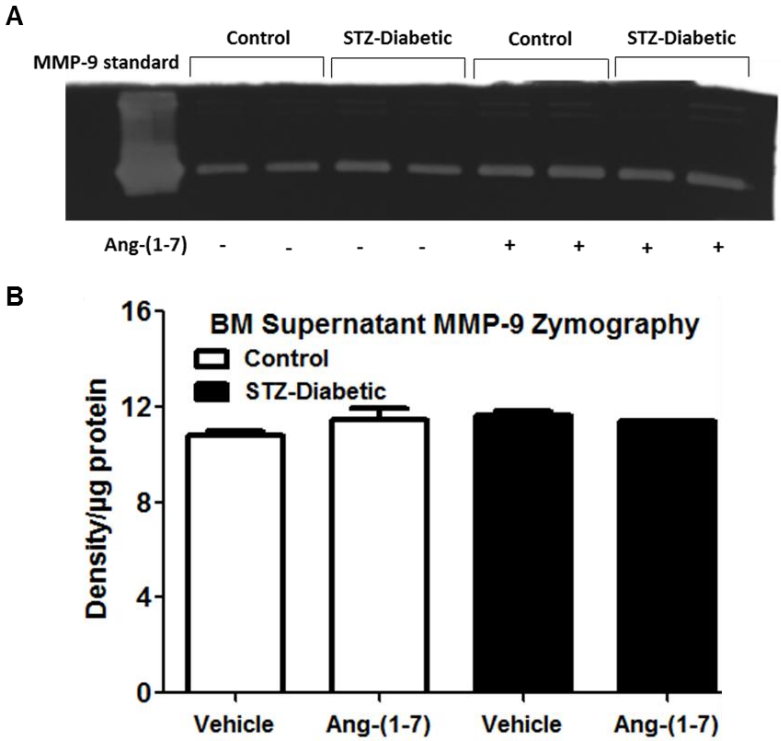


Figure 26 – MMP-9 activity in bone marrow supernatant of experimental type 1 diabetic mice was unchanged before or after treatment with Ang-(1-7). *A*: Representative images of MMP-9 gel zymography in bone marrow supernatants of different treatment groups. B) Quantitative data of zymography from different treatment groups (n=4).

2.4.5. Ang-(1-7) Stimulates Slit3 Secretion from MSCs Which in Turn Activates ROCK in BMPCs

BM-MSCs were shown to secrete Slit3 which activates angiogenic signaling (169; 180) and among the three Slit homolog proteins, it was shown to mediate endothelial migration partly by enhancing ROCK activity (169). However, Slit2 was shown to inhibit migration (181). Therefore, I tested whether Slit proteins are involved in Ang-(1-7)-induced ROCK activation in mouse LSK cells. Bone marrow supernatant Slit1 levels were increased in STZ-diabetic mice but were unaffected by Ang-(1-7) treatment (Fig. 27*A*). Slit2 levels decreased after Ang-(1-7) treatment in STZ-diabetic mice, although unaffected by diabetes itself (Fig. 27*B*). Interestingly, I observed an increase in Slit3 levels in BM supernatants derived from Ang-(1-7) treated control mice, which was even more prominent in STZ-diabetic mice compared to their respective untreated mice. Whereas, diabetes itself does not have any effect on BM Slit3 levels (Fig. 27*C*). These results indicate that most likely Slit3 is involved in mediating Ang-(1-7)-induced ROCK activity.

I further investigated whether BM-MSCs are the source of Ang-(1-7) induced Slit3 increase in BM supernatants. MSCs were cultured in monolayers and treated in vitro with Ang-(1-7) for 18 hours. Cell culture supernatants were isolated and analyzed for Slit3 protein levels. I observed a constitutive Slit3 secretion by MSCs which was enhanced by Ang-(1-7) ($P<0.05$) (Fig. 28). Then I tested whether Slit3 or Slit2 can enhance LSK cell mobilization. Slit3 or Slit2 itself had no effect on migration of LSK cells, whereas, they potentiated the migration induced by SDF or VEGF in a concentration-dependent manner up to 0.4 µg/mL and stabilized thereafter (Fig. 29). Surprisingly, at 1.6 µg/mL concentration, SDF- or VEGF-induced migration in response to Slit2 came back to basal levels (Fig. 29*B*). At present, I am unable to explain this

anomaly, but keen observation of the data shows that the Slit3 protein is more abundant relative to Slit2 in mice bone marrow and its influence on migration might dominate the effects of Slit2 on BMPCs.

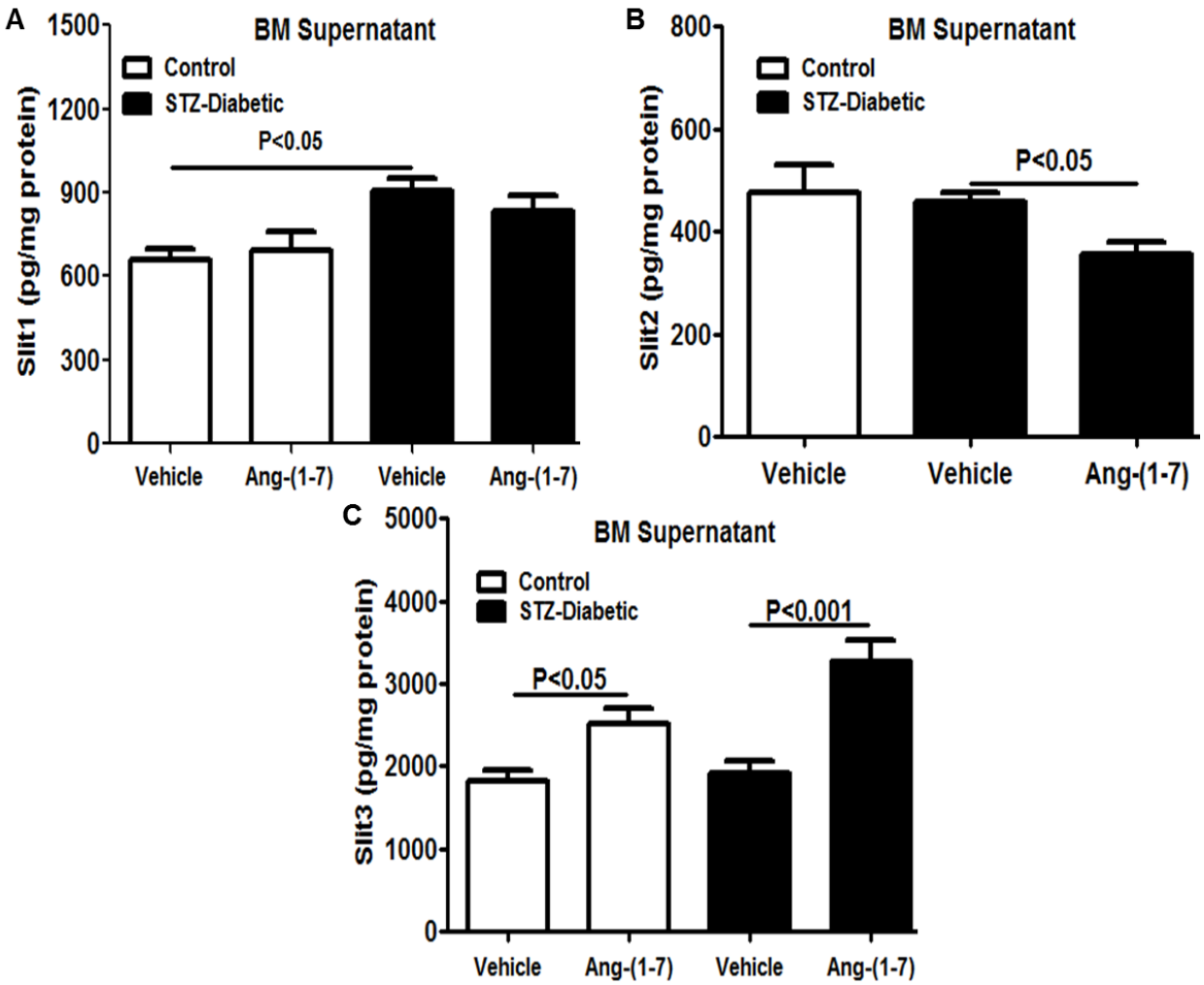


Figure 27 – In vivo Ang-(1-7) treatment has no effect on Slit1 but decreased Slit2 levels and increased Slit3 levels in bone marrow supernatant of STZ-diabetic mice. BM supernatant *A*: Slit1 (n=3), *B*: Slit2 (n=5), and *C*: Slit3 (n=5-7) levels from different treatment groups.

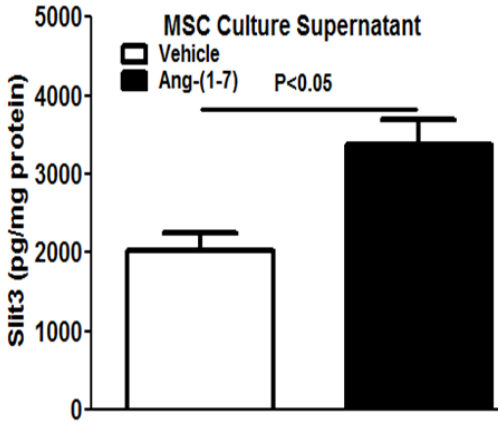


Figure 28 – In vitro Ang-(1-7) treatment enhanced Slit3 protein secretion from cultured MSCs (n=4).

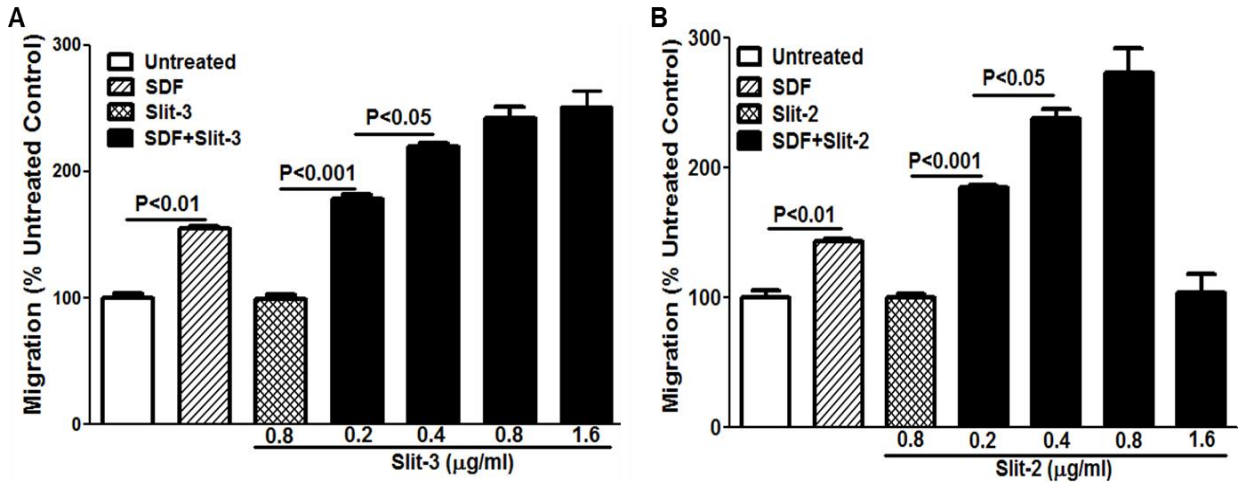


Figure 29 – Slit3 or Slit2 did not induce migration but potentiated migration induced by SDF in a concentration-dependent manner. Effect of *A*: Slit3 or *B*: Slit2 on SDF-induced migration of BM LSK cells (n=4).

Notably, in vitro ROCK activity in LSK cells was also stimulated by Slit3 or Slit2 after 18 hours of treatment (Fig. 30). In my observation, at the concentrations I used, this Slit3- or Slit2-induced ROCK activity was in fact higher than that was induced by a known potent ROCK activator, LPA (182), where it was used as a positive control. These results indicate that the sensitivity of BMPCs to hypoxia-regulated factors, SDF or VEGF is potentiated by Ang-(1-7) towards mobilization, in effect through stimulation of MSCs to release Slit3, which in turn triggers ROCK activity in progenitor cells, and thus reverses mobilopathy in diabetes. The role of Slit2 in Ang-(1-7)-mediated diabetic BMPC mobilization still need to be clarified.

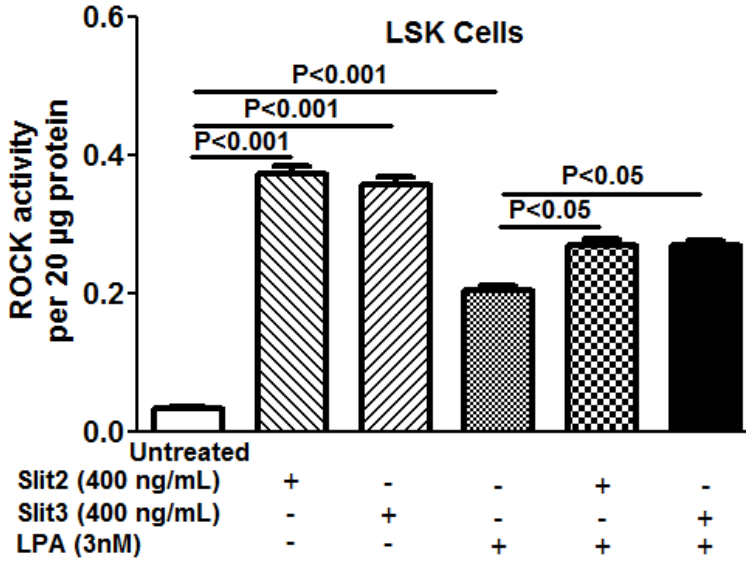


Figure 30 – Slit3 or Slit2 stiumlated ROCK activity in bone marrow LSK cells (n=3).

2.4.6. Endogenous Mas Receptor Expression Is Essential for Mobilization of BMPCs and Their Functions

Finally, I tested whether MasR is essential for in vivo mobilization and in vitro functions of BMPCs. Interestingly, I found that MasR-KO mice have lower number of circulating LSK cells when compared to the age-matched WT mice ($P<0.01$, n=6) (Fig. 31*A*), although the BMPC reserve was comparable in both groups (Fig. 31*B*). This suggests that impaired mobilization of BMPCs is independent of BMPC reserve and genetic ablation of MasR prevents egress of progenitor cells.

