8-22-2013 12:00 AM

Vascular Stem Cells in Diabetic Complications

Emily C. Keats
The University of Western Ontario

Supervisor
Dr. Zia A. Khan
The University of Western Ontario

Graduate Program in Pathology

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

© Emily C. Keats 2013

VASCULAR STEM CELLS IN DIABETIC COMPLICATIONS

(Thesis format: Integrated-Article)

by

Emily C. Keats

Graduate Program

in

Pathology

A thesis submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

The School of Graduate & Postdoctoral Studies

The University of Western Ontario

London, Ontario, Canada

ABSTRACT

Diabetes leads to a variety of secondary complications. At the heart of these complications lies endothelial cells (ECs) – cells that take up unregulated plasma glucose, experience various biochemical alterations, and provide the basis for whole organ vascular dysfunctions. With the purpose of generating new vascular networks for the treatment of these chronic complications, my initial work focused on vascular stem cells (VSCs). VSCs have the ability to differentiate into both endothelial (EPC) and mesenchymal (MPC) progenitor cells, both of which are necessary for the creation of stable and functional blood vessels. To establish whether these progenitor populations retain their integrity in diabetes, we investigated their cellular activity in a high glucose (HG) setting. Contrary to our expectations, EPCs evaded the negative effects of HG while MPCs displayed some functional alterations. Importantly, we noted MPCs in HG to be skewed towards the adipocyte lineage, while differentiation to both osteoblasts and chondrocytes was suppressed.

MPC alterations exposed in our study are reminiscent of phenotypic changes that occur in the bone marrow of long-term diabetic patients. To elucidate the mechanism behind this alteration in MPC differentiation we examined the Wnt signaling pathway in a comprehensive manner. The results of this study have revealed a novel finding. We have demonstrated the autogenous upregulation of non-canonical Wnt11 in HG-treated MPCs, that signals through the Wnt/Ca^{2+}/protein kinase C (PKC) pathway to stimulate adipogenesis. Increase in adipocytes in human diabetic marrow samples correlated with a decrease in the number of stem cells. We have also shown that enhanced

adipogenesis in marrow samples may disrupt the stem cell niche by altering

Angiopoeitin/Tie signaling axis. Taken together, targeting the conversion of stem cells to

adipocytes could be an effective means to combat many chronic diabetic complications.

Preventing adipogenesis may restore stem cell numbers in diabetic patients enabling

endogenous repair.

Key words: diabetes, stem cell, vasculogenesis, hyperglycemia, progenitor cells,

differentiation, adipocyte, Wnt signaling, stem cell niche

CO-AUTHORSHIPS

Manuscript: Vascular stem cells in diabetic complications: evidence for a role in the pathogenesis and the therapeutic promise.

Cardiovasc Diabetol. 2012; 11:37.

Emily C. Keats	Drafted the manuscript.
Zia A. Khan	Supervisor; edited and finalized the manuscript.

Manuscript: Unique responses of stem cell-derived vascular endothelial and mesenchymal cells to high levels of glucose.

PLoS One. 2012; 7(6):e38752.

Emily C. Keats	Designing and conducting all experiments.
Zia A. Khan	Supervisor; assisted in all aspects of the experiments; all experiments conducted under his grant approval.

Manuscript: Glucose activates non-canonical Wnt signaling leading to enhanced adipogenesis in mesenchymal progenitor cells

Emily C. Keats   Designing and conducting all experiments.

James M. Dominguez, 2nd   Maintenance of diabetic animals.

Maria B. Grant   Collaborator; animal experiments conducted under

                                   her grant approval.

Zia A. Khan   Supervisor; assisted in all aspects of the experiments;

                                   all experiments conducted under his grant approval.

DEDICATION

I would like to dedicate this thesis to my supervisor, Dr. Zia Khan – because I am certain I have learned more in the past four years than others will in a lifetime. It has been a privilege working with such a dedicated researcher, inspiring teacher, and wonderful friend. Thank you for seeing the potential in me, and for always practicing good science.

ACKNOWLEDGEMENTS

I would like to acknowledge my advisory committee, Dr. Subrata Chakrabarti and Dr. Jack Bend, who offered support and guidance throughout my graduate studies. Your genuine excitement pertaining to my work and your confidence in my abilities made the difficult tasks much more bearable.

To my family – thank you for your unwavering love, and for always providing just the right amount of humour. I am fortunate to have three generations of strong women in my life, and I could not possibly have accomplished any of this without each of you. To the newest member – Blue, I am looking forward to the day we discuss the contents of this thesis, and to spending the next chapter of my life with you in it.

TABLE OF CONTENTS   **PAGE**

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α-SMA	Alpha-smooth muscle actin
abEPC	Adult blood-derived endothelial progenitor cell
ADP	Adenosine diphosphate
AGE	Advanced glycation end-product
AM	Adrenomedullin
Ang1/2	Angiopoietin-1/2
AP-1	Activating protein-1
APC	Adenomatous polyposis coli protein
ATP	Adenosine-5'-triphosphate
BADGE	Bisphenol-A-diglycidyl
bFGF	Basic fibroblast growth factor
BM	Basement membrane
bmMPC	Bone marrow-derived mesenchymal progenitor cell
BMP	Bone morphogenic protein
Ca^{2+}	Calcium
CaMKII	Calcium/calmodulin-dependent kinase II
CAT	Catalase
cbEPC	Cord blood-derived endothelial progenitor cell
CBP	Cyclic-adenosine monophosphate response element binding protein (CREB)-binding protein
CCND1-4	CyclinD1-D4

c/EBP	CCAAT/enhancer binding protein
Che	Cheletherine chloride
CREB	cAMP-responsive element-binding protein
CTBP	COOH-terminal binding protein
CTNNBIP1	β-catenin interacting protein-1
CXCL12	CXC-chemokine ligand 12
CXCR4	Receptor for CXCL12
DAG	1,2-diacylglycerol
DCCT	Diabetes Control and Complications Trial
Dkk1	Dickkopf-1
DMEM	Dulbecco's Modified Eagle's Medium
DN	Dominant-negative
Dsh	Disheveled
EB	Embryoid body
EBM-2	Endothelial basal media-2
EC	Endothelial cell
ECM	Extracellular matrix
EDV	Endothelial-dependent vasodilation
EGF	Epidermal growth factor
EPC	Endothelial progenitor cell
ER	Endoplasmic reticulum
E-selectin	Endothelial selectin
ET-1	Endothelin-1

FABP4	Fatty acid-binding protein 4
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FN	Fibronectin
Fzd	Frizzled receptor
Glut	Glucose transporter
GPx	Glutathione peroxidase 1
GSK3β	Glycogen synthase kinase 3β
HDMEC	Human dermal microvascular endothelial cell
HG	High glucose
HGF	Hepatocyte growth factor
HO-1	Heme oxygenase-1
HSC	Hematopoietic stem cell
HUVEC	Human umbilical vein endothelial cell
ICAM1	Intercellular adhesion molecule-1
IGF	Insulin-like growth factor
IP3	Inositol 1,4,5-triphosphate
iNOS	Inducible-nitric oxide synthase
IWR-1	IWR-1-endo
JAK2	Janus kinase-2
JNK	c-Jun N-terminal kinase
KDR	Kinase insert domain receptor (also known as VEGFR2)
LDL	Low density lipoprotein

LEF	Lymphoid-enhancer-binding factor
LOX-1	Receptor for oxidized-low density lipoprotein
LRP	Lipoprotein receptor-related protein
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
Mel-CAM	Melanoma cell adhesion molecule (CD146)
MHC	Myosin heavy chain
MMP	Matrix metalloproteinase
MNC	Mononuclear cell
MPC	Mesenchymal progenitor cell
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Reduced NAD phosphate
NFAT	Nuclear factor of activated T cells
NG2	Neuron/glial type 2 antigen
Nkx3.2	NK3 homeobox 2
NO	Nitric oxide
NOS	Nitric oxide synthase
NOS2	Inducible NOS (iNOS)
ox-LDL	Oxidized-low density lipoprotein
p22 Phox	NADPH oxidase
PARP	Poly (ADP-ribose) polymerase
PCP	Planar cell polarity

PDGF	Platelet-derived growth factor
PDGFR-β	Platelet-derived growth factor receptor β
PECAM-1	Platelet endothelial cell adhesion molecule-1 (CD31)
PKB	Protein kinase B
PKC	Protein kinase C
PKCε	Epsilon isoform of PKC
PLC	Phospholipase-C
PNU	PNU74654
PPARγ	Peroxisome proliferator-activated receptor γ
PPARγ2	Peroxisome proliferator-activated receptor γ2
p-PKC	Phospho-PKC
PSF	Penicillin-streptomycin-fungizone
Rac1	Ras-related C3 botulinum toxin substrate 1
RAGE	Receptor for AGE
RhoA	Ras homolog gene family, member A
ROCK	RHO-associated kinase
ROR	Receptor tyrosine kinase-like orphan receptor
ROS	Reactive oxygen species
Runx2	Runt-related transcription factor 2
SDF-1	Stromal cell-derived factor-1
SETDB1	Histone methyltransferase SET domain bifurcated 1
sFRP	Secreted frizzled-related protein
siRNA	Small interfering-RNA

SOD-1	Superoxide dismutase 1
Sox9	Sex determining region Y-box 9
SP7	Osterix
TCF	T-cell-specific transcription factor
TGF-β	Transforming growth factor-β
Tie	Receptor for ANG
TLE-1/2	Transducin-like enhancer protein 1/2
TNFα	Tumor necrosis factor-α
uaSMC	Umbilical artery smooth muscle cell
UKPDS	United Kingdom Perspective Diabetes Study
VE-cadherin	Vascular endothelial cadherin (CD144)
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor-2
VSC	Vascular stem cell
vWF	Von Willebrand factor
WISP1	Wnt1-inducible-signaling pathway protein 1
Wnt	Wingless-type MMTV integration site family member
WST-1	Tetrazolium salt reagent
WT	Wild type

CHAPTER 1 – INTRODUCTION[a]

1.1   Chronic diabetic complications

Diabetes is a chronic and debilitating metabolic disease that presently has no cure. Currently, the total number of people with diabetes is upwards of 221 million and in North America alone, more than 10% of the population is affected[1]. This amounts to a staggering economic burden, estimated to be above $6 billion yearly in Canada[2] and close to $245 billion in the United States[3]. Although the incidence in North America is quite alarming, close to 80% of diabetes-related deaths occur in low- and middle-income countries due to poor management of complications and lower standards of healthcare[4]. Despite great efforts to combat this disease, the World Health Organization projects that diabetes-related deaths will more than double by the year 2030[4].

The most important discovery in the diabetes field was that of insulin in 1921. Exogenous insulin significantly alleviated diabetic coma and ketoacidosis, saving millions of lives. However, diabetic patients today still experience morbidity and mortality due to the chronic secondary complications that arise over time. These long-term complications manifest as micro- (retinopathy, neuropathy, nephropathy, and cardiomyopathy) and macro- (atherosclerosis) vascular dysfunctions[5]. Although the clinical features of the complications are quite varied, the underlying cause is an

---

[a] Parts of this chapter have been published: **Keats EC**, Khan ZA. Vascular stem cells in diabetic complications: evidence for a role in the pathogenesis and the therapeutic promise. Cardiovasc Diabetol. 2012; 11:37. doi: 10.1186/1475-2840-11-37. The BioMed Central applies the *Creative Commons Attribution License (CC-BY Attribution 2.0)* to works. Under this license, authors retain ownership of the copyright for their content. No permission is required from the publishers.

aberration in the vasculature of the target organ involved. Two major clinical trials paved the way to better understanding the cause of the diabetic complications: the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Perspective Diabetes Study (UKPDS), completed in 1993 and 1997 respectively. In both trials, type 1 and type 2 diabetic patients were put under intensive glycemic control, and in both cases, there was delayed progression and/or inhibition of the onset of diabetic complications[6,7]. Other factors, such as hyperlipidemia and hyperinsulinemia, undoubtedly contribute to the pathogenesis of diabetic complications. However, clinical trial results and years of *in vitro* and *in vivo* research confirm the notion that hyperglycemia is the primary cause of micro- and macro-angiopathy seen in long-term diabetes.

1.2   Molecular basis of the vascular dysfunction in diabetic complications

Endothelial cells (ECs) are a critical component of the vascular unit. These specialized cells form the inner lining of blood vessels, sit on a basement membrane, and are surrounded by supportive perivascular cells (pericytes or smooth muscle cells) (figure 1.1). ECs not only function as a barrier – producing an interface between circulating blood and the perfused tissue – but also play a prominent role in tissue functioning as well as organogenesis. These cells are involved in important vascular processes such as regulating blood flow and pressure, permeability, blood fluidity, the thrombotic/fibrinolytic balance, and leukocyte traffic[8,9].

Due to their anatomical location in the blood vessel, ECs are not surprisingly the first to encounter circulating plasma glucose. Glucose transporters (Gluts) facilitate the uptake of glucose[10-12]. The predominant Glut in the vascular ECs is Glut1[13,14]. Although Gluts are typically expressed in a tissue-specific manner, Glut1 is ubiquitously expressed under normal growth conditions[15]. Unlike other glucose transporters, Glut1 activity and expression level does not change with altered plasma glucose levels. This indicates that hyperglycemia may have profound detrimental effects on vascular ECs specifically, as glucose uptake is not actively regulated[13,14]. There are certain conditions, however, that may alter Glut1 expression. For example, hypoxia increases Glut1 levels in ECs[16]. This additive effect of hyperglycemia and hypoxia may be one mechanism behind the uncontrollable dysfunction of vascular ECs in diabetic complications.
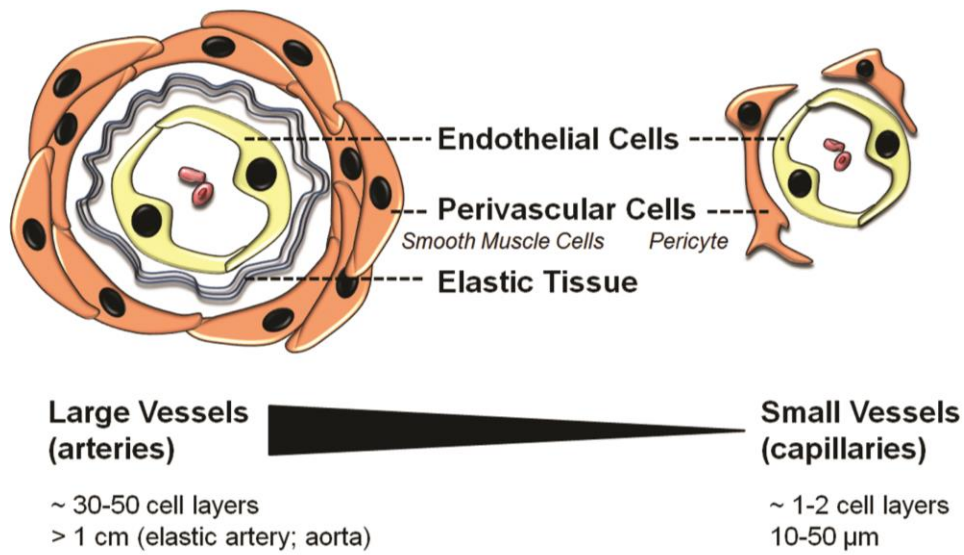
Figure 1.1: Schematic of large and small blood vessels. Large vessels contain a prominent elastic tissue and multiple layers of the contractile cells (smooth muscle cells). Small capillaries may or may not have contractile cell (pericyte) coverage. Endothelial cells in capillaries sit on a thin basement membrane.

In vitro studies have shown that exposure to high levels of glucose lead to biochemical alterations in mature vascular ECs[17]. These alterations manifest as increased production of extracellular matrix proteins, such as collagen and fibronectin, increased production of the procoagulant protein von Willebrand Factor (vWF), and altered cellular activities[18-20]. In addition to a reduction in proliferation and migration[21], a number of studies have provided evidence that hyperglycemia can directly promote EC apoptosis[22-24]. This apoptotic pathway is believed to be activated by increased oxidative stress, increased intracellular Ca^{2+}, mitochondrial dysfunction, changes in intracellular fatty acid metabolism, activation of mitogen activated protein kinase (MAPK) signaling pathways, and impaired phosphorylation/activation of protein kinase B (also known as Akt)[25,26].

One of the earliest functional changes, which precedes any structural change in the vasculature of the target organs, is the impairment of endothelial-dependent vasodilation[19]. This impairment arises because of two inter-regulated mechanisms: decreased production of vasodilators and increased production of vasoconstrictors. Diminished levels of nitric oxide (NO) and increases in endothelin-1 (ET-1), the most potent endogenous vasoconstrictor, have been demonstrated in vascular ECs cultured in high glucose[19]. We, and others, have shown that the enzymes involved in NO production are up-regulated in ECs upon glucose challenge[27]. However, uncoupling of the enzymatic reaction and possible sequestration of NO by oxidative stress leads to significantly reduced NO[19,28]. Another well-established pathway leading to increased EC damage in diabetes is the oxidative stress pathway. Hyperglycemia produces reactive oxygen species (ROS) in ECs, such as hydroxyl radicals, superoxide anions,

and hydrogen peroxide[18] . The overproduction of ROS may also be attributed to the activation of alternate metabolic/signaling pathways such as the polyol pathway and hexoseamine pathway, and signaling through protein kinase C (PKC), advanced glycation end-product (AGE) formation, and Poly (ADP-ribose) polymerase (PARP) activation[17,28,29] (figure 1.2). Each of these pathways may potentiate each other, culminating in increased ET-1 activity, reduced NO bioavailability, oxidative stress, and EC dysfunction. Remarkably, the biochemical changes that we see in high glucose-treated ECs are reminiscent of the chronic complications that present in diabetic patients.

We now know that altered ECs provide a backbone for the long-term vascular dysfunctions that arise in diabetic patients (figure 1.3). Changes in the structure and function of ECs leads to subsequent aberration of entire vascular networks, and tissues will begin to shows signs of poor blood flow and ischemia[30]. Normally, an adaptive response would be expected under these conditions. While there is a vascular response in diabetic patients, it is not uniform and varies depending on the organ system involved. The retina and kidneys typically exhibit enhanced blood vessel formation, while this process is impaired in the heart and lower limbs[30,31]. The selectivity in the target organ system in diabetes suggests the importance of both the tissue microenvironment and the intrinsic properties of the ECs[19].