Moreover, blood glucose concentrations and glucose tolerance were unchanged in MasR-KO mice when compared to the WT mice (Fig. 32). This confirms that stem cell mobilopathy in MasR-KO is also independent of glucose conditions. It is important to note that a previous study has reported glucose intolerance in MasR deficient mice (183), where the genetic background of the mice used was FVB/N, which could be the reason for this difference.

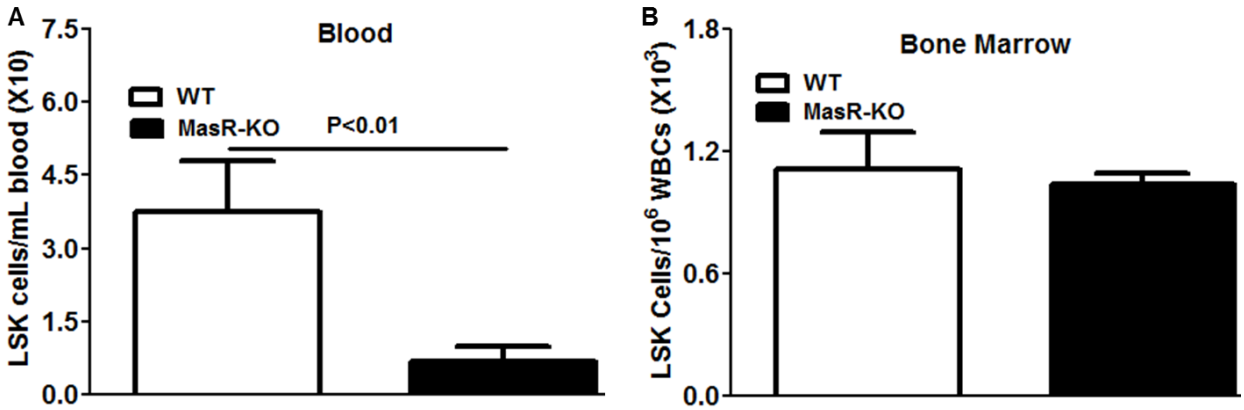


Figure 31 – Circulating LSK cells were decreased in MasR-KO mice despite unaltered bone marrow reserve. LSK cells enumerated in *A*: peripheral blood (n=6) and *B*: bone marrow (n=3) of WT and MasR-KO mice.

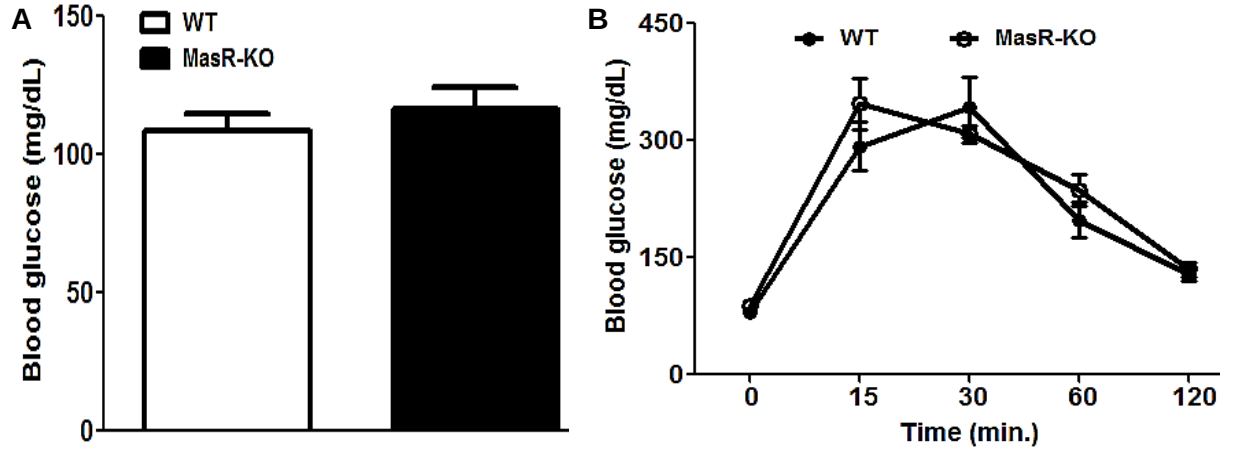


Figure 32 – Glucose conditions were unaltered in MasR-KO mice. *A*: Blood glucose levels and *B*: glucose tolerance were not different in MasR-KO mice compared to the WT mice (n=6).

I also observed the in vitro functions of BMPCs such as proliferation (P<0.05, n=4) (Fig. 33*A*) and migration (P<0.05, n=4) (Fig. 33*B*) of BM LSK cells obtained from MasR-KO mice were significantly lower in basal conditions, or in response to SDF or VEGF when compared to that of WT cells. These results indicate that the lower number of circulating BMPCs might be due to impaired migration and proliferation in response to hypoxia-regulated factors in MasR-KO mice. This also signifies that MasR is essential for mobilization of BMPCs to maintain circulating BMPC levels.

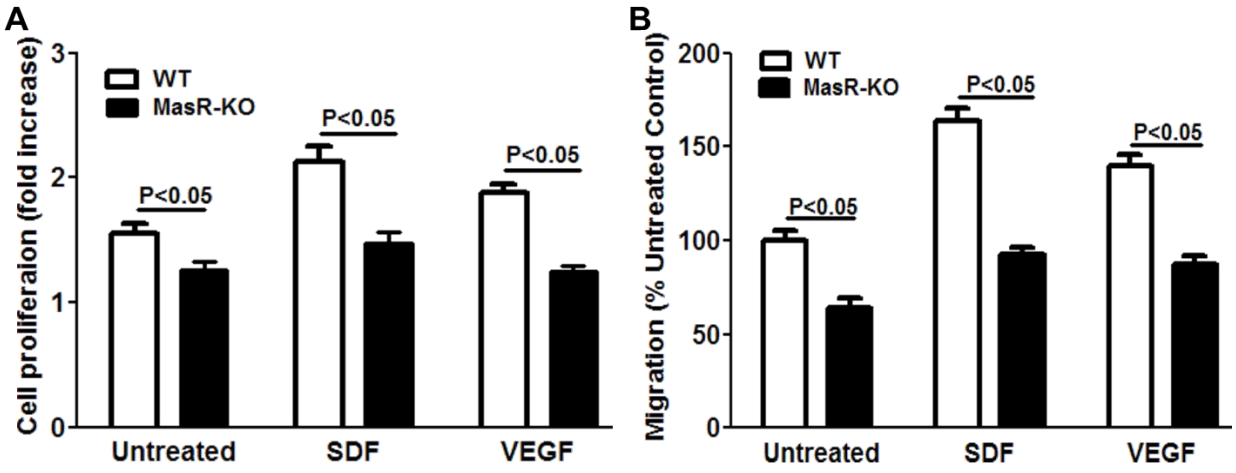


Figure 33 – LSK cells derived from MasR-KO mice showed decreased in vitro proliferation and migration in basal conditions, or in response to SDF or VEGF. *A*: Proliferation and *B*: migration of LSK cells obtained from bone marrow of WT and MasR-KO mice (n=4).

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CHAPTER 3. ANG-(1-7) REVERSES DIABETIC STEM CELL MOBILOPATHY IN RESPONSE TO PLERIXAFOR OR G-CSF

3.1. Rationale and Preliminary Results

Mobilization of progenitor cells in response to clinically used mobilizers such as plerixafor or G-CSF is a critical step in autologous cell therapies. Previous reports showed mobilopathy in response to G-CSF, but not plerixafor in diabetic individuals and in experimental diabetes. This pathological condition precludes diabetic individuals from undergoing cell-based therapies that have the potential to treat cardiovascular complications. In this part of the study, I sought to find out the duration of experimental diabetes that causes mobilopathy aspect of decreased mobilized progenitor cells in response to G-CSF or plerixafor, and I investigated whether Ang-(1-7) treatment reverses mobilopathy in response to these clinical mobilizers.

3.1.1. Plerixafor-Mobilized BMPCs Were Decreased in Long-Term but Not in Short-Term Type 1 Diabetes

Firstly, STZ-diabetic mice were treated with plerixafor at 5 mg/kg, s.c., which was similar to previous studies and the blood samples were analyzed for LSK cells before and after one hour of treatment, where peak mobilization was reported (152) and observed (data not shown). I also tested higher doses of plerixafor at which mice became distressed, therefore I continued using 5 mg/kg dose.

I observed that plerixafor-mobilized WBCs (n=8), LSK cells (n=8) and CFUs (n=8) were not different in short-term STZ-diabetic mice when compared to that of age-matched controls (Fig. 34*A*). In contrast, long-term STZ-diabetic mice showed significantly lower number of plerixafor-mobilized WBCs ($P<0.05$, n=6), LSK cells ($P<0.01$, n=6) and CFUs ($P<0.01$, n=6) when compared to that of age-matched controls (Fig. 34*B*). These results indicate that short-term

diabetes does not have an effect in STZ-diabetic mice to preclude plerixafor-induced mobilization, consequently, mobilopathy in response to plerixafor is not established. Therefore, I used long-term STZ-diabetic mice for further studies with plerixafor.

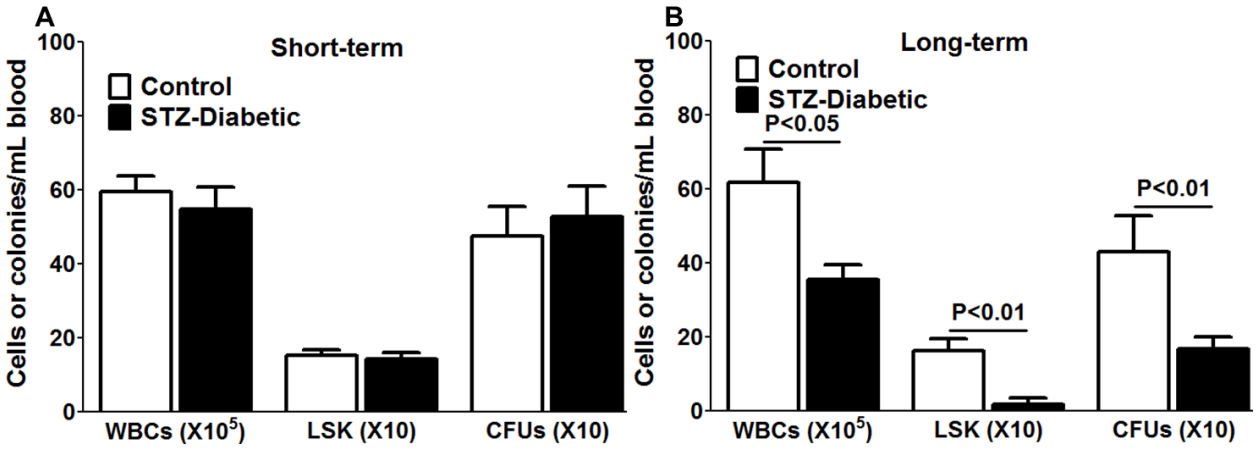

Figure 34 – Plerixafor-induced mobilization of BMPCs was impaired in long-term but not short-term diabetes. Plerixafor-mobilized WBCs, LSK and CFUs in *A*: short-term (n=8) and *B*: long-term (n=6) STZ-diabetic mice.

3.1.2. G-CSF-Mobilized BMPCs Were Decreased in Long-Term but Not in Short-Term Type 1 Diabetes

Secondly, STZ-diabetic mice were treated with G-CSF at 125 µg/kg, twice a day, s.c., for 4 days, which was similar to previous studies (153) and the blood samples were analyzed for LSK cells before and after 12 hours of last dose. I also tested two-day administration with same dose of G-CSF at which significantly lower number of mobilized cells were observed (data not shown).

I observed that G-CSF-mobilized WBCs (n=6), LSK cells (n=6) and CFUs (n=6) were not different in short-term STZ-diabetic mice when compared to that of age-matched controls (Fig. 35*A*). In contrast, long-term STZ-diabetic mice showed significantly lower number of G-CSF-mobilized WBCs (P<0.001, n=6), LSK cells (P<0.001, n=6) and CFUs (P<0.05, n=6) when compared to that of age-matched controls (Fig. 35*B*). These results indicate that short-term

diabetes does not have an effect in STZ-diabetic mice to preclude G-CSF-induced mobilization and mobilopathy in response to G-CSF is not established. Therefore, I used long-term STZ-diabetic mice for further studies with G-CSF.

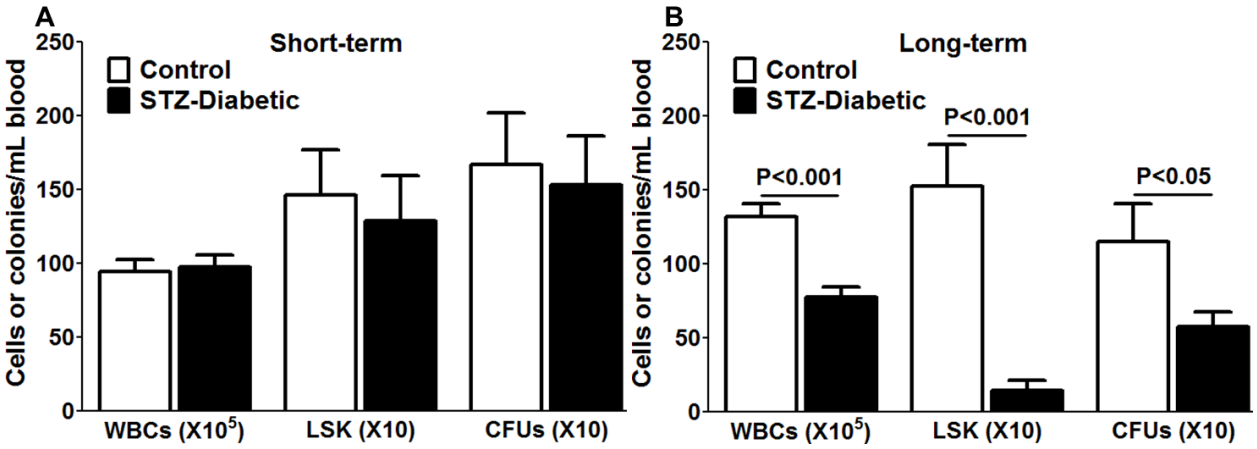

Figure 35 – G-CSF-induced mobilization of BMPCs was impaired in long-term but not short-term diabetes. G-CSF-mobilized WBCs, LSK and CFUs in *A*: short-term (n=6) and *B*: long-term (n=8) STZ-diabetic mice.

3.1.3. Mobilization by Plerixafor or G-CSF Was Not Impaired in db/db Mice

Then, I tested whether mobilopathy in response to plerixafor or G-CSF in long-term diabetic db/db mice is comparable to that of observed in age-matched STZ-diabetics. Surprisingly, in contrast, I observed a significant increase in BMPC mobilization after treatment with plerixafor or G-CSF irrespective of the duration of diabetes. WBCs ($P<0.05$, n=7), LSK cells ($P<0.001$, n=7) and CFUs ($P<0.05$, n=7) were significantly increased in db/db mice after plerixafor treatment when compared to that of lean mice (Fig. 36*A*). Comparably, after G-CSF treatment, WBCs ($P<0.01$, n=8) and LSK cells ($P<0.01$, n=8) were significantly increased and CFUs (n=7) were not different when compared to that of lean mice (Fig. 36*B*). These results indicate that total loss of leptin receptors in db/db mice when compared to the lean mice with 50% of leptin receptors not only dilutes the effect of diabetes on BMPC mobilization but boosts it significantly (154). Therefore, db/db mouse model is not a suitable model to study diabetic

stem cell mobilopathy in response to plerixafor or G-CSF. Type 2 diabetic mouse model with intact leptin receptor expression such as diet-induced diabetes, KK-A^y, or a polygenic model is a suitable model for this study. This was not possible to pursue as a part of the dissertation in the interest of time.

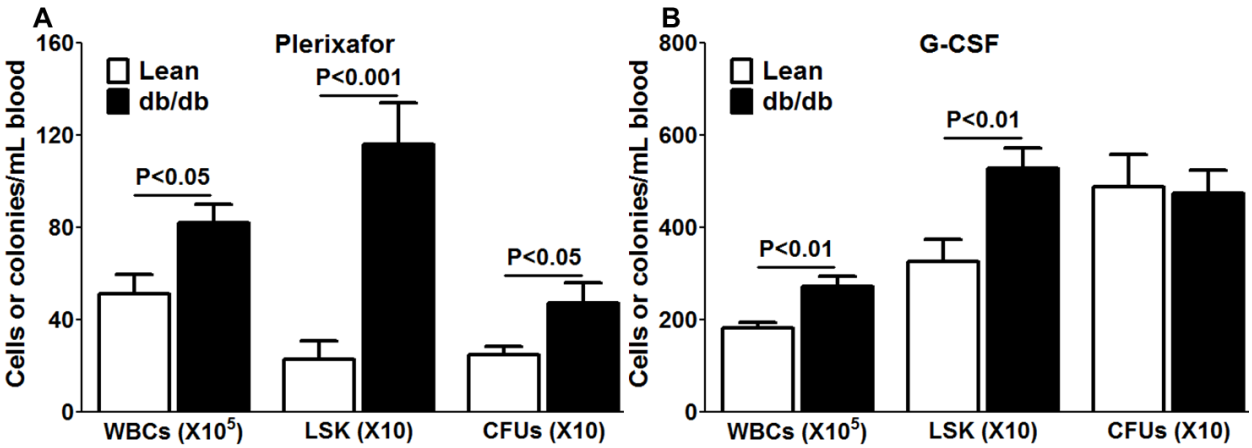

Figure 36 – Plerixafor or G-CSF-induced mobilization of BMPCs was not impaired but enhanced in long-term diabetic db/db mice. *A*: Plerixafor or *B*: G-CSF mobilized WBCs, LSK and CFUs in long-term diabetic db/db mice (n=8).

3.2. Working Hypothesis

I hypothesized that increasing Ang-(1-7) levels reverses BMPC mobilopathy in response to plerixafor or G-CSF in long-term experimental type 1 diabetes.

3.3. Experimental Design

Experimental design for the specific aim 2 is depicted in Fig. 37*A* and 37*B*. The influence of Ang-(1-7) on plerixafor- or G-CSF-induced mobilization of stem/progenitor cells was further determined using MasR-KO mice, which also helped us understand the physiological significance of MasR in mediating BMPC mobilization.

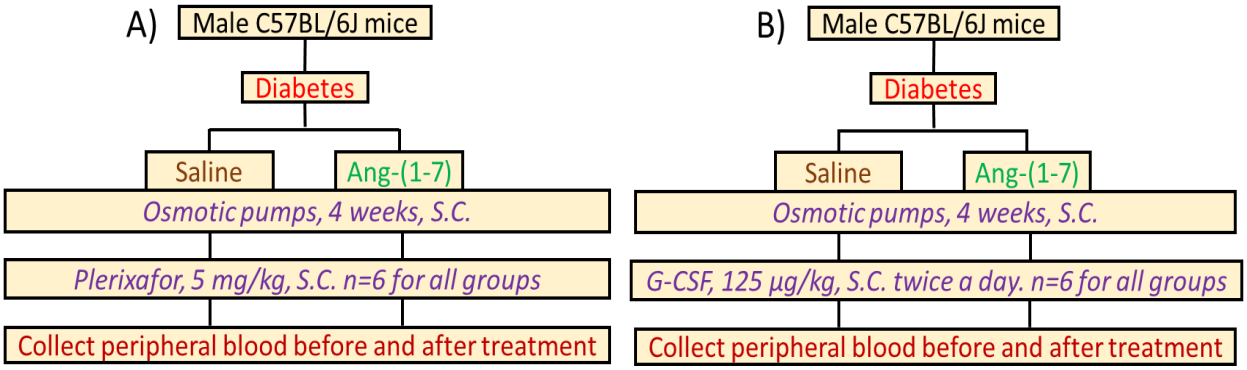

Figure 37 – Flow charts depicting experimental design for specific aim 2 – using *A*: Plerixafor and *B*: G-CSF as mobilizers of BMPCs.

3.4. Results and Discussion

3.4.1. Ang-(1-7) Reverses Diabetic Stem Cell Mobilopathy in Response to Plerixafor

I tested whether Ang-(1-7) treatment can stimulate mobilization of BMPCs in diabetic conditions in response to clinical mobilizers. Ang-(1-7) infusion enhanced plerixafor-mobilized WBCs ($P<0.001$, n=6), LSK cells ($P<0.001$, n=6) and CFUs ($P<0.01$, n=6) in STZ-diabetic mice (Fig. 38). Surprisingly, Ang-(1-7) normalized levels of plerixafor-mobilized BMPCs in STZ-diabetic mice are comparable or higher to that of age-matched untreated control mice, whose data was shown in Fig. 34*B*. This indicates that Ang-(1-7) reverses diabetic stem cell mobilopathy in response to plerixafor.

3.4.2. Ang-(1-7) Reverses Diabetic Stem Cell Mobilopathy in Response to G-CSF

Similarly, I tested whether Ang-(1-7) treatment can stimulate mobilization of BMPCs in diabetic conditions in response to G-CSF. Comparable to the potentiation of plerixafor, Ang-(1-7) infusion enhanced G-CSF-mobilized WBCs ($P<0.01$, n=6), LSK cells ($P<0.001$, n=6) and CFUs ($P<0.01$, n=6) in STZ-diabetic mice (Fig. 39). Surprisingly, Ang-(1-7) normalized levels of G-CSF-mobilized BMPCs in STZ-diabetic mice are comparable or higher to that of age-

matched untreated control mice, whose data was shown in Fig. 35*B*. This indicates that Ang-(1-7) reverses diabetic stem cell mobilopathy in response to G-CSF.

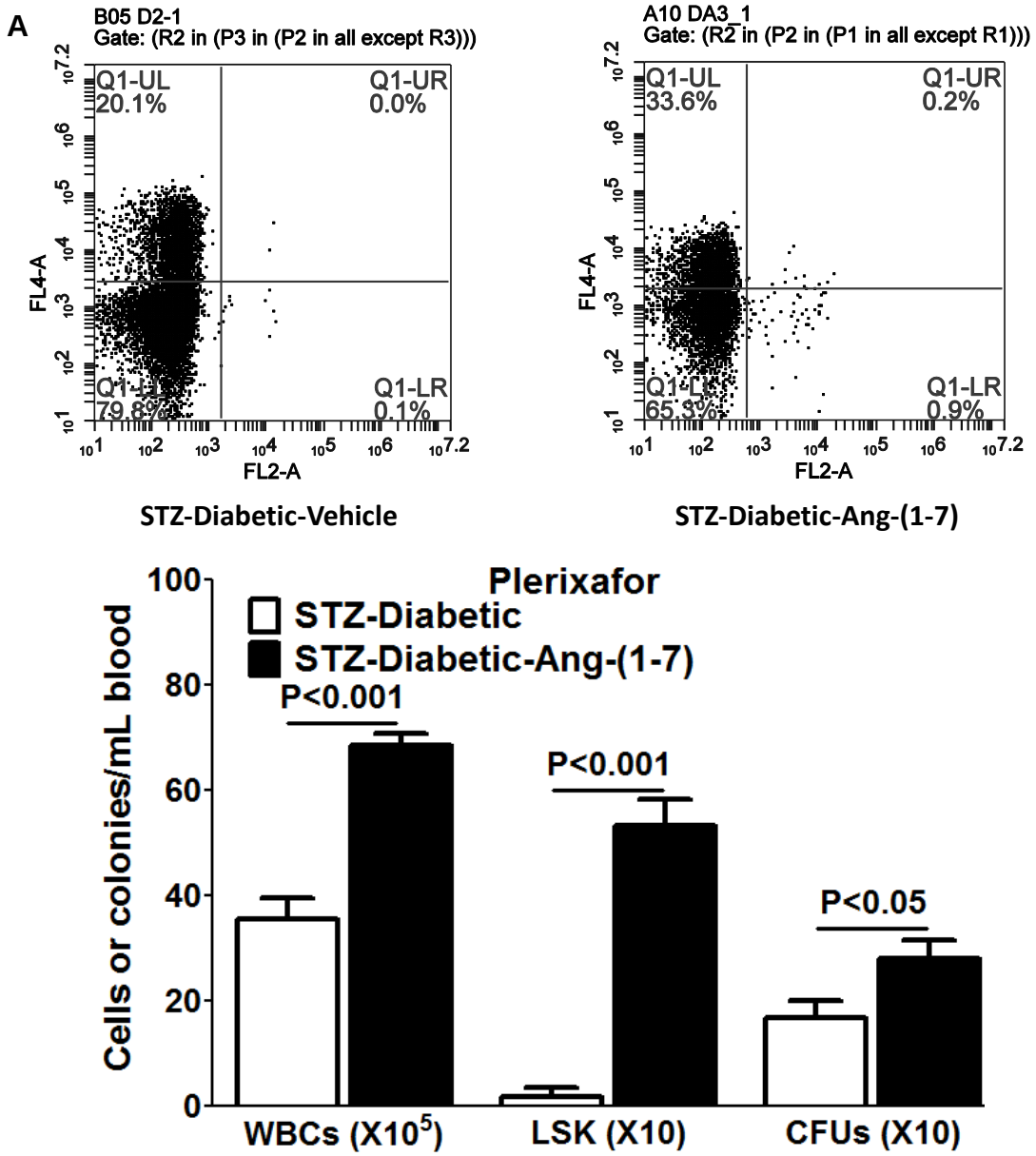

Figure 38 – Ang-(1-7) normalized decreased plerixafor-induced mobilization of BMPCs in experimental type 1 diabetes. *A*: Representative dot plots of flow cytometric enumeration of LSK cells. *B*: Plerixafor-mobilized WBCs, LSK cells and CFUs in STZ-diabetic and STZ-diabetic-Ang-(1-7) treatment groups (n=6).

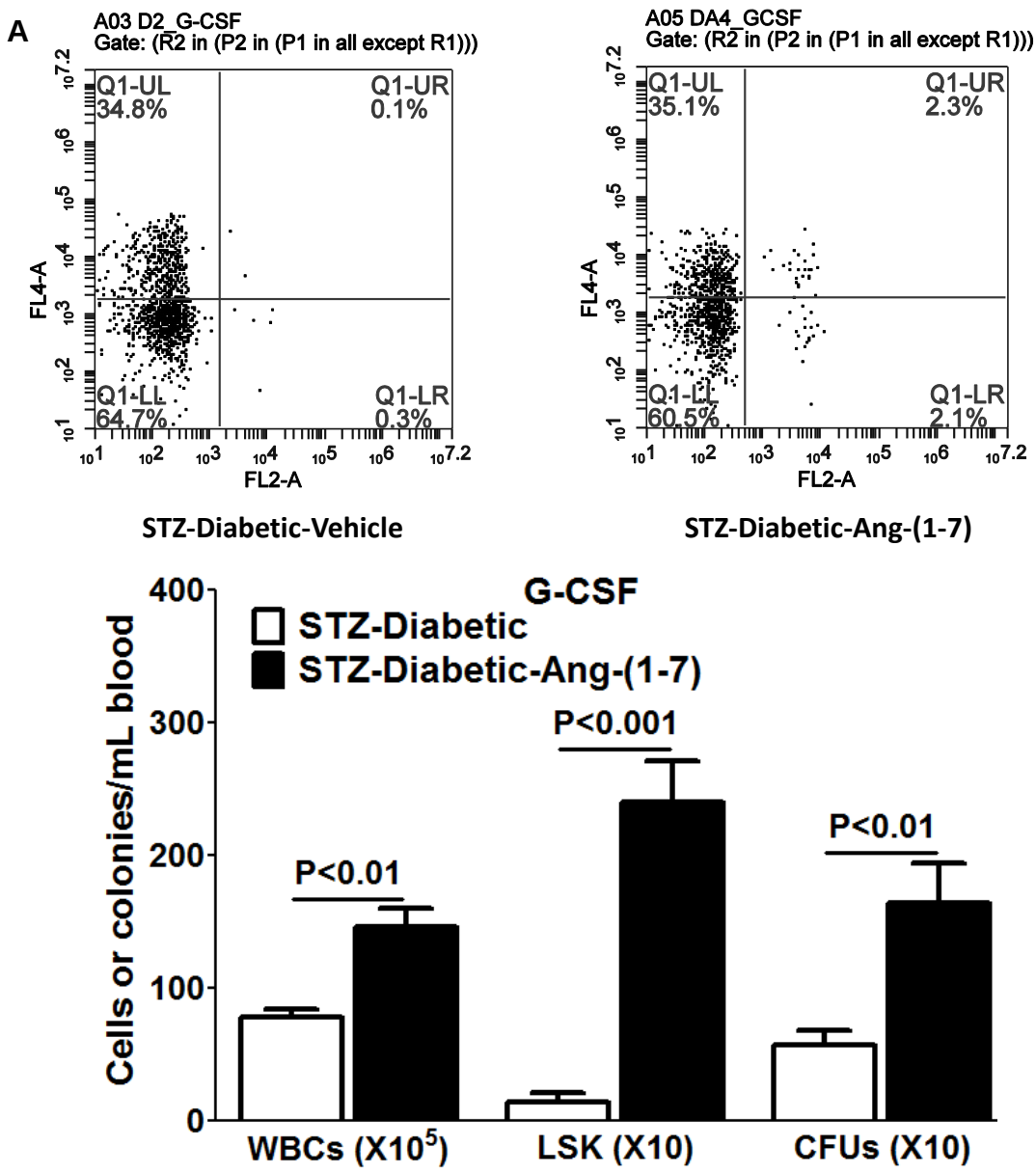

Figure 39 – Ang-(1-7) normalized decreased G-CSF-induced mobilization of BMPCs in experimental type 1 diabetes. *A*: Representative dot plots of flow cytometric enumeration of LSK cells. *B*: G-CSF-mobilized WBCs, LSK cells and CFUs in STZ-diabetic and STZ-diabetic-Ang-(1-7) treatment groups (n=6).