Glucose

- ❑ Glucose auto-oxidation
- ❑ Mitochondrial O_2^-
- ❑ Polyol Pathway ($\downarrow$NADPH, $\uparrow$NADH:NAD$^+$)
- ❑ Hexoseamine Pathway (protein glycosylation)
- ❑ NADPH oxidase induction
- ❑ PARP activation ($\downarrow$NAD$^+$, $\downarrow$ATP)
- ❑ AGE/RAGE interaction
- ❑ Ox-LDL/CD36/LOX-1 interaction
- ❑ Heme oxygenase induction
- ❑ Nitric oxide (iNOS)

$\uparrow$ **ROS Production**
$\downarrow$ **ROS Clearance**
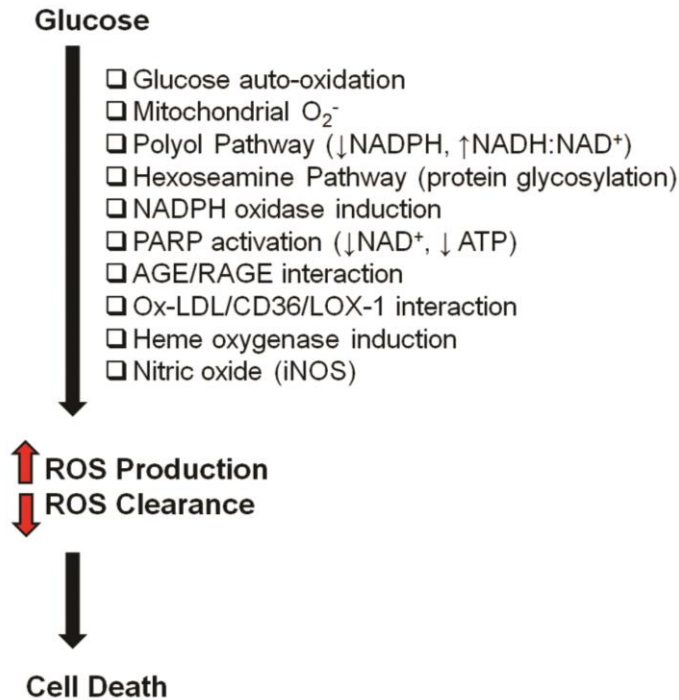
Cell Death

Figure 1.2: Mechanisms of glucose-induced oxidative stress in ECs.
Hyperglycemia leads to cell death by the overproduction of ROS and impairment in the ROS neutralizing enzymes. Multiple pathways may lead to ROS production. A consequence of activating these oxidant pathways is the depletion of co-factors required by the anti-oxidant enzyme systems. The net effect is an imbalance in ROS production and ROS clearance [AGE=advanced glycation end product; ATP=adenosine-5'-triphosphate; iNOS=inducible nitric oxide synthase; LOX-1=receptor for oxidized-low density lipoprotein; NAD=nicotinamide adenine dinucleotide; NADPH=reduced NAD phosphate; ox-LDL=oxidized-low density lipoprotein; PARP=poly (ADP-ribose) polymerase; RAGE=receptor for AGE].

Glucose
- ❑ Unregulated glucose uptake (ECs)

Biochemical/Molecular Changes
- ❑ ET-1 ↑
- ❑ Aberrant activation PKC, PKB, MAPK
- ❑ Redox changes (↑ ROS production, ↓ clearance)
- ❑ Protein modifications (e.g. AGE formation)
- ❑ ↑ ECM protein synthesis

Functional Changes
- ❑ EDV Impairment
- ❑ Impaired EC-pericyte interactions

Structural Changes
- ❑ ECM expansion
- ❑ Loss of vascular cells (ECs and pericytes)

Outcome: Vascular Dysfunction
- ❑ Acellular capillaries (BM tubes)
- ❑ Ischemia

Reaction/Response
- ❑ Neovascularization (retina)
- ❑ Scar/fibrosis (heart)

Figure 1.3: Mechanisms leading to vascular disruption in diabetes. High glucose causes various biochemical and molecular changes in the vascular ECs, resulting in functional and structural alterations of the target organ vascular bed. Impaired vasoregulation and loss of vessel integrity leads to reduced blood flow and ischemia. In response, the target organ exhibits neovascularization (diabetic retinopathy/nephropathy) or fibrosis (diabetic cardiomyopathy/neuropathy) [AGE=advanced glycation end product; BM=basement membrane; EC=endothelial cell; ECM=extracellular matrix; EDV=endothelial-dependent vasodilation; ET-1=endothelin-1; MAPK=mitogen-activated protein kinase; PKB=protein kinase B; PKC=protein kinase C].

Growth factors and extracellular matrix (ECM) proteins are two major regulators of the balance that exists between neovascularization and scar formation/fibrosis in diabetic complications. Vascular endothelial growth factor (VEGF) is an EC-specific mitogen that promotes angiogenesis in a number of disease models. In parallel with a lack of angiogenesis that occurs in the heart in diabetes, a reduced expression of VEGF and its receptors is reported in the myocardium[32]. This is in direct contrast to elevated VEGF levels in the retina[33], correlating with uncontrolled retinal neovascularization. In addition to growth factors, the ECM regulates the vascular cells and may contribute to the differential effects of hyperglycemia in diabetic complications. In a normal setting, binding of EC surface integrins to the ECM proteins regulates cell survival/apoptosis, growth, and cytoskeletal changes[34]. Therefore, angiogenesis is highly dependent on the interactions between the cellular components of the vascular unit and the surrounding scaffolding proteins. In fact, ECM changes that are believed to promote neovascularization during tumorigenesis are mimicked in retinal vascular development, and include increases in fibronectin and laminin[35]. Retinal basement membranes of diabetic animals show a similar protein profile- with elevated collagen IV, laminin and fibronectin as early as 8 weeks following the onset of diabetes[36]. Along with ECM protein heterogeneity, increased ECM deposition in the heart may contribute to the impaired angiogenic response. Cardiac fibroblasts, which are present in significant numbers, may be responsible for this unregulated deposition of ECM proteins through the action ET-1, which has been shown *in vitro* to increase production of ECM components by fibroblasts[37,38].

Regardless of the organ system involved, vascular ECs are the primary mediators of hyperglycemic damage and they undergo significant structural and functional alterations. Subsequently, impaired vasoregulation, increased permeability, ECM expansion, and dysfunction of entire vascular networks causes reduced blood flow to the target organ, setting the stage for uncontrolled advancement of complications. To stop the progression and repair the hyperglycemic damage, altered ECs would need to be replaced or, alternatively, brand new vascular networks created. Considering mounting evidence of stem/progenitor cells in various tissues including the blood vessel wall[39], one can speculate that these stem/progenitor cells are also affected and lead to an impaired repair mechanism in diabetes.

1.2 Vascular stem cells (VSCs): current evidence and promise

Stem cells are defined by their ability to both self-renew and differentiate into functionally mature cells[40]. The potential of the cells is determined by the hierarchy and specialization level, with embryonic stem cells being the most versatile. Stem cells have been identified in a variety of post-embryonic tissues, including bone marrow, blood, fat, and skin[41,42]. Discovering these stem cell populations has presented the opportunity for non-invasive tissue repair and tissue regeneration, including the vascular tissue. However, to generate new vascular networks (de novo formation), a suitable cell source must first be identified. Ideally, it would be one cell type or subpopulation that could produce both the endothelial and supportive perivascular cells that make up the vascular unit. The notion of a specific vascular stem cell (VSC) able to produce mature/functional cells of the blood vessel wall is slowing gaining momentum. Several groups have demonstrated the existence of a common vascular precursor cell in both mouse and human studies. Kattman et al. used cell tracing studies in mice and showed that cardiomyocytes arise from a cell population expressing the VEGF receptor-2 (VEGFR2/Flk1)[43], indicating that they develop from a progenitor that also has vascular potential. They followed up these studies with an embryonic stem cell differentiation model, in which they isolated cardiovascular progenitors (brachyury+; VEGFR2+) from human embryoid bodies (EBs) and successfully demonstrated their potential for generating cardiomyocytes, endothelial cells, and vascular smooth muscle cells[43]. Yamashita and colleagues showed that VEGFR2+ cells, derived from embryonic stem cells, could differentiate into both endothelial and mural cells through differing culture

conditions[44]. These cells were also able to reproduce the vascular organization process when placed in three-dimensional culture systems[44]. Similarly, Ferreira et al. demonstrated that vascular progenitors (CD34+) isolated from EBs will give rise to endothelial and smooth muscle-like cells, and have the ability to form vascular networks when implanted *in vivo*[45].

Although the exact identity of the VSC is not yet unanimous, there is evidence that this cell type is found in the bone marrow and circulation and is functionally distinct from hematopoietic stem cells (HSCs). Selection of CD133+ cells from the circulation purifies a population(s) of cells that under different culture conditions will produce lineage-restricted endothelial progenitor cells (EPCs) and mesenchymal/mesodermal progenitor cells (MPCs)[40,46-49]. However, cells expressing pan hematopoietic marker CD45 fail to yield endothelial cells[50-52]. It is unknown thus far whether one or more stem cell subtypes reside within this CD133+ population that are limited in their capacity to produce endothelial and mesenchymal cell types. Importantly, CD133+ stem cell-derived EPCs and MPCs form functional vascular networks[44,45,51]. Whether this is a feasible avenue for diabetic patients is just recently being probed.

1.3 Endothelial progenitor cells (EPCs) and diabetic complications

Progenitor, or precursor, cells are committed (lineage-restricted) and highly proliferative derivatives of stem cells. These cells may be capable of doubling their population every 10-15 hours[53]. Unlike fully mature cells, progenitors express markers of full maturity in addition to select stem cell markers[53]. For example, EPCs share markers of both stem and mature endothelial lineages[54]. With the potential use of EPCs, either clinically or as a biomarker, accurate identification and reproducible classification is of great importance. Despite advances in research on this subject, a lack of consensus remains on how EPCs should be defined. Traditionally, EPCs have been identified as the spindle-shaped or polymorphic cells that appear within 2-4 days in culture after isolation of the mononuclear cell (MNC) fraction from blood or bone marrow[55](figure 1.4). These cells are characterized by Ulex europaeus agglutinin binding and DiI-labeled acetylated-low density lipoprotein (LDL) uptake[56-58], two properties that are typically considered functional characteristics of ECs. However, along with expressing some EC markers, these 'short-term' EPC colonies also express monocyte-specific marker CD14 and/or hematopoietic cell marker CD45. Further, acetylated-LDL uptake was identified in 1979 as a known feature of monocytes[59]. Ulex europaeus agglutinin is a lectin which binds to the EC surface via fucose resides. These fucose residues are not specific to the ECs[60]. Taken together, these early outgrowth colonies encompass a variety of cell types, making EPC identification challenging.
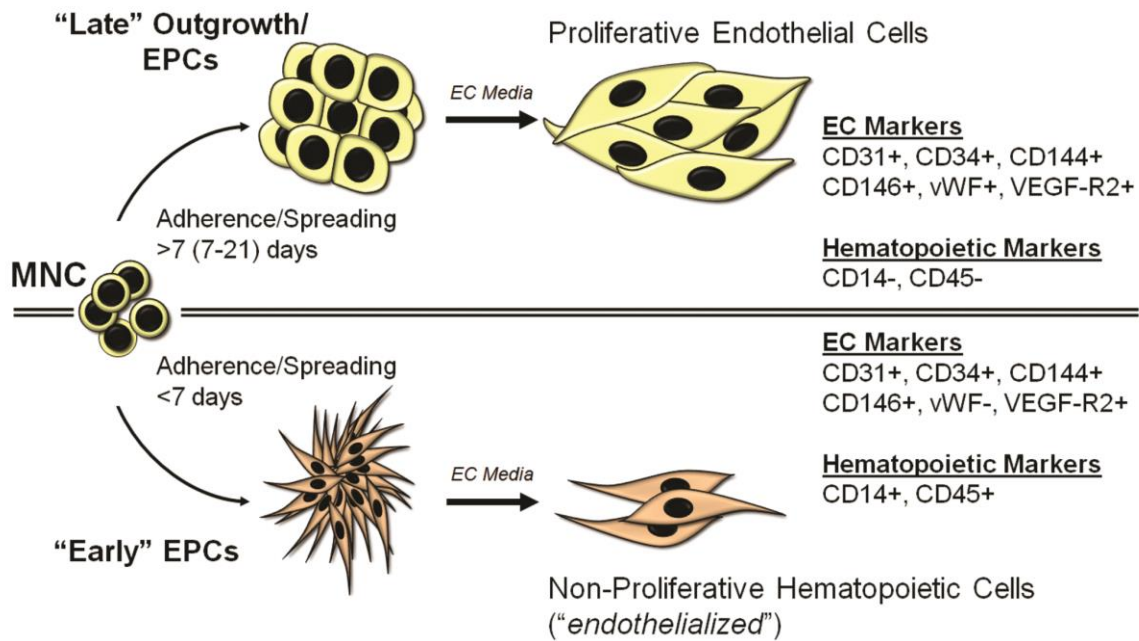
Figure 1.4: Early and late EPCs. Schematic illustrating the two major types of cell colonies arising from blood and bone marrow mononuclear cells. Short term colonies appear within 7days of culture and are comprised of spindle shape or polymorphic cells. These early EPCs (also called angiogenic EPCs) express a number of endothelial and hematopoietic markers but fail to proliferate in culture. Late colonies, appearing from 7 – 21 days, are comprised of epitheliod cells with high proliferative capacity. These late EPCs (also called vasculogenic EPCs) express all markers of mature endothelial cells but lack hematopoietic marker expression.

How then do we define EPCs? The single most important property (or functional attribute) of late outgrowth EPCs is their ability to incorporate into blood vessels. In other words, they participate in *vasculogenesis*[61,62]. In comparison, early EPCs are considered angiogenic because they facilitate angiogenesis through the elaboration of growth factors. We, and others, have extensively characterized marker expression and cellular activities of vasculogenic EPCs. EPCs display properties of both mature ECs and unipotent progenitor cells. However, EPCs differ from mature ECs in CD133 expression (positive on EPCs but readily lost upon culture)[47,63], proliferation/growth kinetics (EPCs show lower population doubling time and higher cumulative population doublings)[50,64], and response to endostatin (EPCs are stimulated whereas mature ECs are inhibited)[47]. Over time, EPCs resemble mature ECs in terms of marker expression and all cellular activities[47,50,51]. Much of the controversy behind angiogenic and vasculogenic EPCs could be negated by performing functional cellular activity tests (summarized in figure 1.5). These include assessing the expression of endothelial-specific markers[47,50,51], activation by cytokine challenge[47], and most importantly, the ability of the cells to create blood vessels[50,51].

EPCs may be involved in vascular dysfunction in chronic diabetic complications. It has been demonstrated that type 1 and type 2 diabetics maintain a lower circulating number of EPCs when compared with healthy subjects[54,65-67]. In two similar studies, flow cytometric analysis was used to quantify EPCs (CD34+/VEGFR2+/CD31+) in diabetic patients. These studies showed that EPCs were reduced by 44% and 40%, respectively[65,68]. More recently, the number of circulating CD34+/VEGFR2+ cells was shown to correlate with glycemic control in type 2 diabetic patients[69]. This study also

15
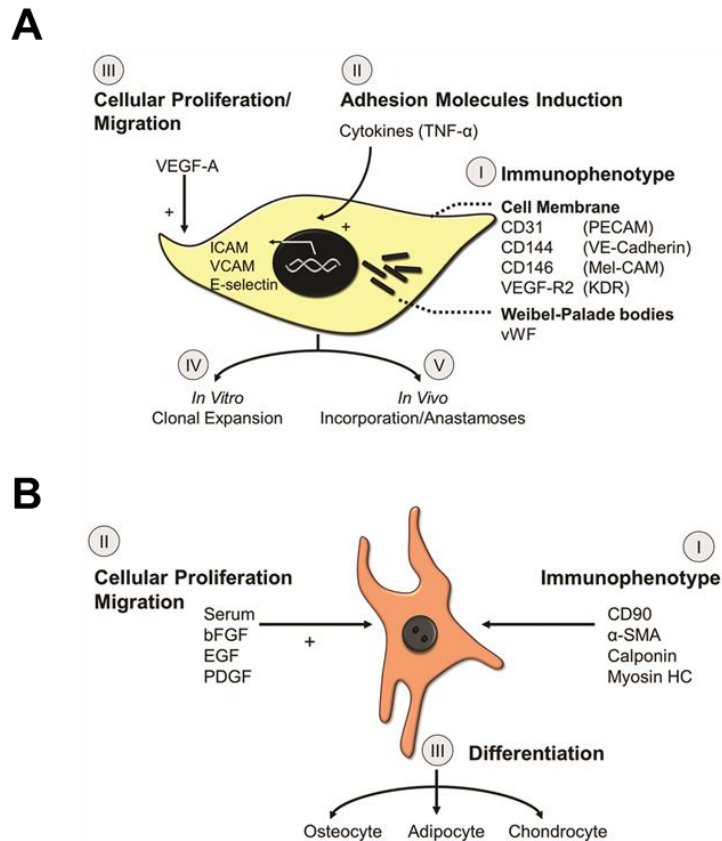
Figure 1.5: Characterization scheme for EPCs and MPCs. (A) EPCs express CD31 and VE-cadherin, localized to the cell membrane, and vWF in the Wieble Palade bodies. VEGF is a mitogen for ECs and EPCs. Also, EPCs induce adhesion molecules when challenged with cytokines, similar to mature ECs. The progenitor properties include clonal growth potential and *in vivo* vasculogenesis. (B) MPCs express CD90, α-SMA, and calponin. Upon treatment with PDGF and EGF, MPCs proliferate and also exhibit chemotaxis. The progenitor phenotype involves the ability of the cells to give rise to adipocytes, osteocytes, and chondrocytes [VEGF-A = vascular endothelial growth factor-A; TNF-α = tumor necrosis factor-α; PECAM = platelet endothelial cell adhesion molecule; VE-cadherin = vascular endothelial-cadherin; Mel-CAM = melanoma cell adhesion molecule; KDR = kinase insert domain receptor (VEGFR2); bFGF = basic fibroblast growth factor; EGF = epidermal growth factor; PDGF = platelet-derived growth factor; myosin HC = myosin-heavy chain; αSMA = α-smooth muscle actin].

16

highlighted the negative relationship between circulating CD34+/VEGFR2+ cells and arterial stiffness in diabetic patients. However, because these surface markers are not exclusive to EPCs, the reduced number may be inclusive of altered levels of hematopoietic stem/progenitor cells. In fact, a fairly large analysis of 120 patients with ischemic heart disease showed reduced levels of bone marrow-derived CD34+/CD45+ cells, which also correlated with glycated hemoglobin HbA1c levels[70]. *In vitro* experimental studies using early EPCs have also shown a lower angiogenic ability[65] and impaired adherence to the mature EC monolayer in diabetes[71,72]. Less work has been done on the late vasculogenic EPCs, which display different cellular properties, and may respond quite differently than early EPCs to the adverse effects of high glucose.

Despite the notion that EPC number may be reduced in long-term diabetes, there is still promise for their therapeutic potential. If the cellular activity is able to remain intact in a diabetic setting, administration of *ex vivo* expanded EPCs to a diabetic patient should essentially work to improve vascular dysfunction. Not only has successful expansion of adult blood-derived EPCs been shown *in vitro*, but their ability to form fully functional vascular networks has also been demonstrated *in vivo*[46,50]. It is important to note that in order to form stable and durable networks, EPCs require co-implantation with a source of perivascular cell. Mesenchymal progenitor cells (MPCs), also derived from the CD133+ stem cell fraction, may be a suitable candidate for this task.

1.4 Mesenchymal progenitor cells (MPCs) and diabetic complications

MPCs are multipotent cells that are derived, along with EPCs, from the CD133+ population of circulating cells. MPCs can be isolated in large quantities from adult human bone marrow[73]. In addition, they have been identified in liver[74], spleen[74], and adipose tissue[75]. Like EPCs and other progenitor cell types, MPCs share properties of both stem and mature cells. They can be characterized by a combination of phenotypic and functional properties, including expression of cell surface markers, cell adhesion molecules, and differentiation potential (figure 1.5). Because there is not one marker that is specific to MPCs, all parameters must be taken into consideration when properly identifying this cell population. Mesenchymal cells in culture typically exude a spindle-like morphology, however, some heterogeneity has been noted depending on the tissue source and especially when arising from differing species. MPCs are negative for both the endothelial marker CD31 and the hematopoietic marker CD45[51]. Analysis of mRNA and/or protein can be used to demonstrate expression of α-smooth muscle actin (α-SMA), calponin, CD90, PDGFRβ, and NG2[46,51]. Functionally, MPCs differentiate into the mesenchymal lineage cells including adipocytes, osteoblasts, and chondrocytes[46,51,73].