3.4.3. Genetic Ablation of Mas Receptor Recapitulates Diabetic Stem Cell Mobilopathy in Relation to Plerixafor or G-CSF

Finally, I sought to test the physiological role of MasR in mediating plerixafor or G-CSF induced mobilization of BMPCs. I observed that plerixafor-mobilized WBCs ($P<0.05$,

n=6), LSK cells (P<0.01, n=6) and CFUs (P<0.05, n=6) were significantly decreased in MasR-KO mice when compared to that of WT mice (Fig. 40*A*). Along similar lines, WBCs (P<0.05, n=6), LSK cells (P<0.05, n=6) and CFUs (P<0.05, n=6) were significantly decreased in MasR-KO mice after G-CSF when compared to the WT mice (Fig. 40*B*). These results indicate that the absence of MasR deters the extent of mobilization in response to clinical mobilizers, thereby simulating stem cell mobilopathy that occurs due to long-term diabetic conditions in non-diabetic mice.

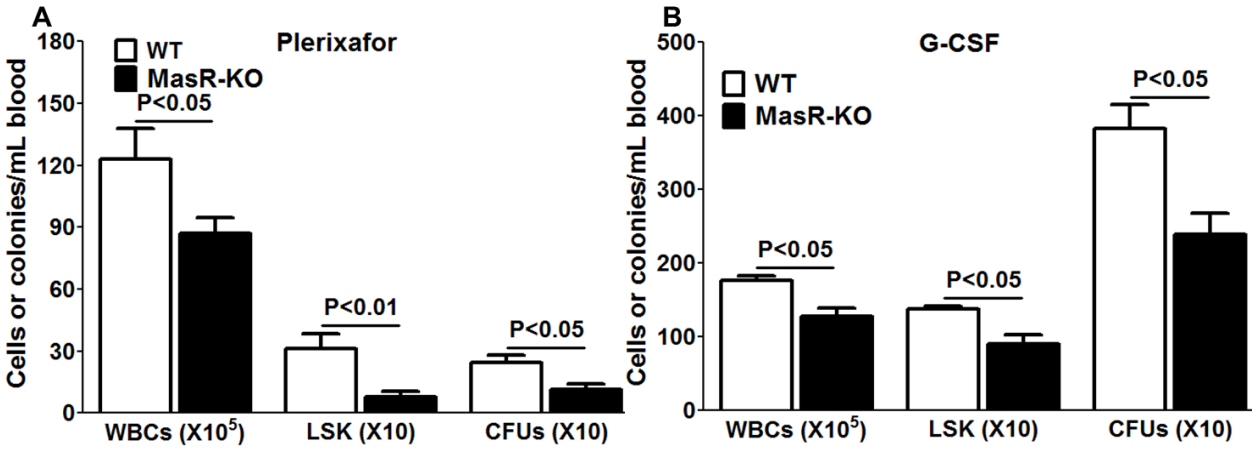

Figure 40 – Plerixafor or G-CSF-induced mobilization of BMPCs was impaired in MasR-KO mice. *A*: Plerixafor or *B*: G-CSF mobilized WBCs, LSK and CFUs in WT and MasR-KO mice (n=3-5).

CHAPTER 4. ANG-(1-7) REVERSES DIABETIC IMPAIRMENT IN ISCHEMIA-INDUCED MOBILIZATION AND VASCULAR REPAIR

4.1. Rationale and Preliminary Results

Ischemia-induced mobilization of BMPCs plays a key role in regular reparative processes to initiate and perform vascular repair, and restore blood flow to the ischemic regions (21; 40). Previous studies reported severe impairment of this essential innate reparative functions in diabetic individuals (7; 185). In this last specific aim, I sought to find out the duration of experimental diabetes that causes mobilopathy aspect of decreased mobilized progenitor cells in response to ischemia and tested the effects of Ang-(1-7) on ischemic vascular repair.

4.1.1. Impaired Blood Flow Recovery of Ischemic Hind Limbs Is More Pronounced in Long-Term Than in Short-Term Type 1 Diabetes

First, I attempted to test the impact of diabetes in ischemia-induced mobilization of BMPCs in short-term and long-term type 1 diabetic mice. HLI was performed in these mice along with their respective age-matched control mice. LSK cell mobilization was estimated in blood samples on days 1, 2, 3, 5, 7 and 10 following HLI with concomitant estimation of blood flow recovery in the ischemic hind limbs by using LDI. In short-term control mice, the peak LSK cell mobilization was observed on day-2 following HLI and the number of LSK cells returned to pre-ischemic levels by day-7. Whereas, in short-term STZ-diabetic mice, LSK cell mobilization was impaired on days 1, 2, 3 and 5 when compared to the age-matched controls (Fig. 41*A*). Irrespective of BMPC kinetics, the blood flow recovery in ischemic hind limbs of short-term STZ-diabetes showed no difference compared to the age-matched controls (Fig. 41*B* and 41*C*). However, I observed severe impairment in blood flow recovery along with mobilopathy in

response to ischemia in long-term STZ-diabetic or db/db mice when compared to their respective age-matched controls (data shown in results section 4.4.).

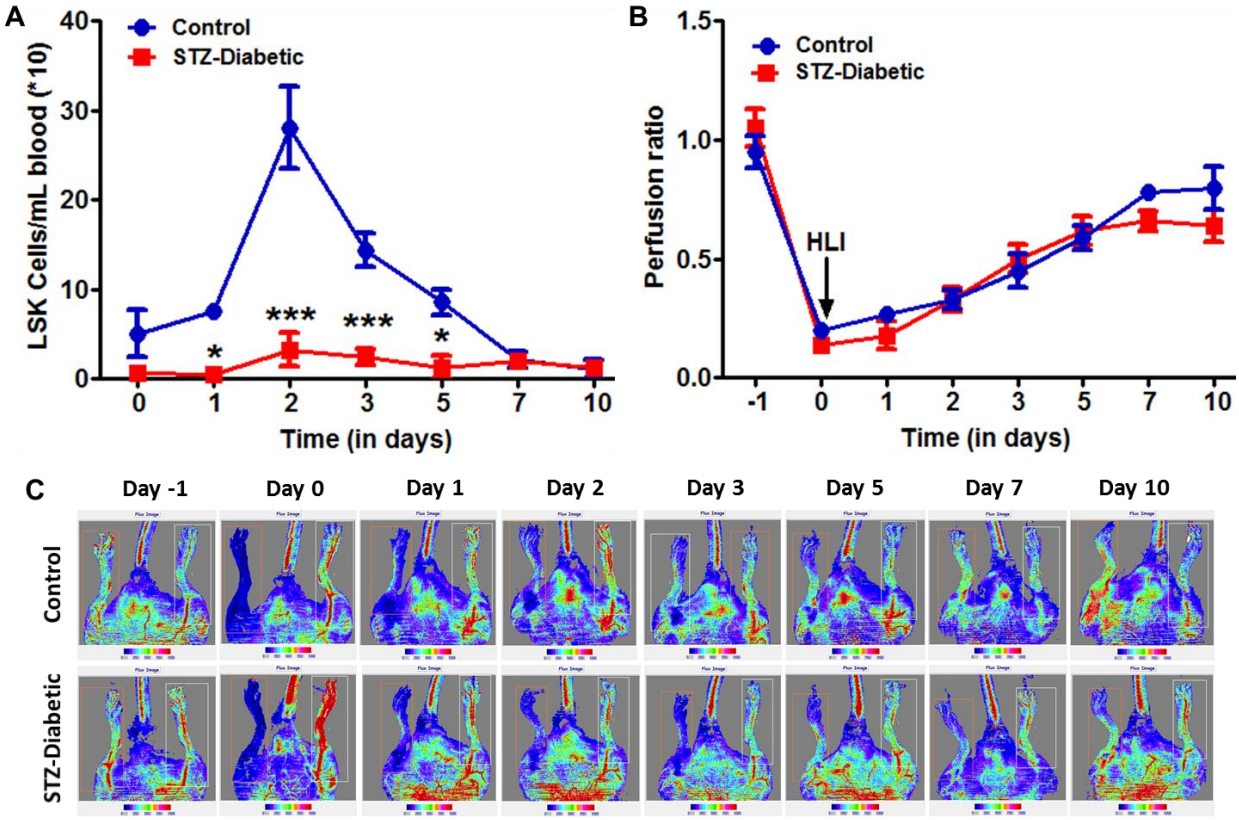

Figure 41 – Ischemia-induced LSK cell mobilization was impaired in short-term experimental type 1 diabetes but not blood flow recovery in ischemic hind limbs following HLI. *A*: LSK cell mobilization and *B*: blood flow recovery in short-term STZ-diabetic mice following HLI (n=3-5). *C*: Representative LDI images of blood flow recovery in ischemic hind limbs. *P<0.05 and ***P<0.001 vs Control as analyzed by using two-way ANOVA.

These results suggest that the effect of diabetes on post-ischemic recovery is more pronounced in long-term than short-term diabetic mice. Therefore, I used only long-term type 1 or type 2 diabetic mice in further studies using HLI model.

4.2. Working Hypothesis

I hypothesized that increasing Ang-(1-7) levels reverses BMPC mobilopathy, and improves blood flow recovery and neovascularization in hind limbs in response to ischemia in experimental type 1 and type 2 diabetes.

4.3. Experimental Design

Experimental design for the specific aim 3 is depicted in Fig. 42*A* and 42*B*. The influence of Ang-(1-7) on ischemia-induced mobilization of stem/progenitor cells was further determined using MasR-KO mice, which also helped us understand the physiological significance of MasR in mediating ischemia-induced BMPC mobilization.

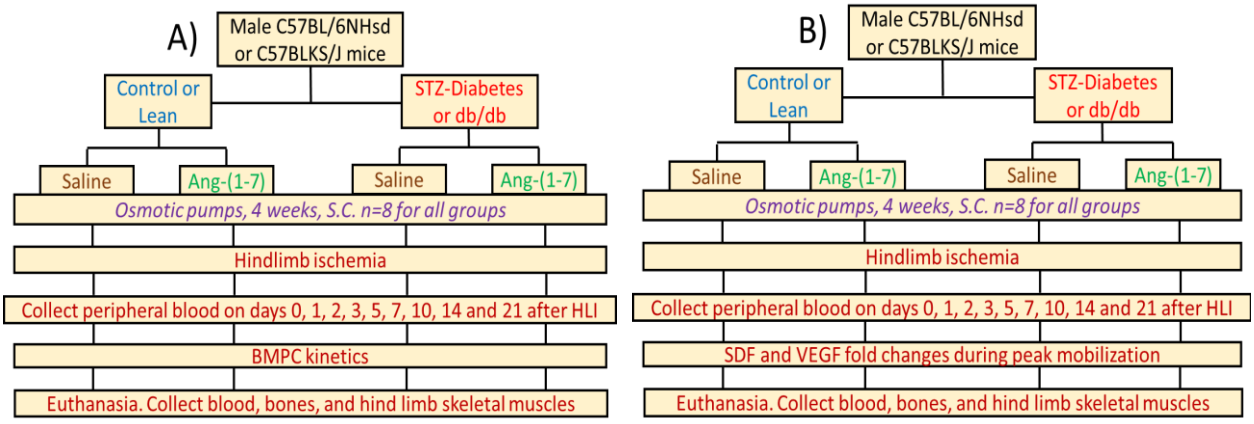

Figure 42 – Flow charts depicting experimental design for specific aim 3 – to study *A*: BMPC kinetics and *B*: SDF and VEGF fold changes in response to hind limb ischemic injury. Blood flow measurements in ischemic hind limbs using LDI and estimation of neovascularization in ischemic skeletal muscle sections was also performed.

4.4. Results and Discussion

4.4.1. Impaired Mobilization of BMPCs in Response to Ischemic Injury Was Reversed by Ang-(1-7) in Type 1 or Type 2 Diabetes

HLI was performed in STZ-diabetic and db/db mice along with their respective controls with or without Ang-(1-7) treatment. Surgery was performed 18 days after Ang-(1-7) treatment

and experimented over a period of 21 days, where single required dose of Ang-(1-7) was given each day after the exhaustion of Ang-(1-7) osmotic pump. Following HLI, blood samples were analyzed on days 1, 2, 3, 5, 7, 10, 14 and 21 for LSK cell mobilization with concomitant estimation of blood flow recovery in the ischemic hind limbs by using LDI.