Not much is known about a possible pathogenic role of MPCs in diabetic complications. In terms of therapeutic benefit, however, recent studies show improvement and amelioration of complications, including cardiomyopathy, nephropathy, neuropathy, and wound healing by MPCs. Using a rat model of diabetic cardiomyopathy, MPCs were administered intravenously and shown to attenuate cardiac remodeling and improve myocardial function through a marked increase in the

18

activity of matrix metalloproteinase (MMP)-2 and decrease in MMP-9[76]. In addition,

reduced levels of VEGF, insulin-like growth factor-1 (IGF-1), adrenomedullin (AM), and

hepatocyte growth factor (HGF) were found[76]. The MPCs differentiated into both

cardiomyocytes and vascular ECs, improving myocardial perfusion and regeneration in

the diabetic heart[76]. MPCs have also successfully improved diabetic nephropathy in

mice. After systemic injection, the precursor cells were shown to engraft in the damaged

kidneys and differentiate into renal cells, improving renal function and the regeneration

of glomerular structures[77,78]. Furthermore, when injected intramuscularly, MPCs

improve diabetic polyneuropathy through increased secretion of angiogenic cytokines

such as basic fibroblast growth factor (FGF) and VEGF[79]. Lastly, in a model of skin

wound healing, administration of MPCs in streptozotocin-induced diabetic rats

completely normalized the delayed wound closure time[80]. This effect was mediated, in

part, through a reduced number of infiltrating CD45+ cells into the wounds. This study

involved 'normal' MPCs (i.e. cells isolated from non-diabetic rats), and the question

remains whether diabetes causes alteration of the functional properties of MPCs.

Although this is still an emerging field, a recent study showed that AGEs may increase

the generation of reactive oxygen species and reduce the proliferation and migration of

MPCs[81]. Whether this plays a role in human diabetes or in animal models of diabetic

complications require further studies.

Given the promising results of the animal studies and the advantages that MPCs

have over other cell types (differentiation potential and capability for regulation of the

immune response), they are likely to be good therapeutic candidates in diabetic

complications. Several studies have also reported that treatment with MPCs can

enhance angiogenesis through paracrine effects[82-84]. This paracrine role may be

important for the restructuring of vascular networks by providing angiogenic factors to

facilitate EPC homing.

1.5 Therapeutic neovascularization in diabetes

As diabetes is beginning to reach epidemic proportions, more efforts must be focused on combatting the vascular dysfunctions that inevitably arise in these patients. Experimental evidence shows that stem/progenitor cells isolated from diabetic mice are able to restore vascular homeostasis[85,86]. If function remains intact, a reduction in stem cell number is the likely deficit in diabetes. This reduction may take place anywhere between the bone marrow and the circulation, and could be caused by a number of factors such as impaired bone marrow release, loss of migratory ability, loss of differentiation potential, or shortened survival time in the peripheral circulation. However, if we can find a way to utilize our EPC and MPC populations to repair vascular damage and restore blood vessel functioning, there is hope that the chronic complications can be attenuated (figure 1.6).

The success of therapeutic vascularization will rely on many factors, one of which being the ability of engineered blood vessels to form stable and functional anastomoses with the host vasculature. Neovascularization has been successfully shown thus far using human umbilical vein endothelial cells (HUVECs), as well as human microvascular endothelial cells (HDMECs)[51,87,88]. However, there are limitations to the clinical use of these particular EC types because of the lower yield. Considering the ease with which CD133+ VSCs can be isolated from adult peripheral blood and bone marrow, an opportunity is presented to obtain these cells non-invasively and in large enough quantities for differentiation and expansion *ex vivo*. Following expansion, the
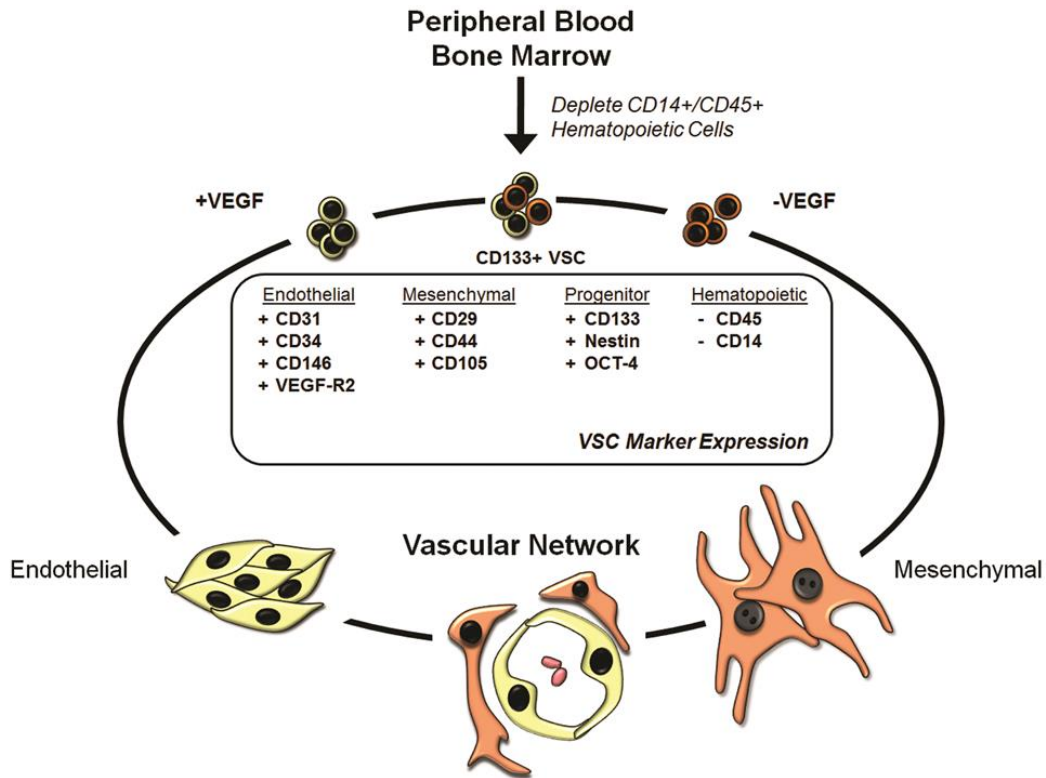
Figure 1.6: The potential of VSCs for therapeutic use in diabetics. A schematic of our working hypothesis showing that CD133+ VSCs are non-invasively isolated from diabetic patients and differentiated into endothelial and mesenchymal lineages by defined media. EPCs and MPCs are then expanded *ex vivo* and re-implanted in the patients to repair the damage and restore vascular homeostasis [middle box shows the immunophenotype of VSCs; VEGF=vascular endothelial growth factor (obligatory factor for endothelial lineage)].

cells can be implanted back into the diabetic patient to restore vascular homeostasis. It has previously been shown that adult and cord blood-derived EPCs can be expanded *ex vivo* and have the ability to form functional vascular networks *in vivo*[50,89]. Importantly, this requires co-implantation with a perivascular or mural cell source in order to maintain stable, functional networks. MPCs are an ideal candidate because, like EPCs, they can be isolated with ease from sites such as bone marrow[73] and even adult blood[90]. We have previously shown the success of subcutaneous co-implantation of EPCs and MPCs into the backs of athymic nu/nu mice, resulting in the creation of human microvessels that formed functional anastomoses with the host vasculature[51]. Although we know VSCs may provide the basis for vasculogenesis in a nude mouse model, whether they are able to restore vascular homeostasis in an in vivo diabetic setting remains to be determined. The next important step is to directly examine the effects of high glucose on both progenitor cell populations.

1.6 Thesis overview

This thesis demonstrates the natural progression of a scientific question. We have previously shown the success of neovascularization in a mouse model[51]. It then remained to be determined if this process is affected in diabetes, and whether VSCs are an appropriate source of EPCs and MPCs for the creation of new vascular networks.

In Chapter 2, I set out to characterize EPCs and MPCs in a high glucose setting. My first aim was to derive both progenitor cell populations. My second aim was to examine the functional characteristics of EPCs and MPCs in a high glucose setting. My studies consisted of the following:

1. Isolation of VSCs that have the required regenerative potential for therapeutic use in diabetic patients
2. Perform directed differentiation of VSCs to the endothelial and mesenchymal lineages
3. Investigate the effect of high glucose on the cellular activity of EPCs and MPCs

The results of these studies provided novel insight into MPC dysfunction in diabetes, highlighting their selective differentiation to the adipocyte lineage. Not only does this alteration reflect the bone phenotype of long-term diabetics, but it hints at an intriguing connection between diabetes and obesity.

In chapter 3, I reviewed potential mechanisms that may regulate adipogenesis – setting the stage for my experimental studies in chapter 4. My first aim was to establish

the alterations that occurred at each level of regulation of Wingless-type MMTV integration site members (Wnt) signaling with the addition of high glucose. My second aim was to manipulate each pathway using both genetic and chemical approaches in an attempt to prevent the high glucose-induced increase in adipogenesis. My final aim was to corroborate these results with *in vivo* data using both human and rat bone marrow specimens in order to establish the relationship of MPC differentiation to the stem cell niche. My studies consisted of the following:

1. Elucidate the high glucose-induced changes in Wnt signaling during adipogenesis.
2. Establish the precise role of β-catenin in the differentiation of MPCs to adipocytes.
3. Determine the involvement of non-canonical Wnt signaling in the skewing of MPC differentiation.
4. Examine alterations in the diabetic bone marrow stem cell niche.

Taken together, these studies highlight novel findings regarding stem/progenitor cell function in a diabetic setting. The knowledge gained from this work will have far reaching consequences should it be expanded and exploited for clinical use.

1.7 References

1   Amos, A. F., McCarty, D. J. & Zimmet, P. The rising global burden of diabetes and its complications: estimates and projections to the year 2010. *Diabet Med* **14 Suppl 5**, S1-85 (1997).

2   Dawson, K. G., Gomes, D., Gerstein, H., Blanchard, J. F. & Kahler, K. H. The economic cost of diabetes in Canada, 1998. *Diabetes Care* **25**, 1303-1307 (2002).

3   Economic costs of diabetes in the U.S. in 2012. *Diabetes Care* **36**, 1033-1046, doi:10.2337/dc12-2625dc12-2625 [pii] (2013).

4   King, H. & Rewers, M. Global estimates for prevalence of diabetes mellitus and impaired glucose tolerance in adults. WHO Ad Hoc Diabetes Reporting Group. *Diabetes Care* **16**, 157-177 (1993).

5   Engerman, R., Bloodworth, J. M., Jr. & Nelson, S. Relationship of microvascular disease in diabetes to metabolic control. *Diabetes* **26**, 760-769 (1977).

6   Albers, J. W. *et al.* Effect of prior intensive insulin treatment during the Diabetes Control and Complications Trial (DCCT) on peripheral neuropathy in type 1 diabetes during the Epidemiology of Diabetes Interventions and Complications (EDIC) Study. *Diabetes Care* **33**, 1090-1096, doi:10.2337/dc09-1941dc09-1941 [pii] (2010).

7   Ratner, R. E. Glycemic control in the prevention of diabetic complications. *Clin Cornerstone* **4**, 24-37 (2001).

8   Florey. The endothelial cell. *Br Med J* **2**, 487-490 (1966).

9   Cines, D. B. *et al.* Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood* **91**, 3527-3561 (1998).

10   Joost, H. G. & Thorens, B. The extended GLUT-family of sugar/polyol transport facilitators: nomenclature, sequence characteristics, and potential function of its novel members (review). *Mol Membr Biol* **18**, 247-256 (2001).

11   Augustin, R. The protein family of glucose transport facilitators: It's not only about glucose after all. *IUBMB Life* **62**, 315-333, doi:10.1002/iub.315 (2010).

12   Thorens, B. & Mueckler, M. Glucose transporters in the 21st Century. *Am J Physiol Endocrinol Metab* **298**, E141-145, doi:10.1152/ajpendo.00712.2009ajpendo.00712.2009 [pii] (2010).

13   Mandarino, L. J., Finlayson, J. & Hassell, J. R. High glucose downregulates glucose transport activity in retinal capillary pericytes but not endothelial cells. *Invest Ophthalmol Vis Sci* **35**, 964-972 (1994).

14   Kaiser, N. *et al.* Differential regulation of glucose transport and transporters by glucose in vascular endothelial and smooth muscle cells. *Diabetes* **42**, 80-89 (1993).

15   Wertheimer, E., Sasson, S., Cerasi, E. & Ben-Neriah, Y. The ubiquitous glucose transporter GLUT-1 belongs to the glucose-regulated protein family of stress-inducible proteins. *Proc Natl Acad Sci U S A* **88**, 2525-2529 (1991).

16   Takagi, H., King, G. L. & Aiello, L. P. Hypoxia upregulates glucose transport activity through an adenosine-mediated increase of GLUT1 expression in retinal capillary endothelial cells. *Diabetes* **47**, 1480-1488 (1998).

17   Brownlee, M. Biochemistry and molecular cell biology of diabetic complications.
      Nature **414**, 813-820, doi:10.1038/414813a414813a [pii] (2001).

18   Khan, Z. A. *et al.* Endothelins: regulators of extracellular matrix protein
      production in diabetes. *Exp Biol Med (Maywood)* **231**, 1022-1029, doi:231/6/1022
      [pii] (2006).

19   Khan, Z. A. & Chakrabarti, S. Therapeutic targeting of endothelial dysfunction in
      chronic diabetic complications. *Recent Pat Cardiovasc Drug Discov* **1**, 167-175
      (2006).

20   Cagliero, E., Maiello, M., Boeri, D., Roy, S. & Lorenzi, M. Increased expression of
      basement membrane components in human endothelial cells cultured in high
      glucose. *J Clin Invest* **82**, 735-738, doi:10.1172/JCI113655 (1988).

21   Graier, W. F., Grubenthal, I., Dittrich, P., Wascher, T. C. & Kostner, G. M.
      Intracellular mechanism of high D-glucose-induced modulation of vascular cell
      proliferation. *Eur J Pharmacol* **294**, 221-229, doi:001429999500534X [pii] (1995).

22   Du, X. L. *et al.* Introduction of apoptosis by high proinsulin and glucose in
      cultured human umbilical vein endothelial cells is mediated by reactive oxygen
      species. *Diabetologia* **41**, 249-256 (1998).

23   Lorenzi, M., Cagliero, E. & Toledo, S. Glucose toxicity for human endothelial cells
      in culture. Delayed replication, disturbed cell cycle, and accelerated death.
      Diabetes **34**, 621-627 (1985).

24   Baumgartner-Parzer, S. M. *et al.* High-glucose--triggered apoptosis in cultured
      endothelial cells. *Diabetes* **44**, 1323-1327 (1995).

25   Yang, Z. *et al.* Critical effect of VEGF in the process of endothelial cell apoptosis induced by high glucose. *Apoptosis* **13**, 1331-1343, doi:10.1007/s10495-008-0257-y (2008).

26   Favaro, E. *et al.* Hyperglycemia induces apoptosis of human pancreatic islet endothelial cells: effects of pravastatin on the Akt survival pathway. *Am J Pathol* **173**, 442-450, doi:10.2353/ajpath.2008.080238S0002-9440(10)61620-3 [pii] (2008).

27   Chen, S., Khan, Z. A., Barbin, Y. & Chakrabarti, S. Pro-oxidant role of heme oxygenase in mediating glucose-induced endothelial cell damage. *Free Radic Res* **38**, 1301-1310, doi:TXM3XDXF235DXFAD [pii]10.1080/10715760400017228 (2004).

28   Nishikawa, T. *et al.* Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* **404**, 787-790, doi:10.1038/35008121 (2000).

29   van den Oever, I. A., Raterman, H. G., Nurmohamed, M. T. & Simsek, S. Endothelial dysfunction, inflammation, and apoptosis in diabetes mellitus. *Mediators Inflamm* **2010**, 792393, doi:10.1155/2010/792393 (2010).

30   Aronson, D. Hyperglycemia and the pathobiology of diabetic complications. *Adv Cardiol* **45**, 1-16, doi:10.1159/0000115118115118 [pii] (2008).

31   Alberti, K. G. & Zimmet, P. Z. New diagnostic criteria and classification of diabetes--again? *Diabet Med* **15**, 535-536, doi:10.1002/(SICI)1096-9136(199807)15:7<535::AID-DIA670>3.0.CO;2-Q (1998).

32   Chou, E. *et al.* Decreased cardiac expression of vascular endothelial growth factor and its receptors in insulin-resistant and diabetic States: a possible explanation for impaired collateral formation in cardiac tissue. *Circulation* **105**, 373-379 (2002).

33   Khan, Z. A. & Chakrabarti, S. Growth factors in proliferative diabetic retinopathy. *Exp Diabesity Res* **4**, 287-301, doi:P9LH3HVTG9D7TK5B [pii]10.1155/EDR.2003.287 (2003).

34   Boudreau, N. J. & Jones, P. L. Extracellular matrix and integrin signalling: the shape of things to come. *Biochem J* **339 (Pt 3)**, 481-488 (1999).

35   Jiang, B., Liou, G. I., Behzadian, M. A. & Caldwell, R. B. Astrocytes modulate retinal vasculogenesis: effects on fibronectin expression. *J Cell Sci* **107 (Pt 9)**, 2499-2508 (1994).

36   Nishikawa, T., Giardino, I., Edelstein, D. & Brownlee, M. Changes in diabetic retinal matrix protein mRNA levels in a common transgenic mouse strain. *Curr Eye Res* **21**, 581-587 (2000).

37   Guarda, E., Katwa, L. C., Myers, P. R., Tyagi, S. C. & Weber, K. T. Effects of endothelins on collagen turnover in cardiac fibroblasts. *Cardiovasc Res* **27**, 2130-2134 (1993).

38   Katwa, L. C., Guarda, E. & Weber, K. T. Endothelin receptors in cultured adult rat cardiac fibroblasts. *Cardiovasc Res* **27**, 2125-2129 (1993).

39   Chandrasekhar, K. S. *et al.* Blood vessel wall-derived endothelial colony-forming cells enhance fracture repair and bone regeneration. *Calcif Tissue Int* **89**, 347-357, doi:10.1007/s00223-011-9524-y (2011).

40   Wu, Y., Wang, J., Scott, P. G. & Tredget, E. E. Bone marrow-derived stem cells in wound healing: a review. *Wound Repair Regen* **15 Suppl 1**, S18-26, doi:10.1111/j.1524-475X.2007.00221.xWRR221 [pii] (2007).

41   Tepper, O. M., Galiano, R. D., Kalka, C. & Gurtner, G. C. Endothelial progenitor cells: the promise of vascular stem cells for plastic surgery. *Plast Reconstr Surg* **111**, 846-854, doi:10.1097/01.PRS.0000039576.63523.08 (2003).

42   Zuk, P. A. *et al.* Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**, 4279-4295, doi:10.1091/mbc.E02-02-0105 (2002).

43   Kattman, S. J., Huber, T. L. & Keller, G. M. Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev Cell* **11**, 723-732, doi:S1534-5807(06)00454-0 [pii]10.1016/j.devcel.2006.10.002 (2006).

44   Yamashita, J. *et al.* Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature* **408**, 92-96, doi:10.1038/35040568 (2000).

45   Ferreira, L. S. *et al.* Vascular progenitor cells isolated from human embryonic stem cells give rise to endothelial and smooth muscle like cells and form vascular networks in vivo. *Circ Res* **101**, 286-294, doi:CIRCRESAHA.107.150201 [pii]10.1161/CIRCRESAHA.107.150201 (2007).

46   Khan, Z. A. *et al.* Multipotential stem cells recapitulate human infantile hemangioma in immunodeficient mice. *J Clin Invest* **118**, 2592-2599, doi:10.1172/JCI33493 (2008).

47   Khan, Z. A. *et al.* Endothelial progenitor cells from infantile hemangioma and umbilical cord blood display unique cellular responses to endostatin. *Blood* **108**, 915-921, doi:108/3/915 [pii]10.1182/blood-2006-03-006478 (2006).

48   Peichev, M. *et al.* Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. *Blood* **95**, 952-958 (2000).

49   Tondreau, T. *et al.* Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity. *Stem Cells* **23**, 1105-1112, doi:2004-0330 [pii]10.1634/stemcells.2004-0330 (2005).

50   Melero-Martin, J. M. *et al.* In vivo vasculogenic potential of human blood-derived endothelial progenitor cells. *Blood* **109**, 4761-4768, doi:blood-2006-12-062471 [pii]10.1182/blood-2006-12-062471 (2007).

51   Melero-Martin, J. M. *et al.* Engineering robust and functional vascular networks in vivo with human adult and cord blood-derived progenitor cells. *Circ Res* **103**, 194-202, doi:10.1161/CIRCRESAHA.108.178590CIRCRESAHA.108.178590 [pii] (2008).

52   Case, J. *et al.* Human CD34+AC133+VEGFR-2+ cells are not endothelial progenitor cells but distinct, primitive hematopoietic progenitors. *Exp Hematol* **35**, 1109-1118, doi:S0301-472X(07)00225-1 [pii]10.1016/j.exphem.2007.04.002 (2007).

53   Zampetaki, A., Kirton, J. P. & Xu, Q. Vascular repair by endothelial progenitor cells. *Cardiovasc Res* **78**, 413-421, doi:10.1093/cvr/cvn081cvn081 [pii] (2008).

54   Fadini, G. P., Agostini, C. & Avogaro, A. Characterization of endothelial progenitor cells. *Biochem Biophys Res Commun* **336**, 1-2, doi:S0006-291X(05)01500-7 [pii]10.1016/j.bbrc.2005.07.119 (2005).

55   Lopez-Holgado, N. *et al.* Short-term endothelial progenitor cell colonies are composed of monocytes and do not acquire endothelial markers. *Cytotherapy* **9**, 14-22, doi:769972714 [pii]10.1080/14653240601047726 (2007).

56   Capla, J. M. *et al.* Diabetes impairs endothelial progenitor cell-mediated blood vessel formation in response to hypoxia. *Plast Reconstr Surg* **119**, 59-70, doi:10.1097/01.prs.0000244830.16906.3f00006534-200701000-00010 [pii] (2007).

57   Kusuyama, T. *et al.* Effects of treatment for diabetes mellitus on circulating vascular progenitor cells. *J Pharmacol Sci* **102**, 96-102, doi:JST.JSTAGE/jphs/FP0060256 [pii] (2006).

58   Wang, C. H. *et al.* Pioglitazone increases the numbers and improves the functional capacity of endothelial progenitor cells in patients with diabetes mellitus. *Am Heart J* **152**, 1051 e1051-1058, doi:S0002-8703(06)00810-6 [pii]10.1016/j.ahj.2006.07.029 (2006).

59   Goldstein, J. L., Ho, Y. K., Basu, S. K. & Brown, M. S. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci U S A* **76**, 333-337 (1979).

60   Jackson, C. J., Garbett, P. K., Nissen, B. & Schrieber, L. Binding of human endothelium to Ulex europaeus I-coated Dynabeads: application to the isolation of microvascular endothelium. *J Cell Sci* **96 (Pt 2)**, 257-262 (1990).

61   Asahara, T. *et al.* Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* **85**, 221-228 (1999).

62   Murohara, T. *et al.* Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. *J Clin Invest* **105**, 1527-1536, doi:10.1172/JCI8296 (2000).

63   Wu, X. *et al.* Tissue-engineered microvessels on three-dimensional biodegradable scaffolds using human endothelial progenitor cells. *Am J Physiol Heart Circ Physiol* **287**, H480-487, doi:10.1152/ajpheart.01232.2003287/2/H480 [pii] (2004).

64   Yoder, M. C. *et al.* Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood* **109**, 1801-1809, doi:blood-2006-08-043471 [pii]10.1182/blood-2006-08-043471 (2007).

65   Loomans, C. J. *et al.* Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes. *Diabetes* **53**, 195-199 (2004).

66   Vasa, M. *et al.* Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* **89**, E1-7 (2001).

67   Fadini, G. P. *et al.* Number and function of endothelial progenitor cells as a marker of severity for diabetic vasculopathy. *Arterioscler Thromb Vasc Biol* **26**, 2140-2146, doi:01.ATV.0000237750.44469.88 [pii]10.1161/01.ATV.0000237750.44469.88 (2006).

68   Fadini, G. P. *et al.* Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. *J Am Coll Cardiol* **45**, 1449-1457, doi:S0735-1097(05)00356-6 [pii]10.1016/j.jacc.2004.11.067 (2005).

69   Yue, W. S. *et al.* Impact of glycemic control on circulating endothelial progenitor cells and arterial stiffness in patients with type 2 diabetes mellitus. *Cardiovasc Diabetol* **10**, 113, doi:10.1186/1475-2840-10-1131475-2840-10-113 [pii] (2011).

70   Bozdag-Turan, I. *et al.* Relation between the frequency of CD34(+) bone marrow derived circulating progenitor cells and the number of diseased coronary arteries in patients with myocardial ischemia and diabetes. *Cardiovasc Diabetol* **10**, 107, doi:10.1186/1475-2840-10-1071475-2840-10-107 [pii] (2011).

71   Tamarat, R. *et al.* Impairment in ischemia-induced neovascularization in diabetes: bone marrow mononuclear cell dysfunction and therapeutic potential of placenta growth factor treatment. *Am J Pathol* **164**, 457-466, doi:S0002-9440(10)63136-7 [pii]10.1016/S0002-9440(10)63136-7 (2004).

72   Tepper, O. M. *et al.* Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation* **106**, 2781-2786 (2002).

73   Pittenger, M. F. *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143-147 (1999).

74   Campagnoli, C. *et al.* Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. *Blood* **98**, 2396-2402 (2001).

75   Traktuev, D. O. *et al.* A population of multipotent CD34-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. *Circ Res* **102**, 77-85, doi:CIRCRESAHA.107.159475 [pii]10.1161/CIRCRESAHA.107.159475 (2008).

76   Zhang, N., Li, J., Luo, R., Jiang, J. & Wang, J. A. Bone marrow mesenchymal stem cells induce angiogenesis and attenuate the remodeling of diabetic cardiomyopathy. *Exp Clin Endocrinol Diabetes* **116**, 104-111, doi:10.1055/s-2007-985154 (2008).

77   Ezquer, F. E. *et al.* Systemic administration of multipotent mesenchymal stromal cells reverts hyperglycemia and prevents nephropathy in type 1 diabetic mice. *Biol Blood Marrow Transplant* **14**, 631-640, doi:10.1016/j.bbmt.2008.01.006S1083-8791(08)00050-5 [pii] (2008).

78   Lee, R. H. *et al.* Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. *Proc Natl Acad Sci U S A* **103**, 17438-17443, doi:0608249103 [pii]10.1073/pnas.0608249103 (2006).

79   Shibata, T. *et al.* Transplantation of bone marrow-derived mesenchymal stem cells improves diabetic polyneuropathy in rats. *Diabetes* **57**, 3099-3107, doi:10.2337/db08-0031db08-0031 [pii] (2008).

80   Kuo, Y. R. *et al.* Bone marrow-derived mesenchymal stem cells enhanced diabetic wound healing through recruitment of tissue regeneration in a rat model of streptozotocin-induced diabetes. *Plast Reconstr Surg* **128**, 872-880, doi:10.1097/PRS.0b013e318217432900006534-201110000-00013 [pii] (2011).

81   Yang, K. *et al.* Advanced glycation end products induce chemokine/cytokine production via activation of p38 pathway and inhibit proliferation and migration of bone marrow mesenchymal stem cells. *Cardiovasc Diabetol* **9**, 66, doi:10.1186/1475-2840-9-661475-2840-9-66 [pii] (2010).

82   Falanga, V. *et al.* Autologous bone marrow-derived cultured mesenchymal stem cells delivered in a fibrin spray accelerate healing in murine and human cutaneous wounds. *Tissue Eng* **13**, 1299-1312, doi:10.1089/ten.2006.0278 (2007).

83   Wu, Y., Chen, L., Scott, P. G. & Tredget, E. E. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* **25**, 2648-2659, doi:2007-0226 [pii]10.1634/stemcells.2007-0226 (2007).

84   Javazon, E. H. *et al.* Enhanced epithelial gap closure and increased angiogenesis in wounds of diabetic mice treated with adult murine bone marrow stromal progenitor cells. *Wound Repair Regen* **15**, 350-359, doi:WRR237 [pii]10.1111/j.1524-475X.2007.00237.x (2007).

85   Naruse, K. *et al.* Therapeutic neovascularization using cord blood-derived endothelial progenitor cells for diabetic neuropathy. *Diabetes* **54**, 1823-1828, doi:54/6/1823 [pii] (2005).

86   Huang, P. *et al.* Autologous transplantation of granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells improves critical limb ischemia in diabetes. *Diabetes Care* **28**, 2155-2160, doi:28/9/2155 [pii] (2005).

87   Koike, N. *et al.* Tissue engineering: creation of long-lasting blood vessels. *Nature* **428**, 138-139, doi:10.1038/428138a428138a [pii] (2004).

88   Nor, J. E. *et al.* Engineering and characterization of functional human microvessels in immunodeficient mice. *Lab Invest* **81**, 453-463 (2001).

89   Au, P. *et al.* Differential in vivo potential of endothelial progenitor cells from human umbilical cord blood and adult peripheral blood to form functional long-lasting vessels. *Blood* **111**, 1302-1305, doi:blood-2007-06-094318 [pii]10.1182/blood-2007-06-094318 (2008).

90   Simper, D., Stalboerger, P. G., Panetta, C. J., Wang, S. & Caplice, N. M. Smooth muscle progenitor cells in human blood. *Circulation* **106**, 1199-1204 (2002).

CHAPTER 2 – UNIQUE RESPONSES OF STEM CELL-DERIVED VASCULAR ENDOTHELIAL AND MESENCHYMAL CELLS TO HIGH LEVELS OF GLUCOSE[b]

This study focused on the characterization of EPCs and MPCs in a high glucose setting in order to establish their therapeutic potential for diabetic neovascularization. My first aim was to derive both progenitor cell populations. My second aim was to examine the functional characteristics of EPCs and MPCs in a high glucose setting. My studies consisted of the following:

1. Isolation of adult human blood-derived VSCs that have the required regenerative potential for therapeutic use in diabetic patients.

2. Perform directed differentiation of VSCs to the endothelial and mesenchymal lineages.

3. Investigate the effects of high glucose on the cellular activity of EPCs and MPCs.

2.1   Introduction

The idea of a specific VSC population is one that is steadily gaining recognition. VSCs are a sub-population of CD133+ cells that are able to differentiate into mature cells of the vascular wall[1,2]. They are predominantly housed in the bone marrow, but can also be derived from the MNC layer of peripheral blood, making them an easily attainable source of cells. The existence of a common vascular stem/progenitor cell that can be derived from adult human blood samples highlights the feasibility of therapeutic vasculogenesis for long-term diabetic patients. Although we know VSCs may provide the basis for vasculogenesis in a nude mouse model[3], whether they are able to restore vascular homeostasis in an *in vivo* diabetic setting remains to be determined. The effects of high glucose on the functionality of both EPCs and MPCs must be elucidated firstly. To date, the role of MPCs in diabetic complications has not been investigated, and much of the work done on EPCs (short-term colonies; 2-4 days in culture) is confounded by the presence of hematopoietic/monocytic cells within the studies[4,5]. These 'early' cells are characterized by Ulex europaeus agglutinin binding and DiI-labeled acetylated-low density lipoprotein (LDL) uptake[4,6,7]. Both of these assays are not specific to ECs[8,9]. Therefore, a combination of both phenotypic and functional properties must be used to unambiguously identify both EPCs and MPCs so as not to impair the results of the studies. We herein determine the precise role of vascular endothelial and mesenchymal progenitor cells in a high glucose setting to highlight their potential application in diabetes therapy.

2.2   Materials and methods

All experiments were approved by the Research Ethics Board at the University of Western Ontario, London Ontario.

2.2.1 Isolation, culture, and differentiation of VSCs

Normal adult peripheral blood (US Biological, Salem, MA; age 26.16±6.79 yrs; n=9) and fresh bone marrow samples (Lonza Inc., Walkersville, MD; n=6) were obtained and mononuclear cell fraction was prepared by the Ficoll-Pacque centrifugation method[10]. To obtain adult blood EPCs (abEPCs), cell suspensions were cultured on fibronectin (FN; 1 μg/cm^2; Millipore, Temecula, CA)-coated plates in modified endothelial cell media: complete Endothelial Basal Media-2 (EBM-2, Lonza Inc.) supplemented with 35% fetal bovine serum (FBS; Lonza Inc.), 1X PSF (antibiotic-antimycotic solution; CellGro Mediatech Inc., Manassas, VA) and SingleQuots (Lonza Inc.; contains human epidermal growth factor, vascular endothelial growth factor, human basic fibroblast growth factor, insulin-like growth factor, hydrocortisone, heparin, ascorbic acid, and gentamicin/amphotericin B). The media was changed every day until colonies began to appear. Thereafter, the media was changed every other day. All subcultures were then performed with EBM2/20% FBS/SingleQuots/PSF media. Bone marrow samples were cultured on FN-coated plates in DMEM (Life Technologies, Burlington, ON) media, supplemented with 20% FBS, 1X PSF and no additional growth factors to yield MPCs. To assess the effect of high levels of glucose on abEPC and

bone marrow MPC (bmMPC) differentiation, the media was supplemented with 25 mmol/L glucose from the initial plating. All other experiments were conducted on passage 2–6 cells with 3 technical and 3–5 biological replicates.

Normal human dermal microvascular endothelial cells (HDMECs; CC-2516, Lonza Inc.) and human cord blood-derived EPCs (cbEPCs; derived from cord blood using the same protocol as mentioned above for adult blood; 2C-150A; Lonza Inc.) were used as control for the abEPCs. Human umbilical artery smooth muscle cells (uaSMCs; CC-2579; Lonza Inc.) were used as control for the bmMPCs.

2.2.2 Cell staining

Cultured cells were trypsinized and plated (10,000 cells/cm^2) on FN-coated 8-chambered slides one day prior to staining to allow for attachment of cells. Immunofluorescence staining for endothelial and mesenchymal cell markers was carried out by incubating the cells with primary antibodies at optimal concentrations (Table 1.1) for 1 hour at room temperature. Following primary incubation, a FITC- or Alexa488-conjugated secondary antibody was applied to the cells for 1 hour at room temperature. Slides were subjected to a nuclei counterstain using DAPI (Vector Laboratories, Burlington, ON) and mounted using Fluoromount (Sigma-Aldrich, Oakville, ON) mounting medium. Images were taken using Olympus BX-51 fluorescent microscope (Olympus Canada Inc., Richmond Hill, ON) and Spot Basic software (SPOT Imaging Solutions, Sterling Heights, MA).

Table 2.1 Antibodies used for immunostaining in chapter 2.

	Antigen	Host	Source (catalogue number)	Dilution
1	CD31	Goat polyclonal	Santa Cruz (sc-1505)	1:200
2	vWF	Rabbit polyclonal	DakoCytomation (A0082)	1:200
3	VE-cadherin	Goat polyclonal	Santa Cruz (sc-6458)	1:200
4	α-SMA	Mouse monoclonal	Sigma Aldrich (A 2547)	1:200
5	PDGF-Rβ	Mouse monoclonal	R&D Systems (MAB1263)	1:200
6	NG2	Mouse monoclonal	Abcam (ab83508)	1:200

2.2.3 RNA isolation and qRT-PCR

Using RNeasy Mini Plus or Micro Plus (Qiagen, Mississauga, ON), total RNA was extracted from the cells grown in culture. Purity of the RNA samples was determined by measuring the absorbance at 260:280 nm in GeneQuant Spectrophotometer (Pharmacia Biotech). The quantity was determined by Qubit® Broad Range RNA assay in the Qubit® Fluorometer (Life Technologies). cDNA synthesis was performed with 200 ng of RNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Primers used for RT-PCR are listed in Table 2.2. RT-PCR reactions consisted of 10 µL 2X SYBR Advantage qPCR premix (Clontech Laboratories, Inc., Mountain View, CA), 2 µL of both forward and reverse primers (at a 10 µM concentration), 2 µL cDNA, and 6 µL of H_2O. All reactions were performed for 40 cycles using the following temperature profiles: 95°C for 5 minutes (initial denaturation); 55°C for 10 seconds (annealing); and 72°C for 12 seconds (extension). 18S rRNA was used as the housekeeping gene. PCR specificity was determined by both the melting curve analysis and gel electrophoresis, and the data was analyzed by standard curve method.

Table 2.2 Primer sequence information for qRT-PCR in chapter 2.

Gene	Length (bp)	Source (catalogue number)
Adipogenesis markers		
CCAAT/enhancer binding protein alpha (C/EBPα)	88 bp	Qiagen (QT00203357)
Peroxisome proliferator-activated receptor γ2 (PPARγ2)	134 bp	5` → 3` ATTGACCCAGAAAGCGATTCC CAAAGGAGTGGGAGTGGTCT
Chondrogenesis markers		
NK3 homeobox 2 (Nkx3.2)	100 bp	Qiagen (QT01079582)
Runt-related transcription factor 2 (Runx2)	102 bp	Qiagen (QT00020517)
Sex determining region Y-box 9 (Sox9)	111 bp	Qiagen (QT00001498)
Endothelial cell markers		
CD31	144 bp	Qiagen (QT00081172)
CD34	106 bp	Qiagen (QT00056497)
Vascular endothelial growth factor receptor 2 (VEGFR-2)	78 bp	Qiagen (QT00069818)
Vascular endothelial cadherin (VE-cadherin)	109 bp	Qiagen (QT00013244)
Von Willebrand factor (vWF)	108 bp	Qiagen (QT00051975)
Endothelial cell activation markers		
Endothelin 1 (ET-1)	166 bp	Qiagen (QT00088235)

| Endothelial selectin (E-selectin) | 96 bp | Qiagen (QT00015358) |
| Intercellular adhesion molecule 1 (ICAM1) | 84 bp | Qiagen (QT00074900) |

Extracellular matrix (ECM) proteins

Fibronectin	119 bp	Qiagen (QT00038024)
Collagen 1	118 bp	Qiagen (QT00037793)
Collagen 3	95 bp	Qiagen (QT00058233)
Collagen 4	119 bp	Qiagen (QT00005250)

Mesenchymal cell markers

Alpha smooth muscle actin (α-SMA)	83 bp	Qiagen (QT00088102)
Calponin	78 bp	Qiagen (QT00067718)
Myosin heavy chain (MHC)	130 bp	Qiagen (QT00069391)
NG2	128 bp	Qiagen (QT00079884)
Platelet-derived growth factor receptor β (PDGFR-β)	102 bp	Qiagen (QT00082327)

Osteogenesis markers

| Osterix (SP7) | 120 bp | Qiagen (QT00213514) |
| Runt-related transcription factor 2 (Runx2) | 102 bp | Qiagen (QT00020517) |

Oxidative stress markers

Catalase (Cat)	60 bp	Qiagen (QT00079674)
Glutathione peroxidase 1 (GPx)	105 bp	Qiagen (QT00203392)
Heme oxygenase 1 (HO-1)	99 bp	Qiagen (QT00092645)
NADPH oxidase (p22 Phox)	106 bp	Qiagen (QT00082481)

Inducible NOS (NOS2)	92 bp	Qiagen (QT00068740)
Superoxide dismutase 1 (SOD-1)	150 bp	Qiagen (QT01671551)

Housekeeping gene

18S rRNA	149 bp	Qiagen (QT00199367)

2.2.4 Cell growth assay

The growth of the abEPCs and bmMPCs was assayed by plating the cells in triplicates on FN-coated multiwell plates at a density of 2500 cells/cm^2. The EPCs were cultured in complete EBM2/20% FBS media with or without the addition of high glucose (25 mmol/L; 450 mg/dL). Cell number was determined on days 1, 6, and 12 using Scepter 2.0 Automated Cell Counter (Millipore). The histogram gating was adjusted to specifically measure live cells. bmMPCs were also plated at a density of 2500 cells/cm^2 in multiwall plates. However, the cells were cultured in DMEM/10% FBS media with or without the addition of 25 mmol/L glucose for 1, 4, and 12 days. The data was presented as cell counts. The results were also confirmed by colorimetric assay utilizing tetrazolium salt reagent (WST-1, Clontech, Mountain View, CA). Following incubation, the absorbance was measured at 450 nm using Multiskan FC Microplate Photometer (Thermo Scientific, USA) with 690 nm absorbance as the reference point.