In response to HLI, the peak mobilization of LSK cells was observed on day-2 following ischemia in control mice, which plateaued over up to day-7 and returned to pre-ischemic levels on day-10 (Fig. 43*B*). Control mice administered with Ang-(1-7) has two-fold increase in peak mobilization of LSK cells on day-2 following ischemia when compared to the untreated ($P<0.001$, n=6-8). Contrarily, in STZ-diabetic mice, HLI-induced mobilization of LSK cells was severely blunted on days 2, 3, 5 and 7 following ischemia when compared to the control mice (Fig. 43*B*). In agreement with this, the area under the curve (AUC) of LSK cell mobilization in STZ-diabetic mice was decreased when compared to the control mice ($P<0.05$, n=6-8) (Fig. 43*C*). This dysfunction was reversed in Ang-(1-7) treated STZ-diabetic mice, where peak mobilization of LSK cells was restored ($P<0.001$, n=6-8) on day-3 following ischemia and AUC of blood flow was restored when compared to the untreated ($P<0.05$, n=6-8) (Fig. 43*B* and 43*C*).

The pattern of LSK cell mobilization in lean mice is distinct from that of control mice and peak mobilization was lower. Whereas, comparable to control mice maximum mobilization was observed on day-2 following ischemia, whereas by day-3 post ischemia LSK cell number returned to the pre-ischemic levels. Ang-(1-7) treatment enhanced LSK cell mobilization in lean mice ($P<0.05$, n=5). Comparable to the STZ-diabetic mice, db/db mice showed blunted mobilization post ischemia ($P<0.01$, n=5) and Ang-(1-7) treatment reversed this dysfunction, where peak mobilization was observed on day-3 compared to the untreated ($P<0.001$, n=5) (Fig. 44*B*). In accordance with this data, AUC of LSK cell mobilization in db/db mice was lower

compared to the lean (P<0.05, n=5), which was reversed after Ang-(1-7) treatment when compared to the untreated (P<0.05, n=5) (Fig. 44*C*). The differential pattern of mobilization in C57BL/6NHsd mice and lean mice might be due to the strain difference or leptin receptor's involvement (154) which requires further investigation.

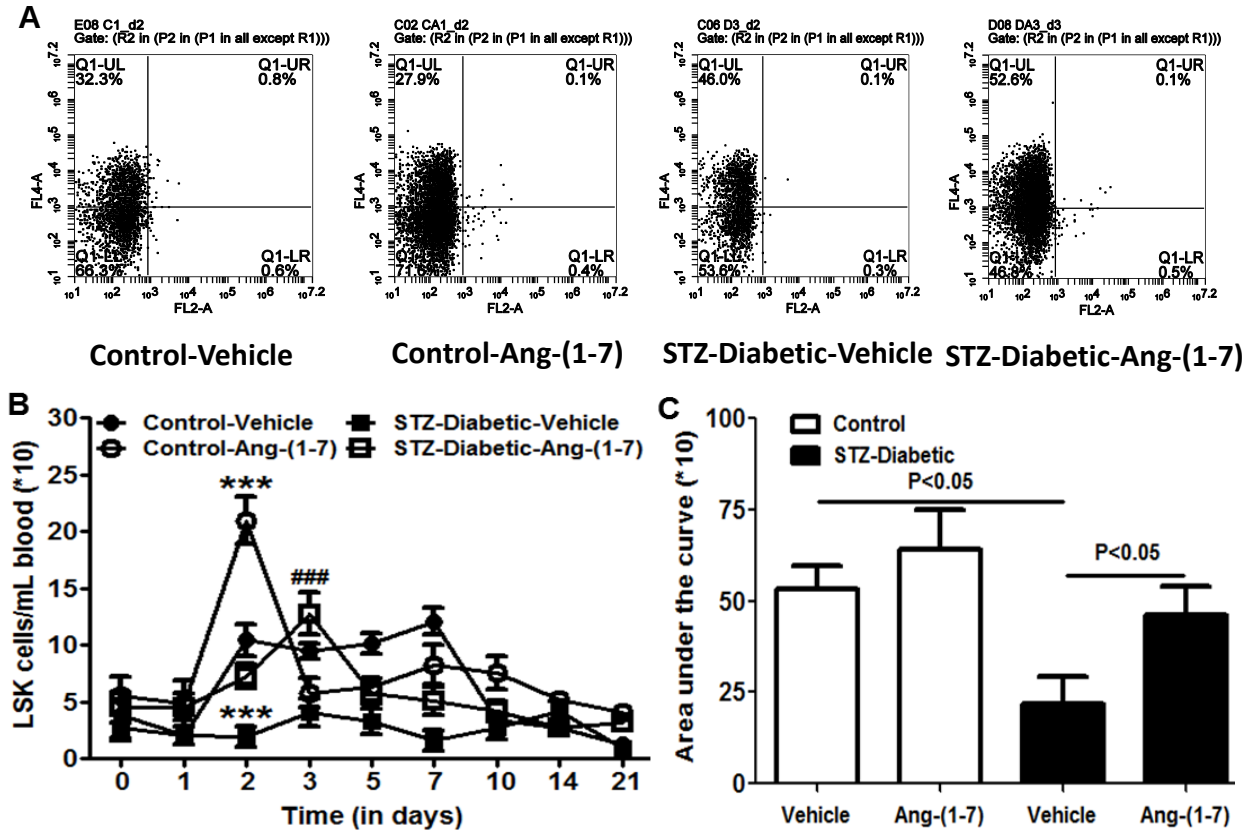

Figure 43 – Reversal of type 1 experimental diabetic dysfunction in the mobilization of BMPCs in response to ischemia by Ang-(1-7). *A*: Representative dot plots of flow cytometric enumeration of LSK cells at peak mobilization, *B*: ischemia-induced LSK cell mobilization kinetics over a period of 21 days following HLI, and *C*: AUC of LSK cell mobilization kinetics in different treatment groups (n=6-8). ***P<0.001 vs Control-Vehicle and ###P<0.001 vs STZ-Diabetic-Vehicle as analyzed by using two-way ANOVA.

These results indicate that Ang-(1-7) reverses mobilopathy in response to ischemia in diabetic conditions as observed in both models of diabetes. The mobilized LSK cells further have the potential to initiate the neovascularization process and enhance blood flow recovery in ischemic regions.

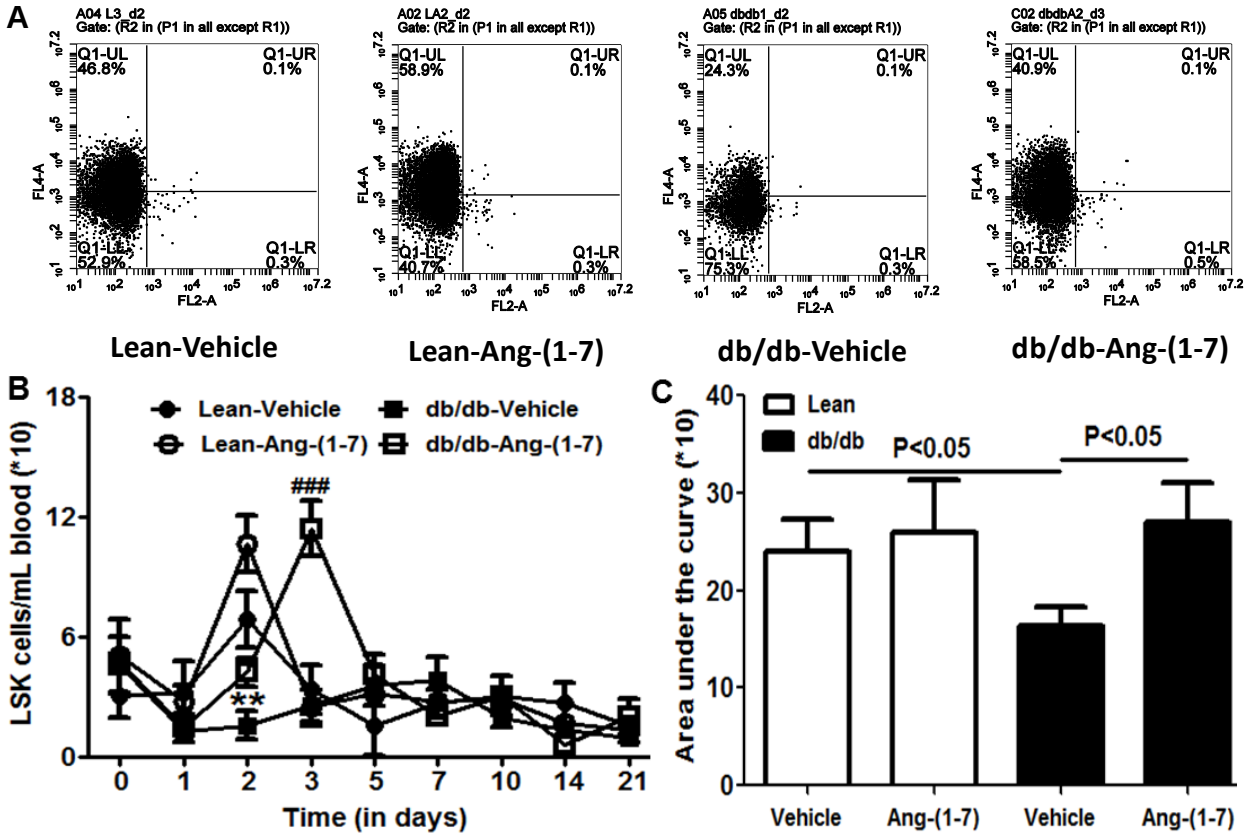

Figure 44 – Reversal of type 2 experimental diabetic dysfunction in the mobilization of BMPCs in response to ischemia by Ang-(1-7). *A*: Representative dot plots of flow cytometric enumeration of LSK cells at peak mobilization, *B*: ischemia-induced LSK cell mobilization kinetics over a period of 21 days following HLI, and *C*: AUC of LSK cell mobilization kinetics in different treatment groups (n=5). **P<0.01 vs Lean-Vehicle and ###P<0.001 vs db/db-Vehicle as analyzed by using two-way ANOVA.

4.4.2. Ang-(1-7) Restores Blood Flow Recovery to Ischemic Limbs in Type 1 or Type 2 Diabetes

Following ischemia, control mice showed 94±2% blood flow recovery by day-14 in ischemic ipsilateral hind limb, which maximized and stabilized at 104±2% by day-21 relative to the non-ischemic contralateral limb. Whereas, blood flow recovery was severely impaired in STZ-diabetic mice which was lower and at slower rate than controls (44±4% on day-10, P<0.001; and 66±10% on day-21, P<0.01, n=6) (Fig. 45*B*). Moreover, 20% of STZ-diabetic mice underwent partial or complete amputation, for which the representative LDI images were shown

in Fig. 45*A*. The impaired blood flow recovery was reversed and accelerated with Ang-(1-7) treatment and completely normalized by day-21 in STZ-diabetic mice when compared to the untreated (81±5% on day-10, P<001; and 102±7% on day-21, P<0.05) (Fig. 45*B*). Notably, there was no amputation of ischemic hind limbs was observed in Ang-(1-7) treated STZ-diabetic mice following HLI.

In a similar manner, following HLI, db/db mice (55±6% on day-10, P<0.001; 68±2% on day-21, P<0.001; n=6) showed lower and slower rate of blood flow recovery compared to lean mice (101±5% on day-10; 105±6 on day-21) (Fig. 46*B*). Moreover, 33.33% of db/db mice underwent partial or complete amputation, or which the representative LDI images were shown in Fig. 46*A*. Ang-(1-7) treatment reversed this dysfunction by accelerating the blood flow recovery in db/db mice (87±5% P<0.05, n=6), however observed no difference in lean mice (105±5%, n=6), when compared to the respective untreated groups (Fig. 46*B*). Importantly, no amputation of ischemic hind limbs was observed following HLI in Ang-(1-7) treated db/db mice.

These results of reversal of impaired blood flow recovery in diabetic mice by Ang-(1-7) treatment is in compliance with the increase in LSK cell mobilization.

4.4.3. Ang-(1-7) Improves Neovascularization in Ischemic Skeletal Muscles in Type 1 or Type 2 Diabetes

At the end of HLI experiments, mice were euthanized, gastrocnemius skeletal muscles were isolated, fixed and processed for immunohistochemistry to estimate neovascularization by staining the vasculature with isolectin-B4 and CD-31 to identify endothelial cell surface and intercellular junctions respectively, where dual positive cells identify the formation of comprehensive vasculature.