2.2.5 Caspase-3 activity

We also tested the effect of high glucose levels on caspase-3 activity (BF3100; R&D Systems, Minneapolis, MN) in serum-reduced media (EBM2/1% FBS for endothelial cell types and DMEM/1% FBS for mesenchymal cell types). The cells were cultured for up to 4 days (longer time periods were not possible as serum reduction itself causes cell death) with or without the addition of 25 mmol/L glucose and cell lysates were prepared. To measure caspase-3 activity, a caspase-specific peptide conjugated to p-nitroaniline was added to the lysates and the cleavage product was quantified by measuring absorbance at 405 nm.

2.2.6 Cell migration assay

A migration assay was performed on both abEPCs and bmMPCs using FN-coated 6.5-mm Transwell inserts with 8.0-µm pores (BD Falcon Cell Culture inserts; BD Biosciences, Mississauga, ON). abEPCs were trypsinized and re-suspended in control (EBM2/1% FBS) or high glucose (EBM2/1% FBS + 25 mmol/L glucose) media. One hundred µL of cell suspension was added to the inserts in triplicates, and at a density of 10,000 cells/insert. The lower chambers contained 10 ng/mL basic fibroblast growth factor (bFGF; 233-FB-025, R&D Systems). The cell density used in this assay was optimized for endothelial cells in our pilot studies to provide a robust measure of cell migration. Twenty-four hours later, cells on the upper insert were removed and cells in the lower chamber that had migrated through the pore were trypsinized. The cell suspension was centrifuged, re-suspended in media, and added to 96-well plates in order to be measured by the Multiskan FC Microplate Photometer. bmMPC migration was measured similarly but with pre-optimized 25,000 cells/cm^2. The lower chambers in bmMPC migration experiments contained 10% serum as the chemoattractant.

2.2.7 MPC differentiation assay

A differentiation assay was conducted on bmMPCs to assess whether they retain their multipotential nature under high glucose conditions. Cells were treated with control (DMEM/10%) or high glucose media (DMEM/10% + 25 mmol/L) for 7 days before the differentiation experiments. To induce differentiation, bmMPCs were seeded at a density of 40,000 cells/cm^2 on 12-well plates in specific differentiation media (StemPro® Adipogenesis/Chondrogenesis/Osteogenesis Differentiation media; Life Technologies).

49

Media was changed every other day. RNA was isolated from cells after 7 and 14 days to perform qRT-PCR in order to quantify the differentiation potential. We measured peroxisome proliferator activated receptor-γ2 and C/EBPα for adipogenesis, Runx2 and osterix (SP7) for osteogenesis, and Sox9, Nkx3.2, and Runx2 for chondrogenesis. These transcription factors are essential for the differentiation of multipotential cells into the lineages indicated[11,12].

2.2.8 Statistical analysis

The data were expressed as means ± SEM. Where appropriate, analysis of variance (ANOVA) followed by two-tailed student's unpaired t-tests were performed. P values <0.05 were considered statistically significant.

2.3   Results

2.3.1   Isolation and characterization of abEPCs

Following isolation of the MNC layer from adult blood, cells were cultured in complete EBM-2 media supplemented with SingleQuots to induce differentiation into the endothelial lineage (abEPCs). Culture of blood-derived cells in high glucose media (HG; 25 mmol/L) did not significantly alter the number of colonies (figure 2.1). No colonies appeared in either control or high glucose level conditions prior to day 14 (data not shown; plates were screened daily using phase contrast microscopy). abEPCs were then characterized through RT-PCR to confirm expression of endothelial cell-selective genes and through immunocytochemistry to properly localize the cellular markers. cbEPCs and HDMECs were used as controls. RT-PCR confirmed the expression of 5 genes of known significance to endothelial cells: CD31, CD34, VEGFR-2, VE-cadherin, and vWF (figure 2.2A). The expression of all endothelial-specific genes tested, except for VEGFR-2, was significantly higher in abEPCs as compared to mature HDMECs (figure 2.2A). Immunostaining showed both CD31 and VE-cadherin localized to the cell membrane of abEPCs, as anticipated (figure 2.2B). vWF, an intracellular protein stored in Weible Palade bodies, showed intracellular localization.
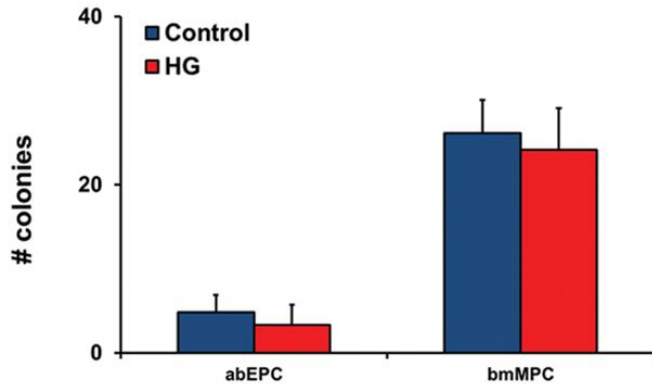
Figure 2.1: EPC and MPC colonies derived from VSCs. abEPCs (n=9) and bmMPCs (n=6) were derived from the mononuclear cell layer of adult peripheral blood samples and bone marrow samples, respectively. No significant change in the number of abEPC and bmMPC colonies was seen with the addition of HG (25 mmol/L) to the culture medium [HG=high glucose].
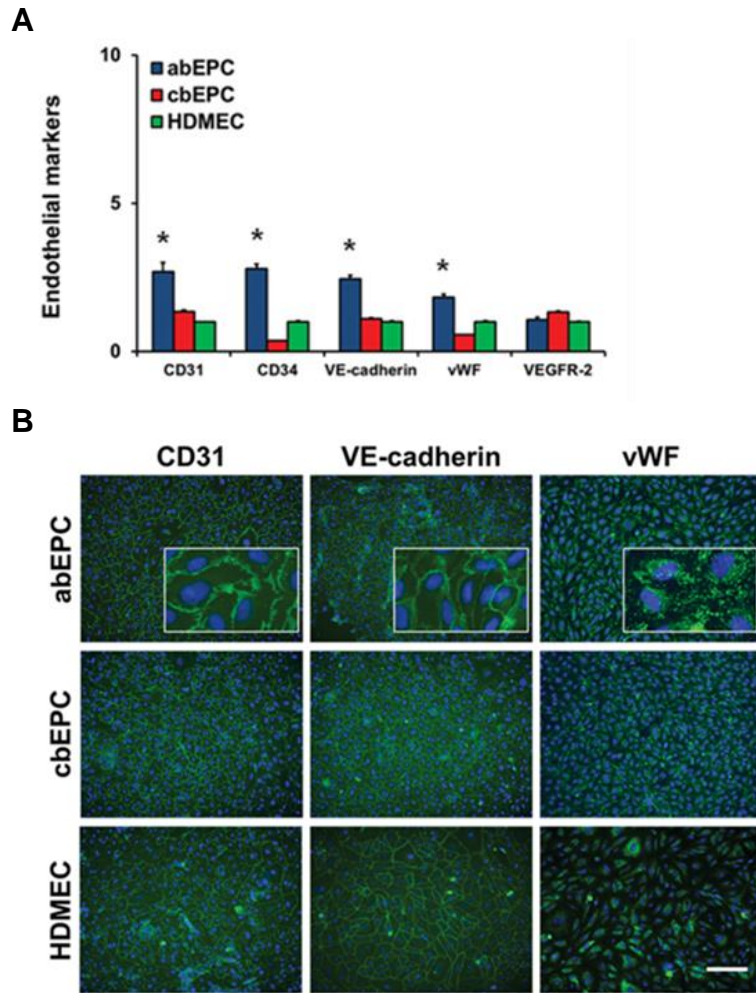
Figure 2.2: Characterization of human abEPCs. (A) abEPCs were characterized through quantitative RT-PCR analysis for expression of known EC-markers: CD31, CD34, VE-cadherin, vWF, and VEGFR-2 [mRNA data normalized to 18S rRNA and presented relative to HDMECs; *$p<0.05$ compared to HDMECs; n = 3]. (B) abEPCs were further characterized through immunostaining for antibodies against cell surface markers CD31 and VE-cadherin, and intracellular marker vWF [Blue: DAPI for nuclear staining; Green: Alexa Fluorochrome 488; scale bar represents 100µm] [VE-cadherin=vascular endothelial-cadherin; vWF=Von Willebrand Factor; VEGFR-2=vascular endothelial growth factor-2].

2.3.2 Isolation and characterization of MPCs

bmMPC colonies appeared within the first 7 days of culture after isolation of MNC from bone marrow. Similar to the abEPC preparations, high levels of glucose did not significantly affect the number of colonies derived (figure 2.1). MPCs were then subcultured and tested for expression of mesenchymal markers, using uaSMCs as a control. bmMPCs expressed mRNA for all mesenchymal markers examined (figure 2.3A). Interestingly, PDGFR-β was highly expressed in bmMPCs as compared to mature uaSMCs (figure 2.3A; $p<0.05$). Other markers of mesenchymal lineage showed significantly lower expression in the bmMPCs. Positive staining was observed for CD90, NG2, PDGFR-β, and α-SMA in bmMPCs (figure 2.3B). CD90, NG2, PDGFR-β were localized to the cell membrane, whereas α-SMA was intracellular, consistent with actin fiber staining.

A
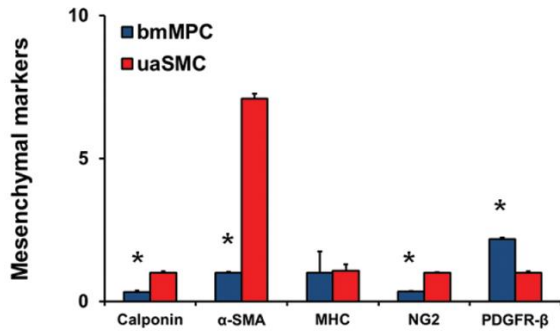
B
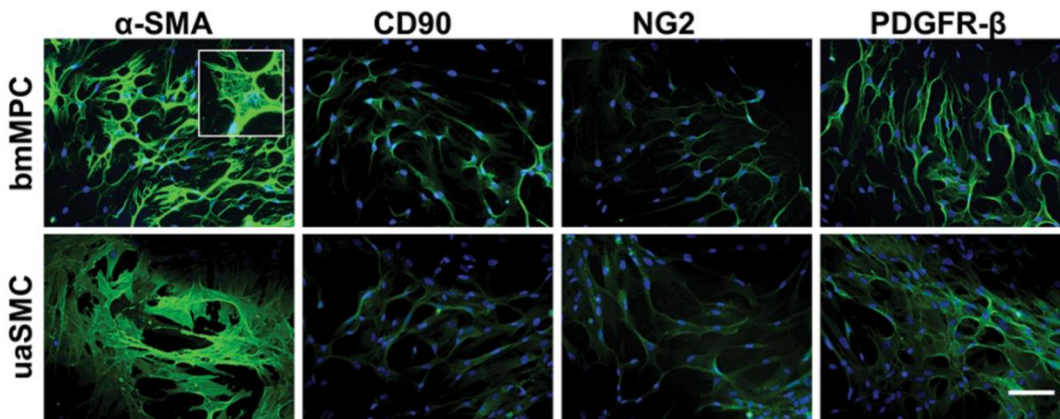
Figure 2.3: Characterization of human MPCs. (A) qRT-PCR analysis of bmMPCs showed expression of mesenchymal markers: calponin, α-SMA, MHC, NG2, and PDGFR-β [data normalized to 18S rRNA and presented relative to uaSMCs; *p<0.05 compared to uaSMCs; n = 3]. (B) MPCs were immunostained for antibodies against membrane-bound proteins CD90, NG2, and PDGFR-β, and cytoplasmic protein α-SMA. [Blue: DAPI for nuclear staining; Green: Alexa Fluorochrome 488; scale bar represents 100µm] [α-SMA=alpha-smooth muscle actin; MHC=myosin heavy chain; NG2=neuron/glial type 2 antigen; PDGFR-β=platelet-derived growth factor receptor-beta].

2.3.3 High glucose levels do not alter abEPC growth or migration

To assess the effect of glucose exposure on abEPC growth, we cultured abEPCs for a period of 12 days in EBM-2/20% FBS, supplemented with either 5 mmol/L glucose (control) or 25 mmol/L glucose (HG). We chose to culture the cells in normal serum levels because this setting is reminiscent of the *in vivo* conditions and would allow us to study the effect of chronically elevated glucose levels without the confounding toxicity associated with serum-free media. Our results show that the growth of abEPCs, and even HDMECs, is not affected by high levels of glucose (figure 2.4A). An increase in total cell number was noted at day 6 in the abEPCs. However, this increase was normalized (relative to control media containing normal glucose levels) by day 12.

We then plated a high density of cells and assessed the cell capacity for survival in depleted media (1% FBS), and how the addition of high glucose might impede this process. Addition of high glucose in 1% serum media reduced the cell number at 24 hours but had no effect at day 4 (data not shown). To confirm that the reduction in cell number was due to apoptosis, we measured caspase-3 activity in the abEPCs and HDMECs. Our results show a slight but significant increase in caspase-3 activity in abEPCs (1.4x increase as compared to control media without high glucose) (figure 2.4B). Interestingly, the same conditions led to a 2.3x increase in caspase-3 activity in the mature HDMECs (figure 2.4B). As the cells were plated under identical conditions (media, cell density, plate coating), these data suggest that abEPCs are more resistant to glucose-induced toxicity as compared to the mature HDMECs.

Next, we tested whether high glucose causes changes in bFGF-induced migration of abEPCs. We treated abEPCs and HDMECs to normal glucose or high glucose levels in EBM-2/1% FBS. The cells were seeded on transwell inserts and the lower chamber contained 10 ng/mL bFGF. Our results show no significant alteration of these cellular processes in both EC types in the presence or absence of high levels of glucose (figure 2.4C).
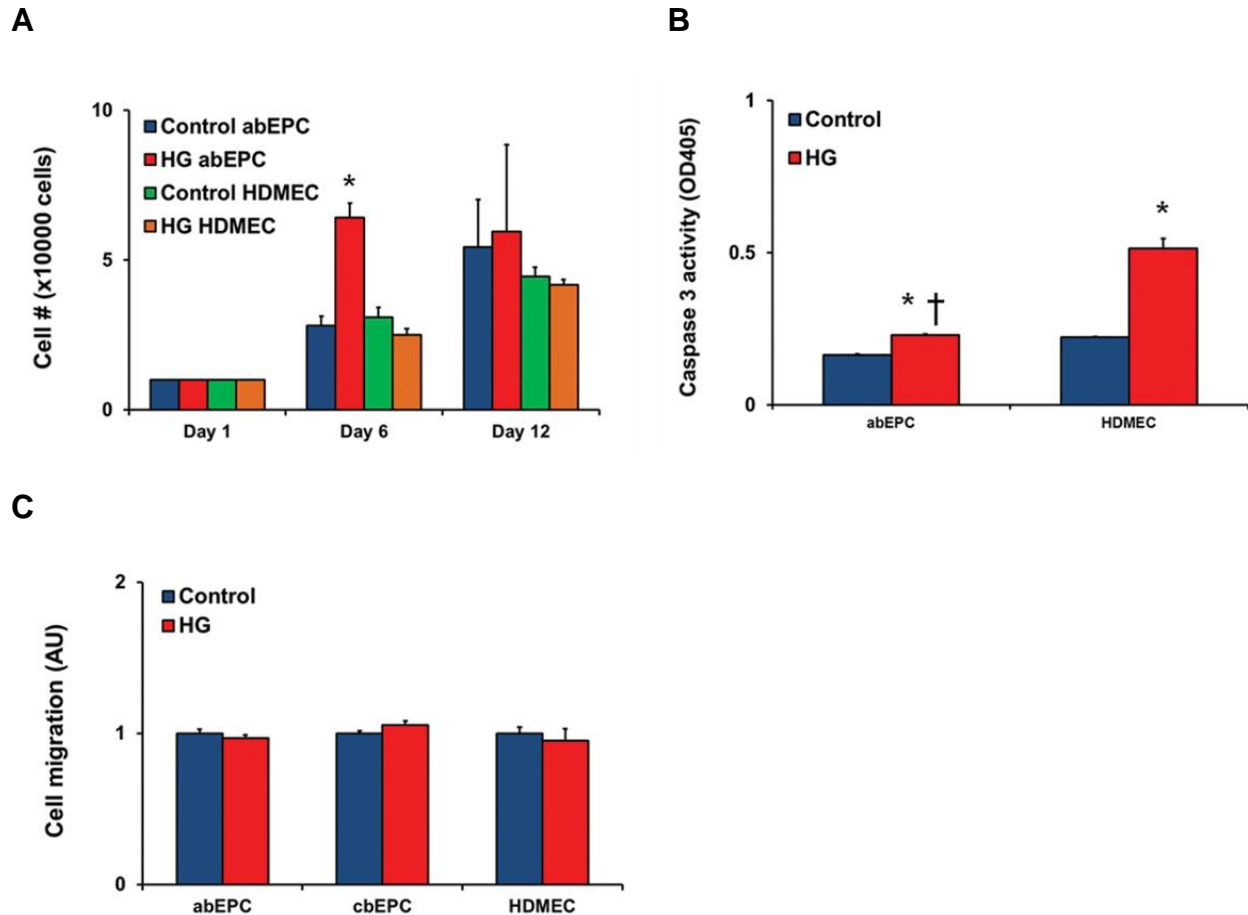
Figure 2.4: EPC functional assays. Cells were cultured in control or HG-treated media. (A) Growth of abEPCs was assessed as cell viability over a 12 day period in high serum media (EBM2/20% FBS). A spike in activity occurred in the hyperglycemic group at day 6 (*$p < 0.05$ compared to cells in control media). However, abEPCs showed no significant differences between control and HG-treated groups over long-term culture. (B) Caspase-3 activity level was measured in abEPCs and HDMECs exposed to control or HG media (in EBM-2/1% FBS) for 24 hours [*$p < 0.05$ compared to respective control media; †$p < 0.05$ compared to HDMECs in HG media; n=3]. (C) A 24-hour migration assay was performed on abEPCs, assessed as their ability to migrate through an 8-µm pore with bFGF as the chemoattractant. HG had no effect on the migratory abilities of abEPCs, cbEPCs, and HDMECs.

2.3.4 HG significantly alters the growth and migration of MPCs

bmMPCs were also subjected to specific cellular activity assays in order to test their functional characteristics in a high glucose setting. Similar to the abEPC experiments, long-term growth of bmMPCs was measured over the course of 12 days. Hyperglycemic bmMPCs showed a significant decrease in cell number on day 1 in the presence of high glucose (figure 2.5A). However, with long-term culture in high glucose media the growth was seemingly unaffected, as cell number had normalized to control cells by day 4. Similarly, no significant changes were observed in serum-depleted media (data not shown). We then measured bmMPC migration using a potent mesenchymal chemoattractant, FBS. Our results show that cell migration of bmMPCs was reduced by nearly 50% in the high glucose group after 24 hours (figure 2.5B).

A
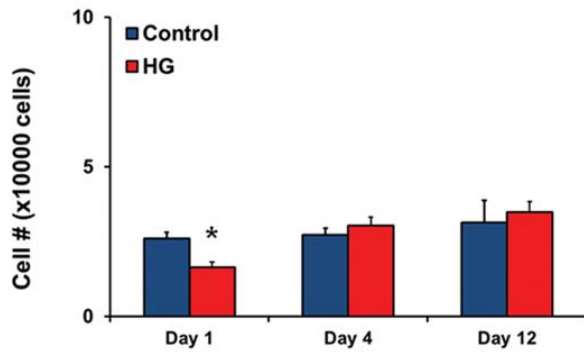
B
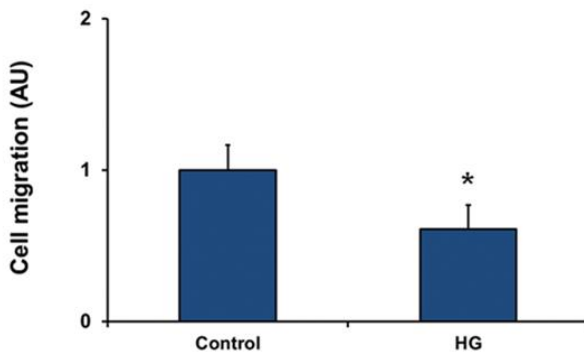
Figure 2.5: MPC functional assays. (A) Growth of bmMPCs was assessed as cell viability over a 12 day period in high serum media (DMEM/20% FBS). HG-bmMPCs showed significantly less growth at day 1 as compared to control (*$p < 0.05$ compared to cells in control media). However, growth over long-term culture appeared to be unaffected by HG. (B) Migration of bmMPCs was assessed in the presence of 10% FBS as chemoattractant. Exposure of bmMPCs to HG significantly reduced the migratory ability towards FBS (*$p < 0.05$ compared to cells in control media).

2.3.5 Effect of glucose on cellular activation, matrix protein expression, and redox-sensitive enzymes

We have previously shown altered cellular activity of ECs in high glucose conditions and in target organs of diabetic complications[13-15]. These altered activities include changes in the redox enzymes, expansion of extracellular matrix, and vasoactive factor alteration. We wanted to determine whether long-term culture of vascular progenitor cells would lead to similar alterations. Therefore, we cultured the cells in complete media (EBM-2/20% FBS with or without 25 mmol/L glucose) and performed a gene expression analyses. We profiled key cell activation genes (endothelin-1, ET-1; E-selectin; and intercellular adhesion molecule-1, ICAM-1), matrix protein genes (collagens 1,3,4; and fibronectin), and oxidative stress genes (catalase, Cat; glutathione peroxidase, GPx; heme oxygenase-1, HO-1; NADPH oxidase, p22 Phox; inducible nitric oxide synthase, NOS2; and superoxide dismutase-1, SOD-1). Interestingly, no significant effect of high levels of glucose was noted in endothelial cell activation, oxidative stress parameters, and matrix proteins at day 1, day 3, or day 14 (figure 2.6). Analysis of bmMPCs also showed no changes in oxidative stress markers (figure 2.7A). Significantly increased levels of matrix proteins (collagen 3, collagen 4, and fibronectin) in high glucose-treated uaSMCs were seen (figure 2.7B). These same matrix protein mRNA levels were significantly reduced in the bmMPCs.

A
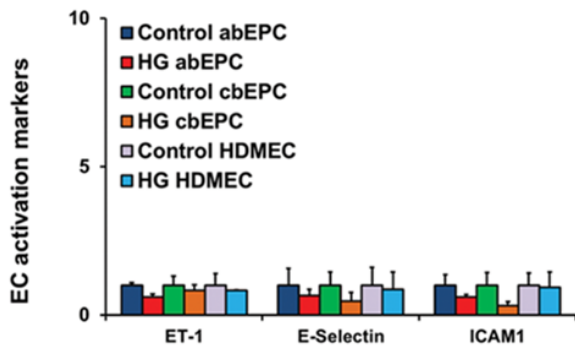
B
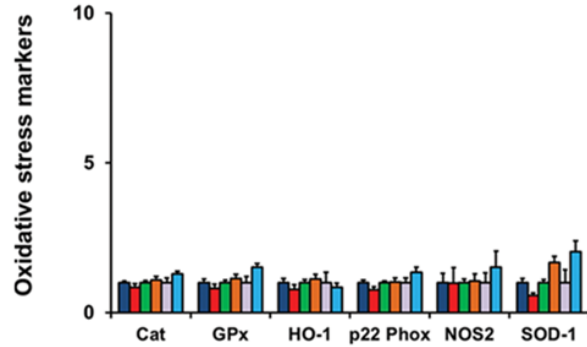
C
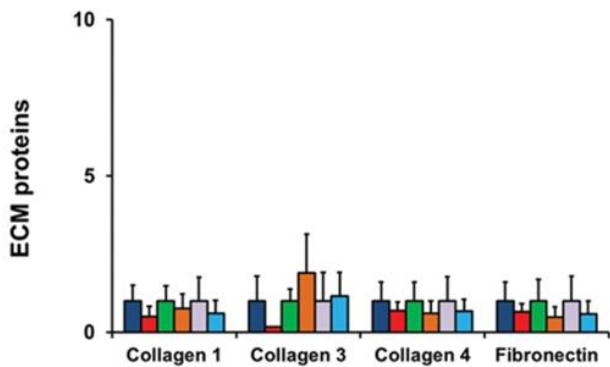
Figure 2.6: Gene expression profiles of abEPCs. Cells were cultured in 5 mmol/L (control) or 25 mmol/L (high glucose; HG) glucose for up to 14 days in high serum media (EBM2/20% FBS). RNA was isolated at day 1, day 3 (data not shown), and day 14 (data not shown) in order to assess changes in gene expression through qRT-PCR. Contrary to what we expected, HG-treated abEPCs, cbEPCs, and HDMECs showed no changes in endothelial cell activation genes (A), redox enzymes (B), or matrix protein genes (C) [ET-1=endothelin-1; E-selectin=endothelial selectin; ICAM1=intercellular adhesion molecule-1; Cat=catalase; GPx=glutathione peroxidase-1; HO-1=heme oxygenase-1; p22 Phox=NADPH oxidase; NOS2=inducible NOS; SOD-1=superoxide dismutase-1].

A
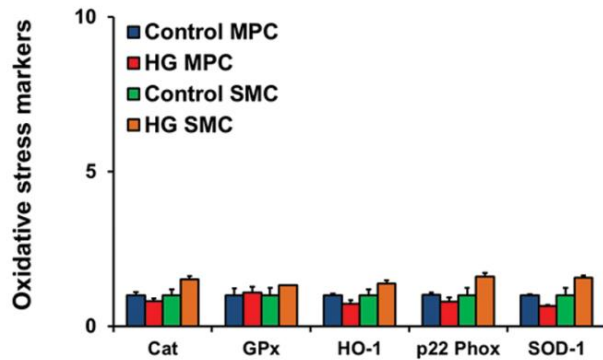
B
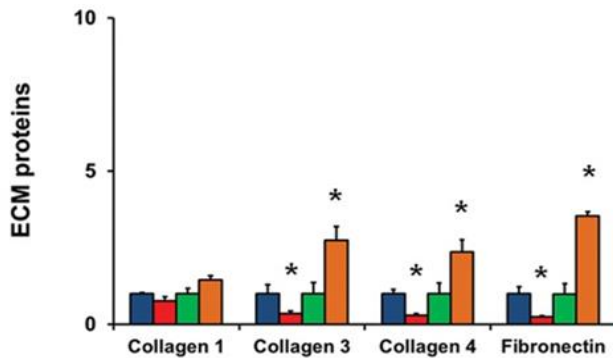
Figure 2.7: Gene expression profiles of MPCs. Cells were cultured in control (5 mmol/L) or HG-treated (25 mmol/L) media for up to 14 days. RNA was isolated at day 1, day 3 (data not shown), and day 14 (data not shown) in order to assess changes in gene expression through qRT-PCR. (A) Oxidative stress marker expression in bmMPCs and uaSMCs showed no significant changes upon exposure to high levels of glucose. (B) HG induced an increase in the production of matrix proteins in mature uaSMCs, but caused downregulation of these same markers in the bmMPCs [*p<0.05 compared to control media; n = 3] [Cat=catalase; GPx=glutathione peroxidase-1; HO-1=heme oxygenase-1; p22 Phox=NADPH oxidase; NOS2=inducible NOS; SOD-1=superoxide dismutase-1].

2.3.6 High glucose enhances adipogenesis of MPCs

We performed mesenchymal differentiation assays on the bmMPCs to assess their ability to differentiate into adipocytes, chondocytes, and osteoblasts with the addition of high glucose. The cells were pre-treated with high levels of glucose for 7 days prior to culture in the differentiation media. qRT-PCR was used to examine the expression of specific transcription factors involved in the differentiation process (adipogenesis was assessed by C/EBPα and PPARγ2; osteogenesis by Runx2 and osterix/SP7; chondrogenesis by Sox9, Nkx3.2, and Runx2). The differentiation scheme for adipogenesis is outlined in figure 2.8A. High glucose drastically increased the differentiation of bmMPCs into adipocytes, as assessed by C/EBPα and PPARγ2 induction at day 7 (figure 2.8B and 2.8C). At day 14, PPARγ2 levels in cells exposed to high glucose were similar to cells in the normal glucose media (figure 2.8D); however, C/EBPα levels remained significantly higher (figure 2.8E).

Figure 2.8: Differentiation of MPCs into adipocytes. bmMPCs were cultured in 5 mmol/L (control glucose) or 25 mmol/L (high glucose; HG) glucose for 7 days prior to differentiation and assessed for induction of PPARγ2 and C/EBPα (A). HG-treated bmMPCs increased adipogenesis at day 7, as demonstrated by upregulated PPARγ2 levels (B) and C/EBPα (C). Analysis of cells at day 14 showed increased PPARγ2 upon differentiation but no differences between control- and HG-treated cells (D). C/EBPα levels (E), on the other hand, were significantly higher in HG-treated cells at day 14 as compared to control glucose treated cells [*p<0.05 compared to control media; †p<0.05 compared to cells treated with control glucose + differentiation media] [c/EBP = CCAAT/enhancer binding protein; PPARγ2 = peroxisome proliferator-activated receptor γ2].

2.3.7 High glucose suppresses differentiation of MPCs to osteoblasts

Next, we assayed for osteogenic differentiation by measuring levels of osteogenic transcription factors Runx2 and osterix/SP7 (figure 2.9A). Analysis of cells exposed to the differentiation media at day 14 showed that high glucose prevented Runx2 induction (figure 2.9B) and significantly reduced osterix/SP7 induction (figure 2.9C).
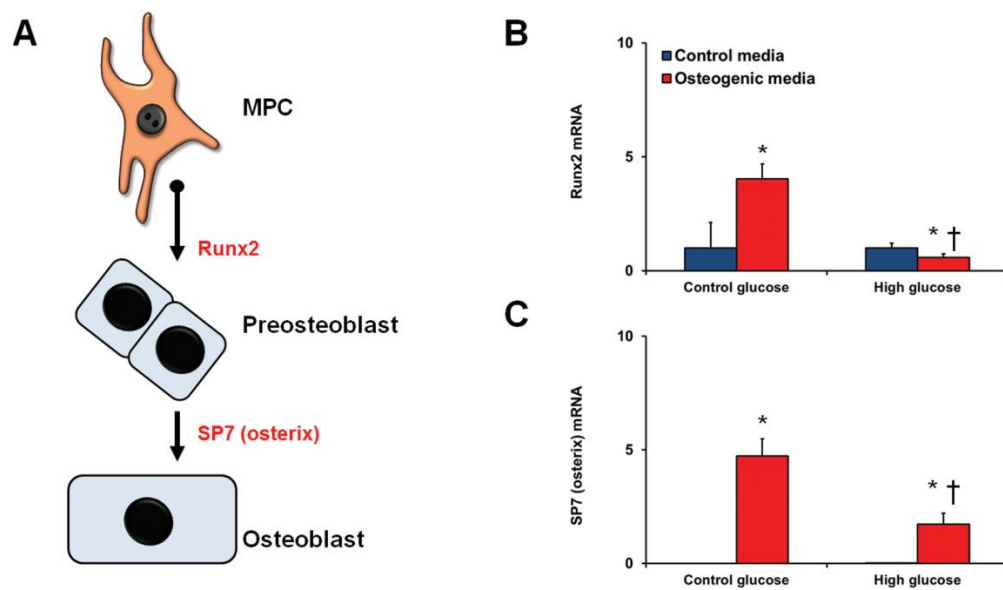
Figure 2.9: Differentiation of MPCs into osteoblasts. bmMPCs were cultured in 5 mmol/L (control glucose) or 25 mmol/L (high glucose; HG) glucose for 7 days prior to differentiation and assessed for induction of Runx2 and SP7 (A). (B) 14-day differentiation of bmMPCs into osteocytes showed significantly depressed expression of Runx2 and SP7 in HG treated cells [*p<0.05 compared to control media; †p<0.05 compared to cells treated with control glucose + differentiation media] [Runx2 = runt-related transcription factor 2; SP7 = osterix].

2.3.8 High glucose suppresses differentiation of MPCs to chondrocytes

Lastly, we determined whether glucose regulates chondrogenesis in bmMPCs. Sox9 mRNA levels were found to be elevated in control cells exposed to the chondrogenic media at day 7, which was not observed in cells exposed to high levels of glucose (data not shown). We then measured these transcription factors at day 14. Contrary to what we expected, we found that cells exposed to differentiation media alone (i.e. normal glucose levels), significantly downregulated early chondrogenesis genes Sox9 and Nkx3.2 (figure 2.10B, C). At this time point, cells exposed to high levels of glucose exhibited significantly higher levels of both Sox9 and Nkx3.2 (figure 2.10B, C). Recently, it has been shown that unlike the adipogenesis-specific and osteogenesis-specific transcription factors (determined above), Sox9 plays an essential stage-specific role in chondrogenesis[11]. To test whether high glucose may be delaying these differentiation steps, we measured Runx2 (late marker of chondrogenesis) in our assay. Our results show that bmMPCs induce Runx2 at day 14, which coincides with repressed Sox9 and Nkx3.2 (figure 2.10D). In contrast, cells exposed to high glucose showed significantly lower Runx2 levels (figure 2.10D).
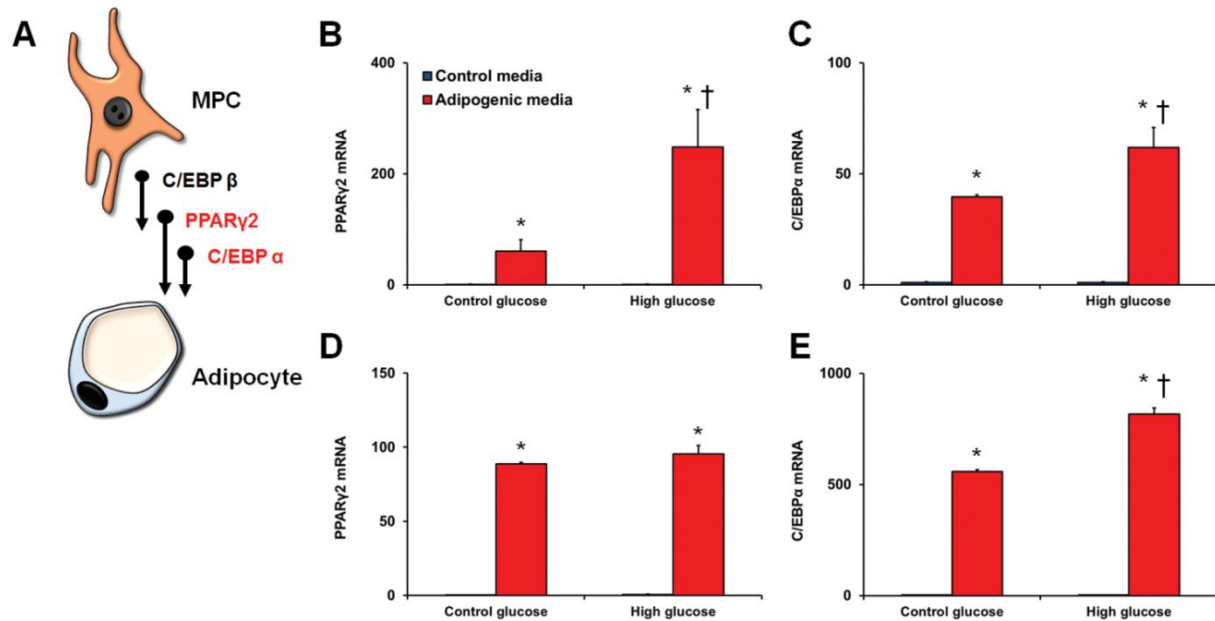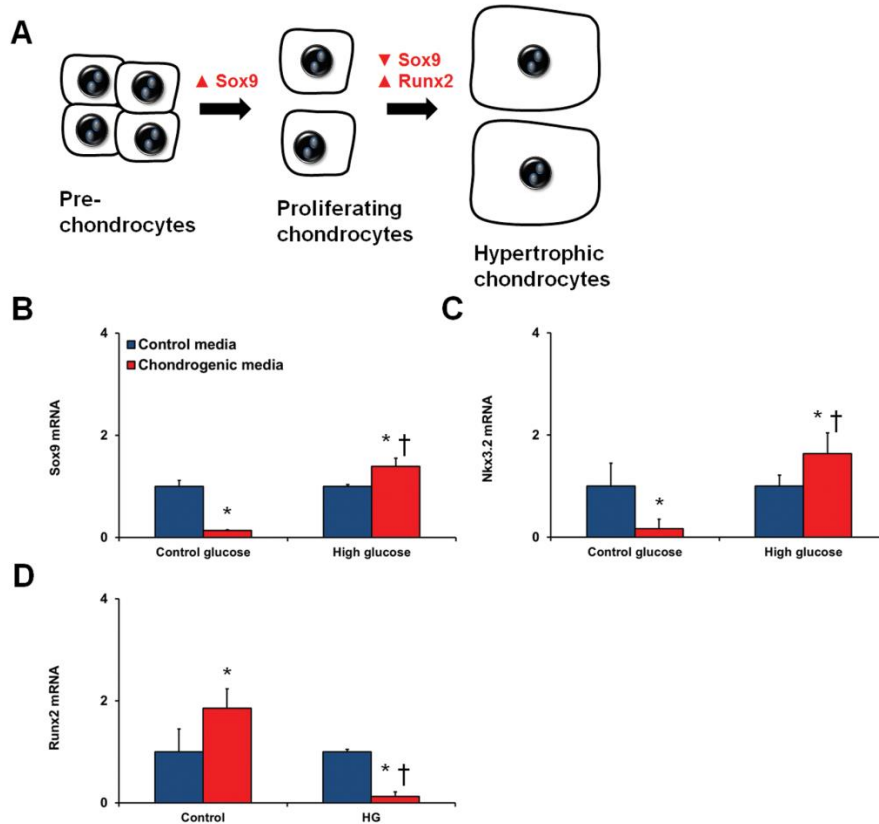
Figure 2.10: Differentiation of MPCs into chondrocytes. bmMPCs, pretreated with 5 mmol/L (control glucose) or 25 mmol/L (high glucose; HG) glucose for 7 days, were cultured in differentiation media and assessed for induction of chondrogenesis transcription factors (A). HG-treated bmMPCs increased expression of Sox9 (B) and Nkx3.2 (C) upon culturing in differentiation media. At this point, cells cultured in control glucose levels showed repressed levels of both Sox9 and Nkx3.2. [*$p<0.05$ compared to control media; †$p<0.05$ compared to cells treated with control glucose + differentiation media]. (D) Runx2 mRNA levels were induced after 14-day differentiation of bmMPCs. Cells treated with HG showed significantly reduced Runx2 expression [*$p<0.05$ compared to control media; †$p<0.05$ compared to cells treated with control glucose + differentiation media] [Sox9 = sex determining region Y-box 9; Runx2 = runt-related transcription factor 2].