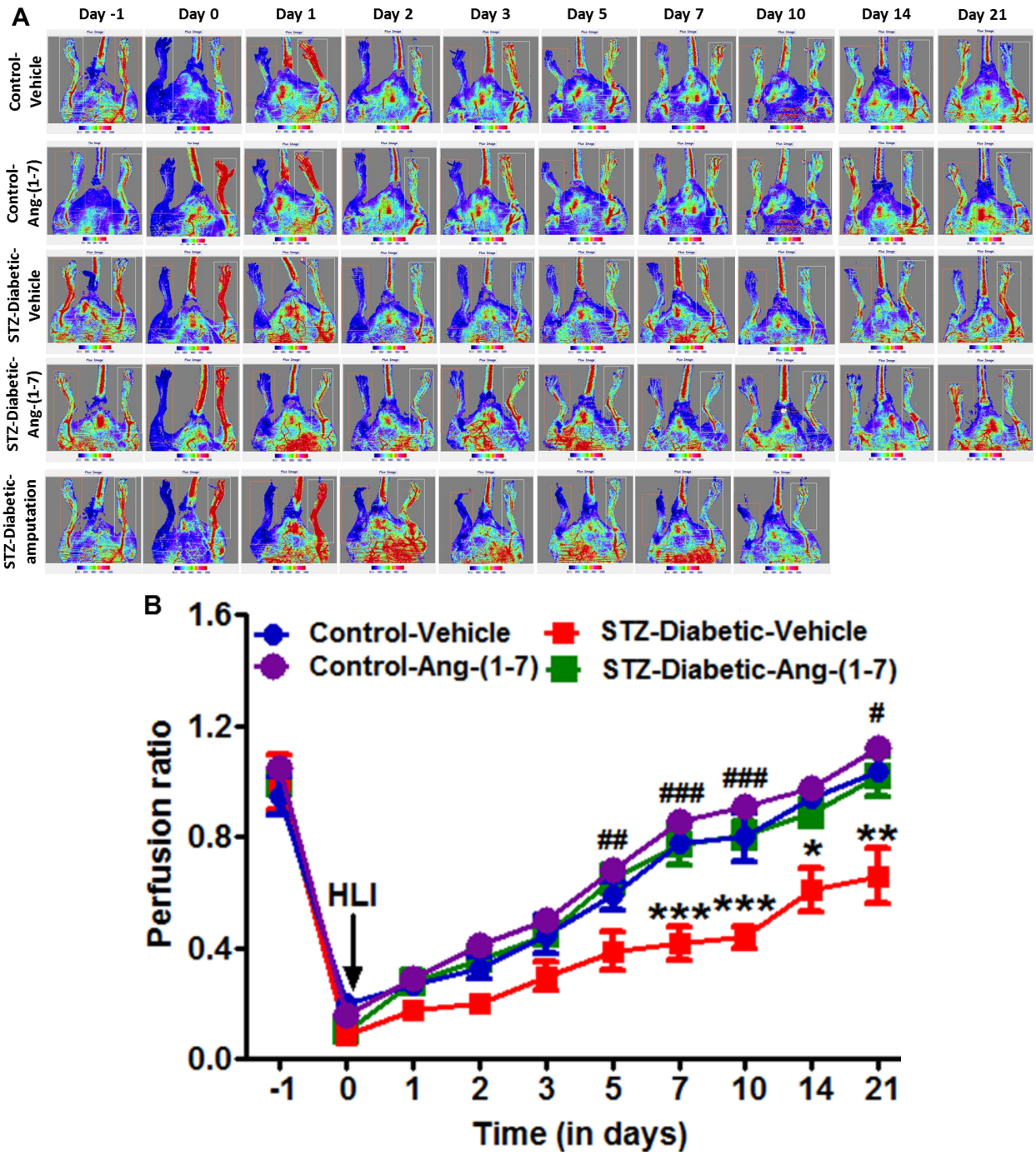

Figure 45 – Reversal of type 1 experimental diabetic impairment in the blood flow recovery of ischemic hind limbs in response to ischemia by Ang-(1-7). *A*: Representative LDI images of blood flow recovery in different treatment groups along with a representation of diabetes-induced auto-amputation of ischemic hind limbs. *B*: Blood perfusion quantified by using RBC flux (blood×area^{-1}×time^{-1}) expressed as percent of the respective contralateral limb (n=6). *P<0.05, **P<0.01 and ***P<0.001 vs Control-Vehicle and #P<0.05, ##P<0.01 and ###P<0.001 vs STZ-Diabetic-Vehicle as analyzed by using two-way ANOVA.

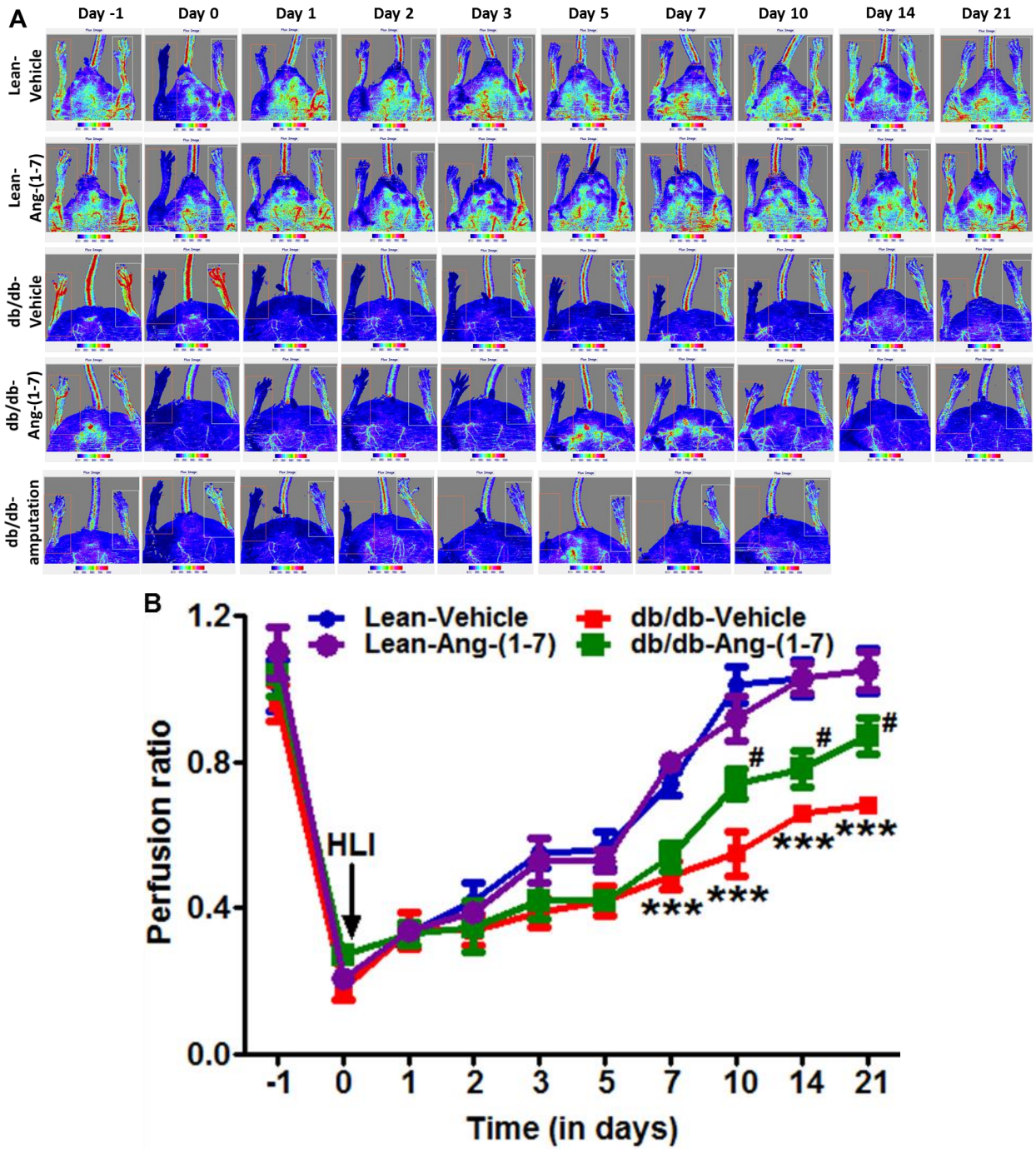

Figure 46 – Reversal of type 2 experimental diabetic impairment in the blood flow recovery of ischemic hind limbs in response to ischemia by Ang-(1-7). *A*: Representative LDI images of blood flow recovery in different treatment groups along with a representation of diabetes-induced auto-amputation of ischemic hind limbs. *B*: Blood perfusion quantified by using RBC flux (blood×area^{-1}×time^{-1}) expressed as percent of the respective contralateral limb (n=5). ***P<0.001 vs Lean-Vehicle and #P<0.05 vs db/db-Vehicle as analyzed by using two-way ANOVA.

In accordance with the above findings, I observed a higher amount of tissue necrosis in ischemic skeletal muscle of STZ-diabetic or db/db mice when compared to their respective controls, which was remarkably reversed after Ang-(1-7) treatment when compared to the respective untreated mice (Fig. 47*A* and 48*A*). In addition, I observed impaired neovascularization of ischemic skeletal muscles of STZ-diabetic mice when compared to the controls ($P<0.001$, n=6). Improved capillary density was observed following Ang-(1-7) treatment in STZ-diabetic mice when compared to the untreated ($P<0.05$, n=6) (Fig. 47*B* – 47*D*). Along similar lines, ischemic skeletal muscles derived from db/db mice showed lower capillary density when compared to the lean ($P<0.001$, n=5) and Ang-(1-7) treatment reversed this dysfunction in db/db mice when compared to the untreated ($P<0.01$, n=5) (Fig. 48*B* – 48*D*).

These results suggest that impaired post-ischemic neovascularization in diabetic conditions can be reversed and improved by Ang-(1-7). This enhanced neovascularization might be due to increased LSK cell mobilization as shown above and/or reduced oxidative stress in the ischemic areas (36). The improved blood flow recovery and decreased amputation rate post ischemia in diabetic mice following Ang-(1-7) treatment might be due to enhanced neovascularization, by which increased oxygen and nutrient supply can be achieved in ischemic regions culminating towards accelerated recovery of ischemic hind limbs.

4.4.4. Ang-(1-7) Reverses Decreased Plasma SDF and VEGF Levels That May Contribute to Peak BMPC Mobilization in Experimental Diabetes

The release of SDF and VEGF by the injured tissue as well as the inflammatory cells into the blood circulation plays a key role in recruitment of stem/progenitor cells. Impaired release in these chemokines may result in stem cell mobilopathy. Therefore, I tested whether SDF or VEGF levels increased during the peak of ischemia-induced mobilization in diabetic conditions.

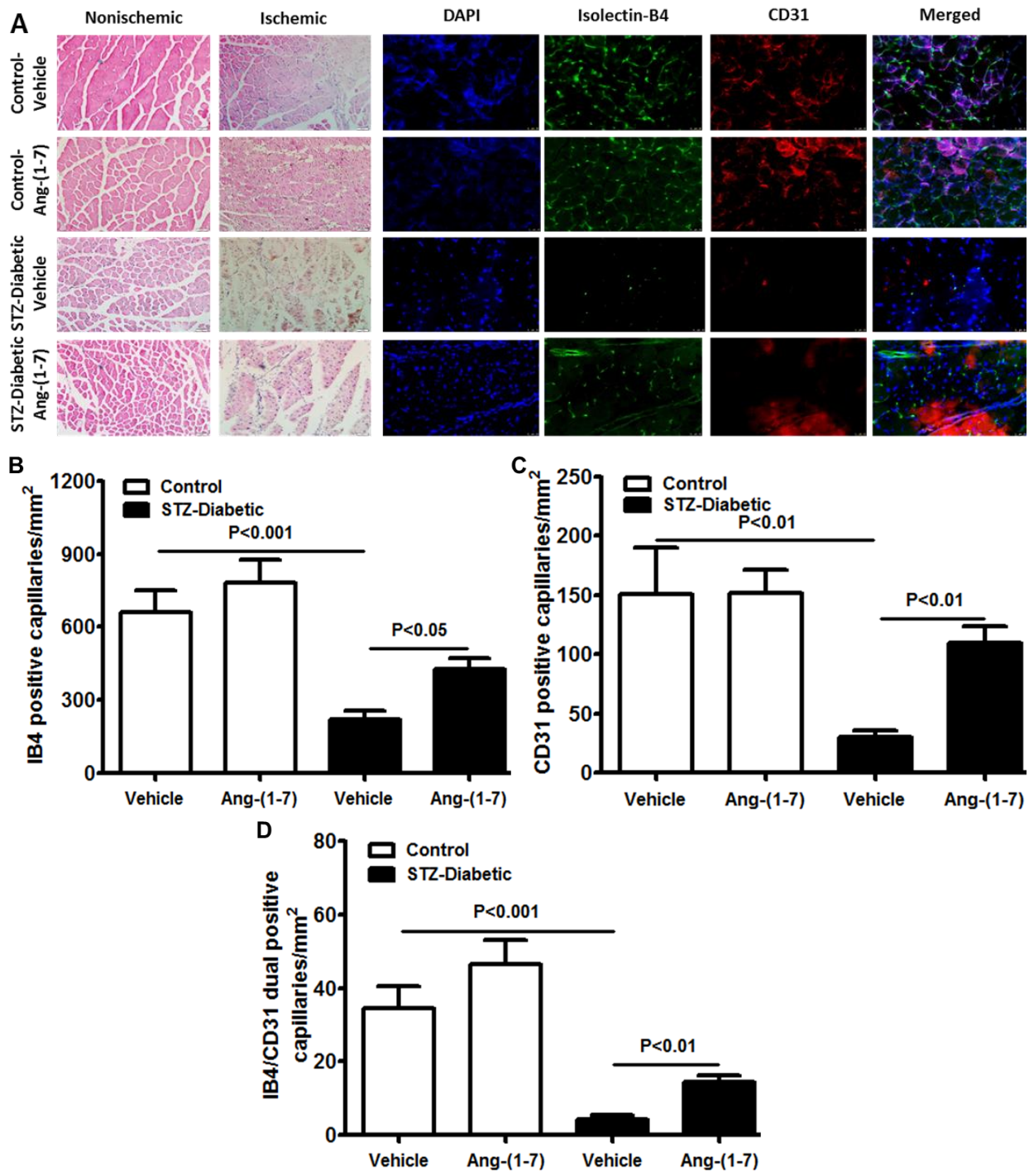

Figure 47 – Reversal of type 1 experimental diabetic impairment in the neovascularization of ischemic areas by Ang-(1-7). *A*: Representative light microscopic images of hematoxylin & eosin staining, and fluorescence images of isolectin-B4 (IB4) or CD31 staining of non-ischemic and ischemic gastrocnemius muscle sections; scale bar = 25 μm. Capillary density was quantified by *B*: IB4, *C*: CD31, or *D*: dual positive capillaries in the muscle sections (n=6).