2.4   Discussion

These studies demonstrate the differential response of vascular progenitor cell populations and mature cells to high levels of glucose. The salient findings of our study are that: 1) high levels of glucose do not alter derivation of *bona fide* EPCs and MPCs, 2) there is no significant cellular dysfunction in the abEPCs, unlike mature ECs, and 3) MPCs undergo significant cellular changes in high glucose conditions displaying altered differentiation potential.

It has been well established that high glucose causes many biochemical alterations in vascular ECs[16], resulting in impaired function[13,16,17]. These changes are evident when ECs are cultured in serum-free media containing high levels of glucose. Our studies do show increased caspase-3 activity (molecular correlate of apoptosis) in HDMECs exposed to high levels of glucose. Under identical conditions, caspase-3 activity level was significantly different in abEPCs as compared to HDMECs. Furthermore, this toxic effect was found to be acute (evident within 24 hours). Long-term culture of abEPCs in high levels of glucose did not alter cell activities. It should be noted that long-term assessment of cellular activity necessitated the use of serum in the media, which may mask the toxic effect of glucose. However, these conditions are consistent with early-stage changes in diabetes. Two studies have demonstrated a lower circulating number of EPCs (CD34+/VEGFR2+/CD31+) in both type 1 and type 2 diabetic patients[18,19]. Further, the number of CD34+/VEGFR2+ cells has been shown to correlate with glycemic control, and negatively associate with arterial stiffness in diabetic patients[20]. This may be because of the acute effect of hyperglycemia as the EPCs are mobilized from the bone marrow. Although compelling, these recent findings

also potentially take into account a reduction in hematopoietic stem/progenitor cells that share some of these same surface markers. A recent study has demonstrated impaired mobilization of EPCs from the bone marrow in diabetic animals[21]. They have also reported dysfunctional bone marrow stroma to be partly responsible for this alteration[21]. A second group has indicated that the bone marrow microenvironment of type 1 diabetic mice can lead to an increase in inflammatory monocytes alongside a decrease in EPCs, further exacerbating vascular dysfunctions in these animals[22]. Taken together, these studies point to the bone marrow as a potential junction of hyperglycemia-induced damage to stem cells, and may provide an explanation for the decreased number of circulating progenitors. An interesting future direction would be to pre-treat abEPCs with high glucose in culture prior to administering in diabetic animals to assess whether the toxic effects of hyperglycemia are evident in the surviving cells.

Unexpectedly, we found that the progenitor population most affected by high glucose is the mesenchymal cell type. When we cultured bone marrow-derived MPCs in high glucose media, we noted a significant reduction in cell numbers at day 1, despite normal serum levels. However, this effect was normalized upon continued exposure. This is a novel finding, as not much research to date has linked diabetes with changes in cells of the mesenchymal lineage. A recent study, however, has indicated that advanced glycation end products may be responsible for an increase in reactive oxygen species and subsequent decrease in proliferation and migration of bone marrow-derived MPCs[23].

MPCs exhibit remarkable plasticity, with the ability to differentiate both *in vitro* and *in vivo* into a number of mesenchymal phenotypes including those that form bone,

cartilage, muscle, fat, and other connective tissues[24-26]. In addition to affecting bmMPC

growth and migration, high glucose caused a very prominent change in their

differentiation potential. MPCs in high glucose exhibited enhanced adipogenesis

(assessed by PPARγ2 and C/EBPα induction levels) when compared to control cells,

while their ability to differentiate into alternate lineages (chondrocytes, osteoblasts) was

impaired. Much research to date has implicated the Wnt/β-catenin signaling pathway as

a major regulator of this process[27,28]. Down-regulation of this pathway increases the

maturation of pre-adipocytes, whereas the use of specific Wnt proteins *in vitro* has been

shown to inhibit differentiation to adipocytes[29]. Whether hyperglycemia directly alters the

Wnt pathway, leading to skewed differentiation into adipocytes, remains to be

determined.

These studies demonstrate the differing response of mature cells and progenitor

cells to high levels of glucose. We have confirmed that high levels of glucose have a

toxic effect on mature ECs, inducing significant increases in apoptosis- a notion that has

been well-established in diabetes research. Conversely, our studies indicate that both

EPCs and MPCs may be useful therapeutic agents in diabetes. Although the cellular

activity (growth and migration) of MPCs was disrupted initially, high glucose had little

effect on both progenitor cell populations over the long-term. Therefore, increasing the

number of vascular stem/progenitor cells and negating the initial toxic effect of

hyperglycemia in diabetic patients may prove to be an effective means of restoring

vascular homeostasis in diabetes. However, before introducing MPCs as a cell therapy

for diabetic patients, it would be useful to uncover the mechanism behind the skewing in

differentiation potential that is seen in high glucose. Finding a way to block the

conversion of MPCs to adipocytes would be necessary in order to negate the possibility

that MPCs may differentiate towards this lineage before performing their role in

repairing vascular dysfunctions.

2.5 References

1   Yamashita, J. *et al.* Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature* **408**, 92-96, doi:10.1038/35040568 (2000).

2   Ferreira, L. S. *et al.* Vascular progenitor cells isolated from human embryonic stem cells give rise to endothelial and smooth muscle like cells and form vascular networks in vivo. *Circ Res* **101**, 286-294, doi:CIRCRESAHA.107.150201 [pii]10.1161/CIRCRESAHA.107.150201 (2007).

3   Melero-Martin, J. M. *et al.* Engineering robust and functional vascular networks in vivo with human adult and cord blood-derived progenitor cells. *Circ Res* **103**, 194-202, doi:10.1161/CIRCRESAHA.108.178590CIRCRESAHA.108.178590 [pii] (2008).

4   Tepper, O. M. *et al.* Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation* **106**, 2781-2786 (2002).

5   Lopez-Holgado, N. *et al.* Short-term endothelial progenitor cell colonies are composed of monocytes and do not acquire endothelial markers. *Cytotherapy* **9**, 14-22, doi:769972714 [pii]10.1080/14653240601047726 (2007).

6   Capla, J. M. *et al.* Diabetes impairs endothelial progenitor cell-mediated blood vessel formation in response to hypoxia. *Plast Reconstr Surg* **119**, 59-70, doi:10.1097/01.prs.0000244830.16906.3f00006534-200701000-00010 [pii] (2007).

7   Kusuyama, T. *et al.* Effects of treatment for diabetes mellitus on circulating vascular progenitor cells. *J Pharmacol Sci* **102**, 96-102, doi:JST.JSTAGE/jphs/FP0060256 [pii] (2006).

8   Jackson, C. J., Garbett, P. K., Nissen, B. & Schrieber, L. Binding of human endothelium to Ulex europaeus I-coated Dynabeads: application to the isolation of microvascular endothelium. *J Cell Sci* **96 (Pt 2)**, 257-262 (1990).

9   Goldstein, J. L., Ho, Y. K., Basu, S. K. & Brown, M. S. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci U S A* **76**, 333-337 (1979).

10   Khan, Z. A. *et al.* Endothelial progenitor cells from infantile hemangioma and umbilical cord blood display unique cellular responses to endostatin. *Blood* **108**, 915-921, doi:108/3/915 [pii]10.1182/blood-2006-03-006478 (2006).

11   Leung, V. Y. *et al.* SOX9 governs differentiation stage-specific gene expression in growth plate chondrocytes via direct concomitant transactivation and repression. *PLoS Genet* **7**, e1002356, doi:10.1371/journal.pgen.100235610-PLGE-RA-3448 [pii] (2011).

12   MacArthur, B. D., Please, C. P. & Oreffo, R. O. Stochasticity and the molecular mechanisms of induced pluripotency. *PLoS One* **3**, e3086, doi:10.1371/journal.pone.0003086 (2008).

13   Khan, Z. A. *et al.* Endothelins: regulators of extracellular matrix protein production in diabetes. *Exp Biol Med (Maywood)* **231**, 1022-1029, doi:231/6/1022 [pii] (2006).

14   Khan, Z. A., Cukiernik, M., Gonder, J. R. & Chakrabarti, S. Oncofetal fibronectin in diabetic retinopathy. *Invest Ophthalmol Vis Sci* **45**, 287-295 (2004).

15   Khan, Z. A. & Chakrabarti, S. Endothelins in chronic diabetic complications. *Can J Physiol Pharmacol* **81**, 622-634, doi:10.1139/y03-053y03-053 [pii] (2003).

16   Khan, Z. A. & Chakrabarti, S. Therapeutic targeting of endothelial dysfunction in chronic diabetic complications. *Recent Pat Cardiovasc Drug Discov* **1**, 167-175 (2006).

17   Cagliero, E., Maiello, M., Boeri, D., Roy, S. & Lorenzi, M. Increased expression of basement membrane components in human endothelial cells cultured in high glucose. *J Clin Invest* **82**, 735-738, doi:10.1172/JCI113655 (1988).

18   Fadini, G. P. *et al.* Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. *J Am Coll Cardiol* **45**, 1449-1457, doi:S0735-1097(05)00356-6 [pii]10.1016/j.jacc.2004.11.067 (2005).

19   Loomans, C. J. *et al.* Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes. *Diabetes* **53**, 195-199 (2004).

20   Yue, W. S. *et al.* Impact of glycemic control on circulating endothelial progenitor cells and arterial stiffness in patients with type 2 diabetes mellitus. *Cardiovasc Diabetol* **10**, 113, doi:10.1186/1475-2840-10-1131475-2840-10-113 [pii] (2011).

21   Westerweel, P. E. *et al.* Impaired endothelial progenitor cell mobilization and dysfunctional bone marrow stroma in diabetes mellitus. *PLoS One* **8**, e60357, doi:10.1371/journal.pone.0060357PONE-D-12-31448 [pii] (2013).

22   Hazra, S. *et al.* Long-term type 1 diabetes influences haematopoietic stem cells by reducing vascular repair potential and increasing inflammatory monocyte generation in a murine model. *Diabetologia* **56**, 644-653, doi:10.1007/s00125-012-2781-0 (2013).

23   Yang, K. *et al.* Advanced glycation end products induce chemokine/cytokine production via activation of p38 pathway and inhibit proliferation and migration of bone marrow mesenchymal stem cells. *Cardiovasc Diabetol* **9**, 66, doi:10.1186/1475-2840-9-661475-2840-9-66 [pii] (2010).

24   Caplan, A. I. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J Cell Physiol* **213**, 341-347, doi:10.1002/jcp.21200 (2007).

25   Caplan, A. I. Mesenchymal stem cells. *J Orthop Res* **9**, 641-650, doi:10.1002/jor.1100090504 (1991).

26   Prockop, D. J. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* **276**, 71-74 (1997).

27   Chung, S. S. *et al.* Regulation of Wnt/beta-catenin signaling by CCAAT/enhancer binding protein beta during adipogenesis. *Obesity (Silver Spring)* **20**, 482-487, doi:10.1038/oby.2011.212oby2011212 [pii] (2012).

28   Muruganandan, S., Roman, A. A. & Sinal, C. J. Adipocyte differentiation of bone marrow-derived mesenchymal stem cells: cross talk with the osteoblastogenic program. *Cell Mol Life Sci* **66**, 236-253, doi:10.1007/s00018-008-8429-z (2009).

29   Rosen, E. D. & MacDougald, O. A. Adipocyte differentiation from the inside out. *Nat Rev Mol Cell Biol* **7**, 885-896, doi:nrm2066 [pii]10.1038/nrm2066 (2006).

CHAPTER 3 – POTENTIAL MECHANISMS REGULATING ADIPOGENESIS IN DIABETES

In the previous chapter, I investigated the effects of high glucose on endothelial and mesenchymal progenitor cell populations. While there is evidence to support a therapeutic role for EPCs in diabetes, MPCs demonstrated some dysfunction in a high glucose setting. Importantly, the differentiation potential of MPCs was skewed towards the adipocyte lineage, while their ability to become both osteoblasts and chondrocytes was reduced[1].

In chapter 3, I will focus on the potential mechanism behind this high glucose-induced alteration, setting the stage for my experimental studies in chapter 4.

3.1   MPC lineage commitment

Lineage commitment of MPCs is a tightly controlled process that is governed by a variety of endogenous and extracellular signals. Along with changes in the cytoarchitecture of the cell, transcription factors, epigenomic modifiers and various signaling cascades are activated and de-activated at the appropriate stage for successful differentiation to a specified lineage.

Adipogenesis is divided into two phases known as commitment and terminal differentiation. In the commitment phase, MPCs differentiate into preadipocytes – cells that no longer have ability to become alternate mesenchymal lineages. Preadipocytes, however, will not undergo spontaneous differentiation to mature cells without the appropriate exogenous stimuli. A number of factors are responsible for initiating commitment of MPCs. Transforming growth factor-beta (TGFβ) superfamily ligands are secreted morphogens that are critical for MPC lineage decisions, though their exact role in adipogenesis remains unclear. TGFβ expression is positively associated with obesity in both human and animal models[2], but inhibits adipogenesis of 3T3-F442A cells *in vitro* through Smad signalling[3]. Paradoxically, Smad3-null mice are resistant to diet-induced obesity[4], highlighting the discrepancy that is often noted when comparing *in vitro* and *in vivo* studies of TGFβ and adipogenesis. Other members of the TGFβ superfamily, bone morphogenic proteins (BMPs), have been shown to stimulate adipogenesis through the activation of both Smads and the p38 kinase pathway[5,6]. In addition to these signalling pathways, studies have shown that the composition and stiffness of the ECM is able to influence cell fate decisions. Human MPCs are less likely to differentiate into adipocytes

when grown on stiff matrices that have a high concentration of collagen I[7,8]. ECM stiffness regulates tissue tension, which can lead to cell stretching through actin and myosin fibre formation. Cell spreading favors osteogenesis, while roundness will stimulate adipogenesis[9,10]. Changes in cell shape are mediated by RHO GTPase-RHO-associated kinase (ROCK) signalling[11]. RHO-GDP, the inactive form, is predominant in rounded MPCs and promotes adipogenesis, whereas RHO-GTP in stretched cells activates ROCK and generates actinomyosin fibre formation[11,12].

In the terminal differentiation phase, preadipocytes will take on all the characteristics of fully functional adipocytes. This requires activating hormone responsiveness and acquiring the machinery necessary for lipid synthesis and transport. The initial phase of adipogenesis is quite poorly characterized, but terminal differentiation has been shown to consist of various well-known transcriptional cascades. Initially, c/EBPβ and c/EBPδ are transiently expressed for the purpose of directly inducing C/EBPα and PPARγ, the two main transcriptional regulators of adipogenesis. Not much is known about c/EBPδ regulation, however, c/EBPβ expression is increased by cAMP agonists that stimulate the transcriptional activator cAMP-responsive element-binding protein (CREB)[13,14]. While mice lacking either c/EBPβ or c/EBPδ protein have only mild developmental defects in adipose tissue[15], it has been shown that terminal differentiation will not take place in the absence of PPARγ[16]. Two isoforms exist – PPARγ1 and PPARγ2, both of which can stimulate adipogenesis. PPARγ2, however, is specific for adipocytes while PPARγ1 is present in other MPC-derived cell types[17,18]. Although an endogenous ligand for this nuclear receptor has not yet been reported, studies in 3T3-L1 cells have revealed cAMP

agonists as positive regulators of PPARγ. Additional *in vitro* work has pointed to

c/EBPs, along with the glucocorticoid receptor, as major regulators of the PPARγ gene

locus upon adipogenic stimuli[19-21]. c/EBPα and PPARγ regulate the expression of many

downstream target genes that are necessary for adipogenesis, as well as feed-back to

induce their own expression, thus maintaining the mature adipocyte phenotype. To be

fully functional, terminal differentiation must also consist of the activation of various

metabolic genes and adipokines, including fatty acid-binding protein 4 (FABP4), glucose

transporter 4 (GLUT4), leptin and adiponectin.

Although osteoblasts and adipocytes are derived from the same parent cell, the

process of osteogenesis is quite varied from its counterpart. TGFβ/BMP signaling is a

major regulator of bone formation during mammalian development, as well as during

adult bone repair[22,23]. Signal transduction through Smad-dependent (TGFβ/BMP

ligands, receptors and Smads) and Smad-independent (i.e. p38 mitogen-activated

protein kinase pathway, MAPK) pathways converge on the master regulator of

osteogenesis, Runx2. Both adipo- and osteogenesis involve a complex cross-talk

between various signaling systems including TGFβ/BMP, Wnt, MAPK, Hedgehog,

Notch, and FGF, though transcriptional regulators, such as PPARγ and Runx2, are

thought to have the final say in lineage commitment.

3.2 Wnt signaling and adipogenesis

Wingless-type MMTV integration site family members (WNTs) are a large family of secreted glycoproteins that play a role in cell fate and development. Wnt signaling was first described in *Drosophila*[24], and has since been shown to be a highly conserved pathway. Wnts are active during embryogenesis, and signal through both autocrine and paracrine mechanisms to regulate adult tissue homeostasis as well. There are 19 known Wnt ligands that bind to transmembrane spanning Frizzled (Fzd) receptors and several co-receptors, including lipoprotein receptor-related protein (Lrp)-5/6[25,26] and receptor tyrosine kinase-like orphan receptor (ROR)[27,28] . This will initiate signaling in either a β-catenin-dependent or –independent manner, also known as canonical and noncanonical signaling, respectively. While the cell type and ligand-receptor combination is what appears to direct cell signaling, it is generally accepted that canonical Wnt ligands include: Wnt2/2b, Wnt3, Wnt7a/b, Wnt8a, Wnt9a/b, and Wnt10a/b[29]. Noncanonical Wnt ligands, then, consist of: Wnt1, Wnt4, Wnt5a/b, Wnt6, Wnt11, and Wnt16[29]. Both pathways are involved in mediating cell proliferation, migration, fate, and behaviour and, as such, have been implicated in regulating the differentiation of multipotent MPCs to specific lineages.