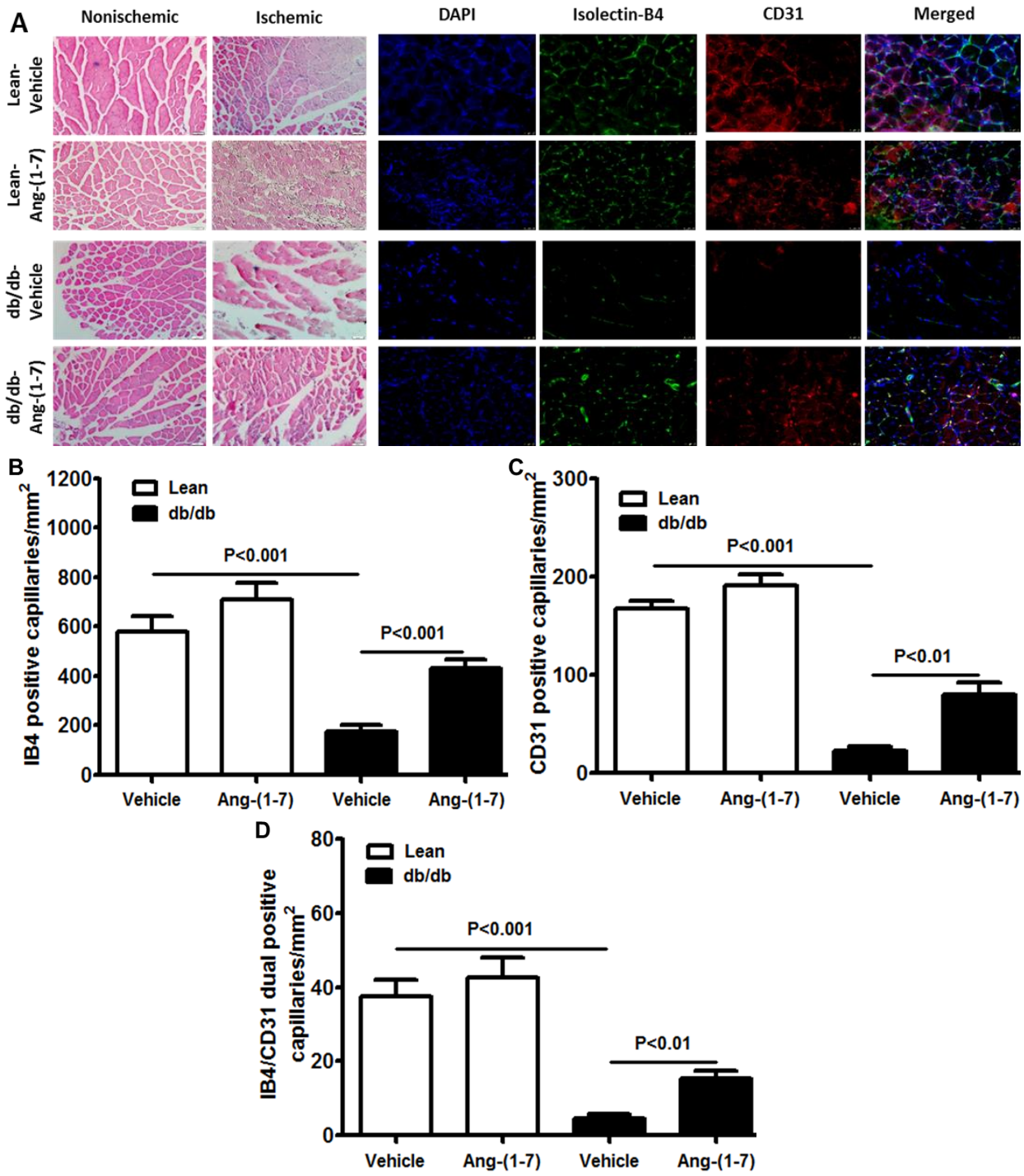

Figure 48 – Reversal of type 2 experimental diabetic impairment in the neovascularization of ischemic areas by Ang-(1-7). *A*: Representative light microscopic images of hematoxylin & eosin staining, and fluorescence images of isolectin-B4 (IB4) or CD31 staining of non-ischemic and ischemic gastrocnemius muscle sections; scale bar = 25 µm. Capillary density was quantified by either *B*: IB4, *C*: CD31, or *D*: dual positive capillaries in the muscle sections (n=5).

Unsurprisingly, I have observed a decrease in plasma SDF (P<0.05, n=3) and VEGF (P<0.01, n=3) fold increase on the day of peak LSK cell mobilization relative to that of day 0 following HLI in STZ-diabetics when compared to that of control mice. Ang-(1-7) treatment has reversed this dysfunction and improved the plasma SDF (P<0.01, n=3) and VEGF (P<0.05, n=3) fold increase during peak LSK cell mobilization in STZ-diabetic mice (Fig. 49*A* and 49*B*).

Similar results were observed in type 2 diabetic mice. Plasma SDF (P<0.05, n=3) and VEGF (P<0.05, n=3) fold increase was blunted in db/db mice during peak LSK cell mobilization in lean mice. Whereas, Ang-(1-7) treatment improved this condition and enhanced fold increase of plasma SDF (P<0.05, n=3) and VEGF (P<0.05, n=3) on the day of peak mobilization (Fig. 49*C* and 49*D*). Notably, there is no real increase in SDF fold change in lean mice (Fig. 49*C*).

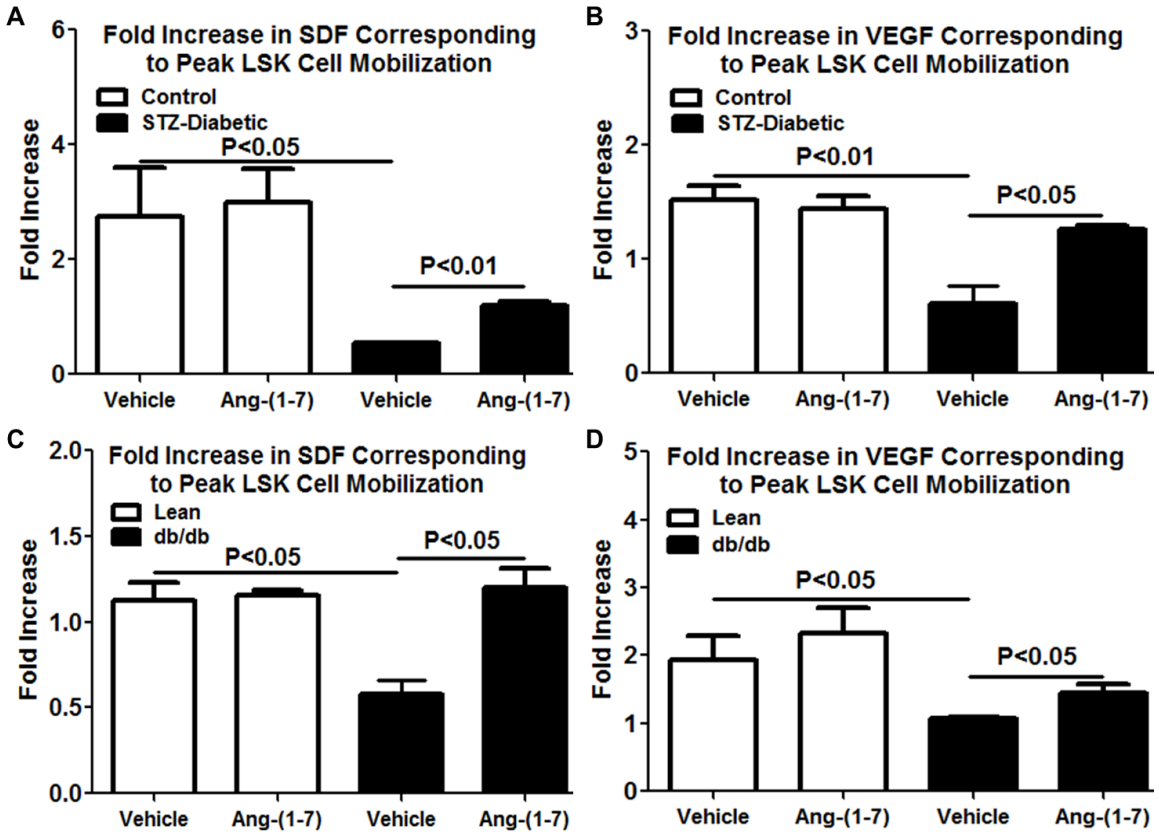

Figure 49 – Reversal of impairment in increase of plasma SDF and VEGF corresponding to peak LSK cell mobilization in experimental type 1 and type 2 diabetes following HLI by Ang-(1-7). Plasma SDF (*A* and *C*) and VEGF (*B* and *D*) measured in various treatment groups (n=3).

4.4.5. Genetic Ablation of Mas Receptor Recapitulates Diabetic Stem Cell Mobilopathy in Non-Diabetic Mice in Response to Ischemia

Lastly, I tested whether BMPC mobilization in response to ischemic injury requires endogenous expression of MasR. In response to HLI, WT mice responded comparable to the control mice, where the peak mobilization of LSK cells was observed on day-3 post ischemia. Whereas, MasR-KO mice showed significant impairment in ischemia-induced mobilization, especially on day-2 and day-3 following HLI when compared to the WT mice (Fig. 50*A*). In a similar manner, MasR-KO mice (64±2%, $P<0.01$, n=4 on day-21) showed lower and slower rate of blood flow recovery when compared to the WT mice, especially on days 5 to 21 (83±5% on day-21) (Fig. 50*B*). In order to confirm whether Ang-(1-7) is mediating its effects in ischemia-induced mobilization and blood flow recovery through MasR, I treated MasR-KO mice with Ang-(1-7) and performed HLI. Unsurprisingly, mobilization of LSK cells (Fig. 50*A*) or blood flow recovery (Fig.50*B*) were unaffected in Ang-(1-7) treated MasR-KO mice when compared to the untreated. These results indicate that MasR plays an important role physiologically in mediating ischemia-induced mobilization and might be an important target in reversing diabetic stem cell mobilopathy in response to ischemia.

Corresponding to this, I observed higher tissue necrosis as observed by using H&E staining in gastrocnemius ischemic skeletal muscle sections of MasR-KO mice when compared to that of WT (Fig. 51*A*). Moreover, neovascularization in these sections was significantly decreased in MasR-KO mice compared to that of WT ($P<0.001$, n=4) (Fig. 51*B* – 51*D*). The lower amount of vascular regeneration signifies the lower blood flow and slower recovery of ischemic hind limbs following ischemia in MasR-KO mice. Altogether, the absence of Mas receptor simulates diabetic stem cell mobilopathy in response to ischemia even in non-diabetic

mice. These results also imply the physiological importance of MasR in ischemia-induced mobilization of stem/progenitor cells, therefore in ischemic vascular repair as well.

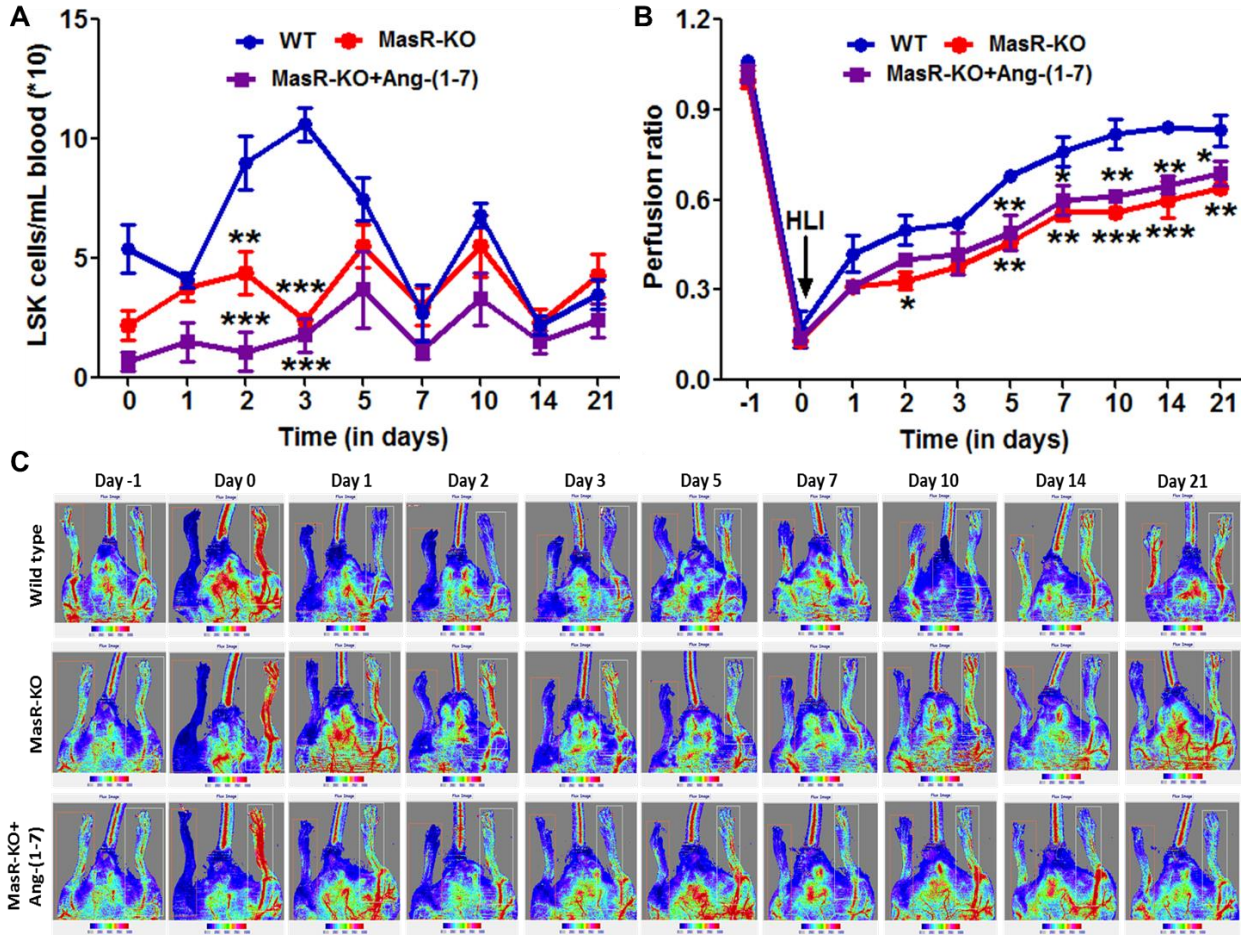

Figure 50 – Ischemia-induced LSK cell mobilization and blood flow recovery of ischemic hind limbs were impaired in MasR-KO mice following HLI and were unaffected by Ang-(1-7) treatment. *A*: LSK cell mobilization and *B*: blood flow recovery in different treatment groups (n=4). *C*: Representative LDI images of blood flow recovery in ischemic hind limbs. *P<0.05, **P<0.01 and ***P<0.001 vs WT mice as analyzed by using two-way ANOVA.

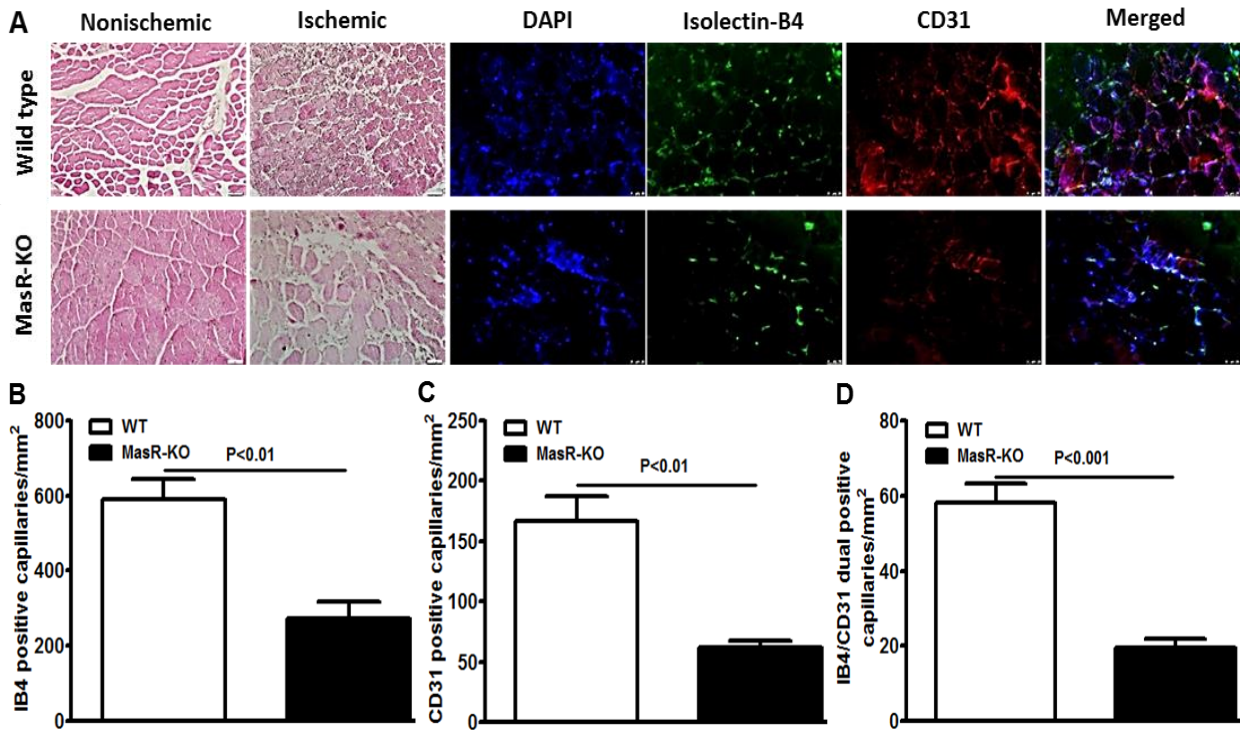

Figure 51 – Impaired neovascularization of ischemic areas in MasR-KO mice following HLI. *A*: Representative light microscopic images of hematoxylin & eosin staining, and fluorescence images of isolectin-B4 (IB4) or CD31 staining of non-ischemic and ischemic gastrocnemius muscle sections; scale bar = 25 µm. Capillary density was quantified by *B*: IB4, *C*: CD31, or *D*: dual positive capillaries in the muscle sections (n=4).

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

5.1. Summary and Conclusions

Efficient healing of injured/ischemic regions or successful neovascularization outcomes of cell-based therapies involve a) proper mobilization of BMPCs in response to ischemia or clinical mobilizers, b) their engraftment after transplantation, c) incorporation of BMPCs into ischemic/injured area or target tissue, and d) paracrine functions of BMPCs that enhance angiogenesis. Although many studies are in disagreement with the incorporation of BMPCs into the ischemic areas due to the existence of lower number of EPCs in the newly formed vessels (186-188), the role of paracrine angiogenic function in postnatal angiogenesis is well corroborated (189). Diabetic individuals suffer poor ischemic vascular repair which is further resistant to cell-based therapies, both because of inefficiency in above-mentioned criteria. The later three criteria were shown to be improved by Ang-(1-7) in experimental diabetic conditions in various studies from our lab and others, where improved engraftment of stem/progenitor cells (126; 141), their homing into ischemic regions (146), and paracrine angiogenic functions (122) were described. This study tested whether Ang-(1-7) treatment reverses mobilopathy in diabetic conditions which can recruit crucial stem/progenitor cells that can initiate, participate and accomplish the vascular repair.

This study reports several novel findings. Ang-(1-7) treatment reversed diabetic mobilopathy in physiological conditions, in response to clinical mobilizers, or ischemic insult. In addition, this study provides evidence for a novel pathway involving MasR/Slit3/ROCK in the mobilization of progenitor cells by Ang-(1-7). Ang-(1-7)-enhanced, ischemia-induced mobilized BMPCs were effective in promoting neovascularization and vascular repair in diabetic

conditions. Importantly, endogenous expression of MasR appears to be indispensable for the mobilization of bone marrow progenitor cells and that genetic ablation of MasR recapitulates diabetic bone marrow mobilopathy and impaired vascular regeneration. Conceptually, this study provides evidence for a novel role of ACE2/Ang-(1-7)/Mas receptor axis of RAS in bone marrow microenvironment and stem/progenitor cell operation. Thus, enhancing Ang-(1-7)/MasR signaling in diabetic conditions restores proper functioning of stem/progenitor cells participating in vascular repair.

In conclusion, collectively, these results demonstrated for the first time that Ang-(1-7) orchestrates progenitor cell mobilization by stimulating Slit3 from BM-MSCs, which in turn causes reversal of stem cell mobilopathy and enhance ischemic vascular repair in diabetic conditions (Fig.53). Therefore, Ang-(1-7) could be a promising pharmacological agent to improve outcomes of cell-based therapies for the treatment of diabetic vascular disease.

5.2. Clinical Relevance

Ang-(1-7) has an orphan drug status currently for the patients requiring accelerated stem cell mobilization during transplantation and pulmonary arterial hypertension (U.S. FDA). Ang-(1-7) and its derivatives passed phase 1 clinical trials in cardiovascular disease and other conditions (ClinicalTrials.gov), emphasizing their safety in cardiovascular disease conditions. Thus, our studies in diabetic stem cell mobilopathy can be quickly translated to clinical studies provided the similar beneficial effects are observed in human population. The serious limitation of Ang-(1-7) is its half-life, which can be superseded by employing more stable analogues such as cyclic Ang-(1-7) and $NorLeu^3$-Ang-(1-7), which are currently being developed and/or by using specific formulation methods. $NorLeu^3$-Ang-(1-7) was shown to be safe and effective in phase 1 and phase 2 clinical trials to treat diabetic foot ulcers (190)

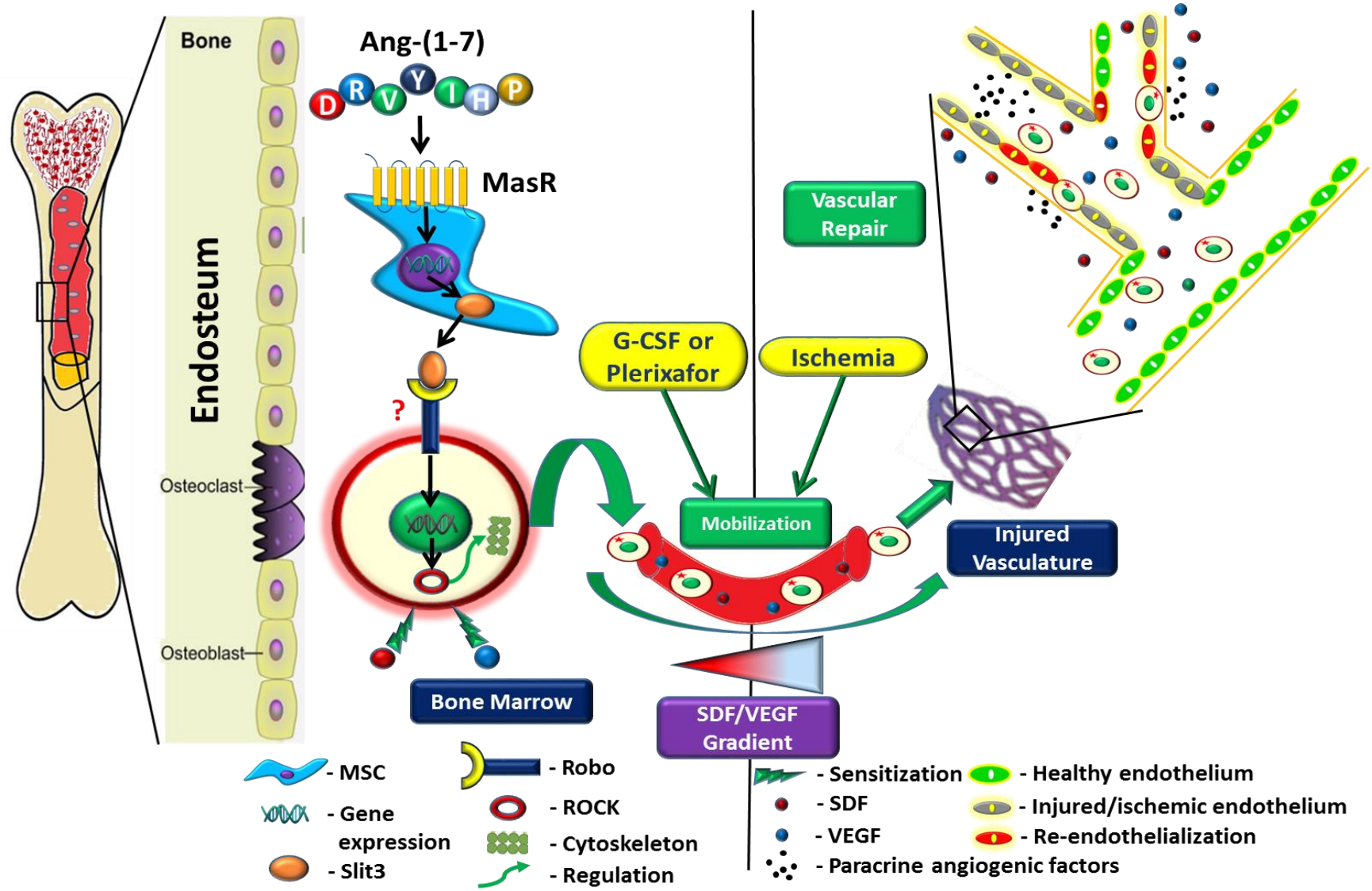

Figure 52 – Summary of results. Further investigation is needed to determine the type of Robo receptor involved (**?**). Figure adapted from (59).

Effect of Ang-(1-7) on mobilization in experimental diabetes is independent of hyperglycemia, therefore, this effect if proven further will have tremendous clinical applications in diabetic conditions as it can be used irrespective of the glycemic condition of the patient. Further, studies confirmed the beneficial effects of Ang-(1-7) in reversing mobilopathy and impaired functions of BMPCs in a mouse model of type 2 diabetes along with type 1 diabetes. Therefore, outcomes of this study would have strong clinical relevance as the prevalence of type 2 is considerably higher than type 1 diabetes. Lastly, the usage of long-term diabetic mice of duration greater than 20 weeks makes this study clinically more relevant as the vascular complications occur following long-term diabetes, at which stage the reparative bone marrow cells become dysfunctional.

5.3. Limitations

This study resulted in several novel findings however not free from limitations, both conceptual and methodological.

I have observed type 1 and type 2 models of diabetes showed significant differences in the circulating levels of cytokines despite similar dysfunctions in bone marrow mobilization in response to ischemia. Plasma SDF levels in db/db mice are almost similar to that observed in lean mice which was in contrast to STZ-model where significant decrease was observed compared to the respective control. SDF generation in response to ischemia was even more intriguing – circulating SDF was not increased in the lean mice in contrast to the C57Bl/6NHsd mice that showed 2.5-fold increases, while diabetes decreased ischemic SDF generation regardless of the model. These findings point to an unknown role of leptin receptor in the regulation of vascular angiogenic signaling. It is worth noting that both lean and db/db mice are deficient of leptin receptors, 50 and 100%, respectively, therefore including a strain control

would help understand these differences. Importantly, high-fat diet induced model of type 2 diabetes with no loss of leptin receptors appear to be a right model for these studies, which stands out as a major limitation.

Diabetes is known to alter the mobilization of inflammatory cells (191), which are known to modify the angiogenic outcomes following ischemia in health and disease (192). This concept was not studied in the current study, however requires additional experimentation, which would lead to novel hypotheses.

Conceptually, I have observed that Slit proteins enhance migratory propensity of progenitor cells by sensitizing the cells to other chemotactic factors. Increased ROCK activity was apparent in response to Slits however which itself did not induce migration. While I believe an unknown molecular mediator is involved in connecting ROCK pathway to migration, I find it in contrast to the previous reports and there was no consensus. For instance, Geutskens et al. (193) have shown that in human stem/progenitor cells Slit3 treatment decreases RhoA, a small GTPase protein which activates ROCK, and inhibits migration. However, the same group showed that Slit3 enhances RhoA and promotes migration in human $CD14^{+}$ monocytes (194). Although species, strain and/or cell type variation might involve in these differential findings, in-depth molecular signaling studies are needed to define the Slit/Robo pathway in the current context. No reliable tools are available to study this pathway, which remained a major limitation of the study.

5.4. Future Directions

Though Ang-(1-7) is found to mediate its actions by activating MSCs to release Slit3 protein, the involvement of Slit2 protein is yet to be answered. Reviewing the literature, I presumed that Robo4 is the only receptor that is expressed at translational level on BMPCs

(195), therefore Slit3 most probably acts via Robo4 signaling. Confirmation studies need to be performed using specific Robo function-blocking antibodies on mouse stem/progenitor cells to accurately determine the type of Robo receptor that is involved in Ang-(1-7)-induced ROCK activity.

Apart from these there are a couple of unexplainable findings which might need further research. The effect of Ang-(1-7) on plasma SDF levels is varied between type 1 and type 2 diabetic mice, which might be a result of ObRb receptor knockout, but further investigation is needed to confirm it. Finally, despite increase in in vitro Slit3 release from MSCs in control mice, in vivo ROCK activity increase was not observed following Ang-(1-7) treatment. This could be because SDF can itself activate ROCK during migration of various cells including HPCs (196-198) and that increase in ROCK activity in Ang-(1-7) treated diabetic BMPCs is an observable effect as SDF levels are gone down in diabetic mice. Yet this is found to be not true in case of type 2 diabetic mice where SDF levels were unchanged compared to the lean control mice, therefore, it needs further investigation.

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