The canonical, or β-catenin-dependent, signaling pathway is contingent on β-catenin stabilization in the cytoplasm[30] (figure 3.1). In the absence of Wnt ligands, β-catenin exists in a degradation complex that consists of Axin, the adenomatous polyposis coli (APC) protein, and glycogen synthase kinase 3β (Gsk3β). Enzymatic
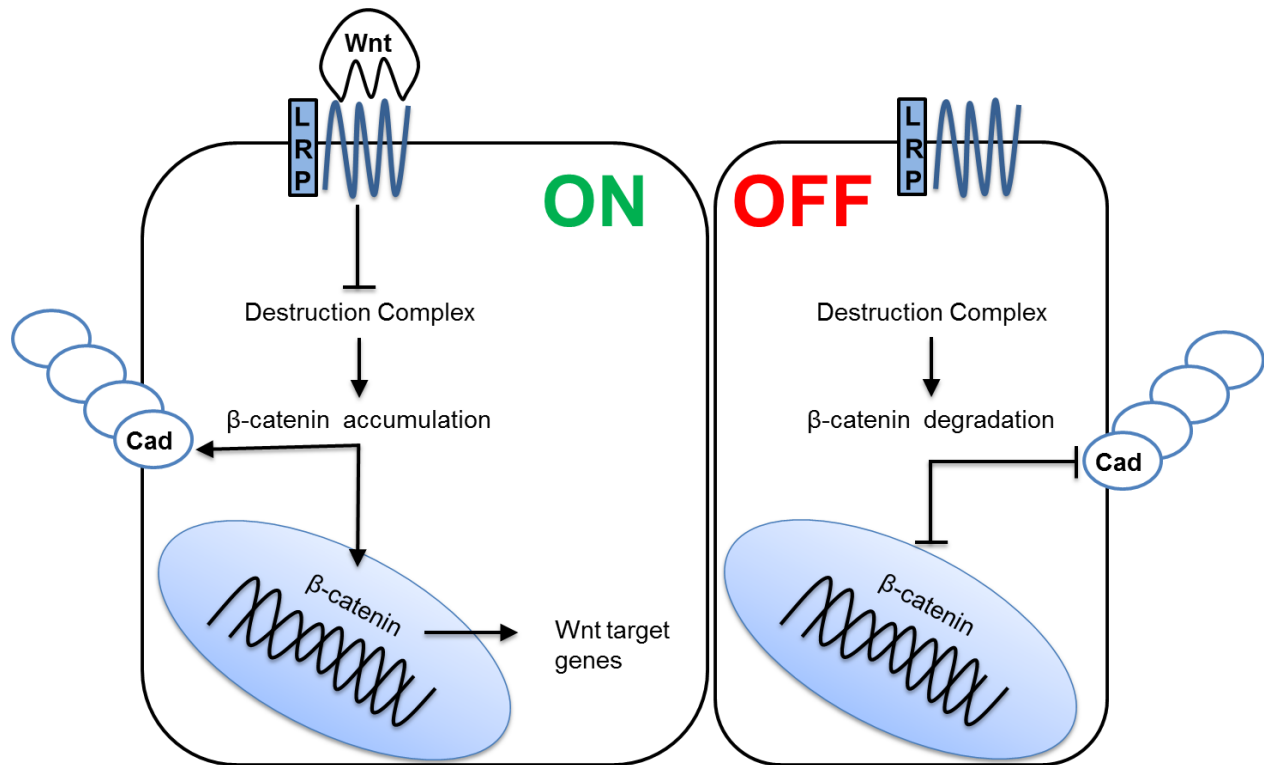
Figure 3.1: Schematic of canonical Wnt/β-catenin signaling. When the pathway is on, a Wnt ligand binds to a Fzd receptor, activating intracellular molecule Disheveled so that the destruction complex is inhibited. β-catenin is free to accumulate and can bind to a cell surface cadherin or can translocate to the nucleus to stimulate the transcription of Wnt target genes. When the pathway is off, the destruction complex member GSK3β phosphorylates β-catenin, marking it for ubiquitination and degradation by the proteasome [Cad=cadherin; LRP=lipoprotein receptor-related protein].

activity by Gsk3β marks β-catenin for ubiquitination, followed by degradation by the proteasome[31]. However, with the binding of Wnt to a receptor complex consisting of a Fzd receptor and Lrp co-receptor, Disheveled (Dsh) is activated and enhances the phosphorylation of Gsk3β, inhibiting its activity. The degradation complex then disassembles and β-catenin is able to accumulate within the intracellular compartment. β-catenin can translocate to the nucleus where it interacts with a complex of transcription factors, including lymphoid-enhancer-binding factor/T-cell-specific transcription factor (LEF/TCF), in order to promote activation of Wnt target genes.

Non-canonical/β-catenin-independent signaling consists of two pathways commonly known as the Wnt/Calcium (Ca^{2+}) pathway (figure 3.2A) and the Wnt/Planar Cell Polarity (PCP) pathway (figure 3.2B). Both are much less well characterized than the β-catenin-mediated pathway. In the Wnt/Ca^{2+} cascade, the binding of Wnt proteins to a Fzd receptor and Ror1/2 co-receptor activates phospholipase-C (PLC) through the action of heterotrimeric G-proteins[32]. PLC can derive inositol 1,4,5-triphosphate (IP3) and 1,2 diacylglycerol (DAG) from the membrane-bound phospholipid phosphatidyl inositol 4,5-bisphosphate. IP3 interacts with calcium channels on the membrane of the endoplasmic reticulum (ER), resulting in the release of calcium ions. These ions, along with calmodulin (which is ubiquitously expressed), activate the Ca^{2+}-sensitive calcium/calmodulin-dependent kinase II (CaMKII)[33]. Ca^{2+}, together with DAG, can also stimulate PKC[34]. Both CaMKII and PKC are responsible for the activation of transcription factors, such as cAMP-responsive element-binding protein (CREB) and nuclear factor-κB (NFκB).
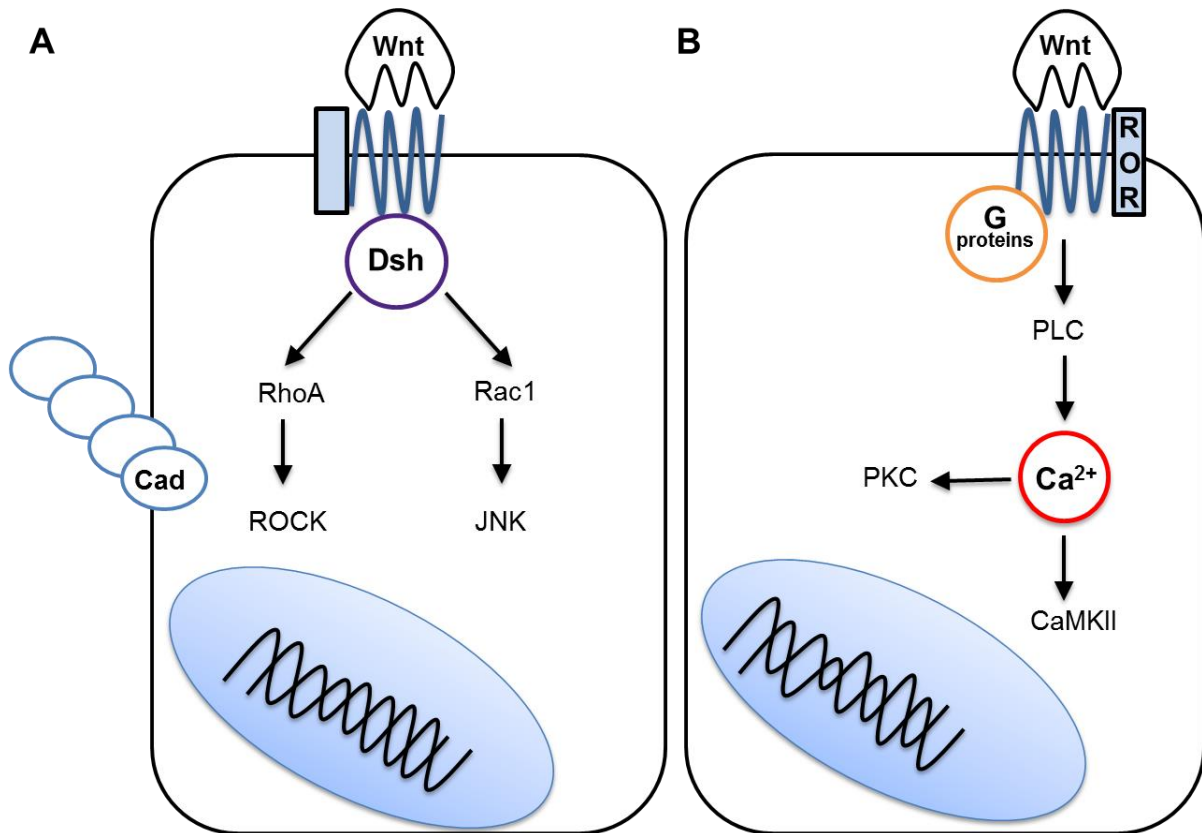
Figure 3.2: Non-canonical/β-catenin independent signaling. (A) The Wnt/PCP pathway consists of a Wnt ligand binding to Fzd receptor to activate Dsh. Dsh can stimulate RhoA, which binds to its downstream effector ROCK to mediate changes to the actin cytoskeleton. Dsh can also stimulate Rac1 which regulates cell survival genes through a series of MAP kinases that terminate on JNK. (B) Wnt/Ca^{2+} mediated signaling relies on the activation of PLC through a Wnt ligand-receptor interaction to increase the production of IP3 and DAG. IP3 increases intracellular calcium concentrations that act in concert with DAG to stimulate PKC, or calmodulin to stimulate CaMKII [Cad=cadherin; Dsh=disheveled; RhoA=Ras homolog gene family, member A; ROCK=RHO-associated kinase; Rac1=Ras-related C3 botulinum toxin substrate 1; JNK=c-Jun N-terminal kinase; Ror=receptor tyrosine kinase-like orphan receptor; PLC=phospholipase C; PKC=protein kinase C; CaMKII=calcium/calmodulin-dependent kinase II].

The PCP pathway was so named for its ability to regulate the polarity of epithelial cells within the plane of the epithelium in *Drosophila*. Wnt/PCP signaling is initiated by specific Wnt ligands binding to Fzd or Ror receptors. Dsh is activated and can directly stimulate the small GTPase, Rac1. The Rac1 branch of the PCP pathway regulates cell survival through a series of MAP kinases that terminate on c-Jun N-terminal kinase (JNK). Upon JNK induction, c-Jun is phosphorylated by the kinase and can form a transcriptional complex with AP-1 and c-fos, resulting in the activation of specific cell survival genes[35]. Dsh can also activate RhoA which, in turn, stimulates downstream effector ROCK. ROCK is responsible for mediating cytoskeletal changes and plays a large role in regulating cell shape and motility.

Some of the first work implicating a role for Wnt signaling in adipogenesis was done by Ross *et al.* in 2000. They were able to maintain preadipoctyes in an undifferentiated state through the use of Wnt10b[36]. They went on to show that Wnt10b exerts its inhibitory actions by blocking the induction of pro-adipogenic transcription factors PPARγ and C/EBPα[36]. To confirm these results, Ross used a dominant negative TCF4 which prevented Wnt signaling and caused enhanced differentiation of preadipocytes[36]. Since, many independent groups have indicated a role for Wnt signaling in the suppression of adipogenesis. Along with Wnt10b, Wnt1[36], Wnt3a[37], Wnt6[38], and Wnt10a[38] have also been deemed negative regulators of this process *in vitro*. Manipulating downstream effectors of the pathway in 3T3-L1 cells appeared to mirror these results. The use of a Gsk3β phosphorylation-defective mutant, and a pharmacological inhibitor of this same protein, were both shown to block adipogenesis[39]. Additionally, mouse embryonic fibroblasts (MEFs) deficient of the LRP6

co-receptor displayed increased adipogenic differentiation[40]. These findings led to the belief that endogenous Wnts act as a switch during adipogenesis; when switched off, differentiation of committed preadipoctyes is able to proceed. Studies have confirmed that Wnt10b signal is highest in preadipocytes, but diminishes upon subsequent differentiation to mature adipocytes[36,41]. Conversely, levels of Dickkopf-1 (Dkk1) and secreted frizzled-related protein-4 (sFRP4), two established inhibitors of canonical Wnt signaling, were shown to increase gradually throughout the process of adipogenesis in human adipose tissue-derived mesenchymal stem cells[42]. *In vivo* studies have allowed a more physiological approach to examining the role of canonical Wnt siganling in adipogenesis. Transgenic mice overexpressing Wnt10b from the adipocyte-specific fatty acid-binding protein-4 (FABP4) promoter have decreased WAT when maintained on a regular diet, and are more resistant to diet-induced obesity[43]. In humans, an inactivating mutation in the Wnt10b gene was identified as a proband for early-onset obesity[44]. Interestingly, this mutation was not detected in over 600 healthy control subjects.

The reciprocal relationship between adipo- and osteogenesis appears to be reflected in the role of canonical Wnt signaling in these two processes. While it is thought that β-catenin must be suppressed for differentiation of preadipocytes to proceed, it is generally accepted that canonical Wnts promote the maturation of osteoblastic precursors into mature osteoblasts[45,46]. The first indication of this inverse association came from the examination of mice expressing Wnt10b from the FABP4 promoter. Alongside having reduced adipose tissue, these mice were unexpectedly found to have increased bone mass[39]. Mice expressing Wnt10b from the osteocalcin promoter in osteoblasts displayed a similar phenotype[47], reinforcing the concept of

canonical Wnt signaling as a positive regulator of osteoblastogenesis and mineralizing

activity. Several other canonical Wnt ligands have since been identified as stimulators of

osteoblast formation *in vitro*, including Wnt6 and Wnt10a[38]. Enhancing β-catenin, either

by the depletion of Wnt antagonists[48] or overexpression of specific Wnt ligands[47], has

been shown to induce bone formation in both mice and humans. It appears that Runx2

is a direct target of beta-catenin/TCF, indicating the mechanism behind these

phenotypic changes[49,50]. Further *in vivo* approaches have demonstrated that osteoblast-

specific deletion of either the β-catenin gene or the APC gene in mice leads to severe

osteopenia with a concurrent increase in osteoclast number[51]. This same group

demonstrated that constitutive activation of β-catenin in the conditional APC-mutant

mice corrected this increase in osteoclast number and produced greater bone

deposition[51]. In a similar study by a different group, stabilization of β-catenin in

osteoblasts led to an increase in bone mass whereas its deletion resulted in a lower

bone mineral density[52]. Taken together, these results indicate a second role for β-

catenin in promoting the inhibition of osteoclast differentiation.

The role of non-canonical signaling in MPC lineage commitment has been

controversial to date. Unlike canonical Wnt10b expression, levels of Wnt5b have been

shown to increase gradually throughout adipogenesis. Adenoviral overexpression of

Wnt5b impaired the nuclear translocation of β-catenin, thus antagonizing the canonical

pathway and enhancing adipogenesis in 3T3-L1 cells[53]. Other groups have reported

non-canonical Wnt signaling to be a negative regulator of adipogenesis. Wnt5a

expression in ST2 cells inhibited this process by directly attenuating PPARγ

transcriptional activity through the activation of histone methyltransferase SET domain

bifurcated 1 (SETDB1)[54,55]. Non-canonical Wnt signaling has proven to be elusive in regards to osteogensis as well. Wnt4 increased bone regeneration of MPCs isolated from human craniofacial tissues both *in vitro* and *in vivo*[56]. MPCs cultured in medium containing Wnt5a-neutralizing antibodies were unable to undergo osteogenesis, indicating a positive role for Wnt5a in this process as well[57]. More recently, Wnt5a was shown to stimulate the osteogenic differentiation of adipose tissue-derived MPCs through changes in the actin cytoskeleton controlled by ROCK[58]. This brings into play the concept of mechanical regulation of cell fate decisions. Arnsdorf et al. demonstrated that non-canonical Wnt5a signaling causes changes in Ror2 and RhoA, coupled with N-cadherin mediated β-catenin signaling alterations, to mechanically induce osteogenic differentiation[59]. Taken together, it appears that MPC lineage commitment may be more complex than initially anticipated, being regulated by both canonical and non-canonical Wnt signaling, as well as the mechanical microenvironment of the cell.

Although we would like to assign discrete roles for the β-catenin-independent and –dependent pathways in MPC lineage commitment and many people have, this is an over-simplified approach to a very complex signaling cascade. Examining a single canonical or non-canonical Wnt ligand may not be a good representation of these signaling systems as a whole. Further, cell lines do not always behave in a similar manner to human-derived multi-potent stem/progenitor cells. As such, many contradictory results have been published concerning the role of Wnt signaling in MPC lineage selection. In fact, Quarto et al. demonstrated opposing roles for Wnt3a in osteogenesis depending on the model system, dosage used, and differentiation state of the recipient cell[60]. This further exemplifies the difficulty of studying Wnt signaling *ex*

vivo and drawing conclusions that convincingly demonstrate a cause and effect scenario. For my remaining studies, I will take an all-encompassing approach to studying Wnt signaling in adipogenesis in order to elucidate the potential mechanism behind the skewing in differentiation potential seen in high glucose.

3.3   References

1   Keats, E. & Khan, Z. A. Unique responses of stem cell-derived vascular

        endothelial and mesenchymal cells to high levels of glucose. *PLoS One* **7**,

        e38752, doi:10.1371/journal.pone.0038752

        PONE-D-12-03648 [pii] (2012).

2   Zamani, N. & Brown, C. W. Emerging roles for the transforming growth factor-

        {beta} superfamily in regulating adiposity and energy expenditure. *Endocr Rev*

        32, 387-403, doi:10.1210/er.2010-0018

        er.2010-0018 [pii] (2011).

3   Choy, L., Skillington, J. & Derynck, R. Roles of autocrine TGF-beta receptor and

        Smad signaling in adipocyte differentiation. *J Cell Biol* **149**, 667-682 (2000).

4   Yadav, H. *et al.* Protection from obesity and diabetes by blockade of TGF-

        beta/Smad3 signaling. *Cell Metab* **14**, 67-79, doi:10.1016/j.cmet.2011.04.013

        S1550-4131(11)00215-4 [pii] (2011).

5   Huang, H. *et al.* BMP signaling pathway is required for commitment of

        C3H10T1/2 pluripotent stem cells to the adipocyte lineage. *Proc Natl Acad Sci U

        S A* **106**, 12670-12675, doi:10.1073/pnas.0906266106

        0906266106 [pii] (2009).

6   Hata, K. *et al.* Differential roles of Smad1 and p38 kinase in regulation of

        peroxisome proliferator-activating receptor gamma during bone morphogenetic

        protein 2-induced adipogenesis. *Mol Biol Cell* **14**, 545-555, doi:10.1091/mbc.E02-

        06-0356 (2003).

7   Winer, J. P., Janmey, P. A., McCormick, M. E. & Funaki, M. Bone marrow-derived human mesenchymal stem cells become quiescent on soft substrates but remain responsive to chemical or mechanical stimuli. *Tissue Eng Part A* **15**, 147-154, doi:10.1089/ten.tea.2007.0388 (2009).

8   Chun, T. H. *et al.* A pericellular collagenase directs the 3-dimensional development of white adipose tissue. *Cell* **125**, 577-591, doi:S0092-8674(06)00447-8 [pii]

        10.1016/j.cell.2006.02.050 (2006).

9   Akimoto, T. *et al.* Mechanical stretch inhibits myoblast-to-adipocyte differentiation through Wnt signaling. *Biochem Biophys Res Commun* **329**, 381-385, doi:S0006-291X(05)00122-1 [pii]

        10.1016/j.bbrc.2005.01.136 (2005).

10   Jakkaraju, S., Zhe, X., Pan, D., Choudhury, R. & Schuger, L. TIPs are tension-responsive proteins involved in myogenic versus adipogenic differentiation. *Dev Cell* **9**, 39-49, doi:S1534-5807(05)00178-4 [pii]

        10.1016/j.devcel.2005.04.015 (2005).

11   McBeath, R., Pirone, D. M., Nelson, C. M., Bhadriraju, K. & Chen, C. S. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev Cell* **6**, 483-495, doi:S1534580704000759 [pii] (2004).

12   Kilian, K. A., Bugarija, B., Lahn, B. T. & Mrksich, M. Geometric cues for directing the differentiation of mesenchymal stem cells. *Proc Natl Acad Sci U S A* **107**, 4872-4877, doi:10.1073/pnas.0903269107

        0903269107 [pii] (2010).

13   Zhang, J. W., Klemm, D. J., Vinson, C. & Lane, M. D. Role of CREB in
      transcriptional regulation of CCAAT/enhancer-binding protein beta gene during
      adipogenesis. *J Biol Chem* **279**, 4471-4478, doi:10.1074/jbc.M311327200
      M311327200 [pii] (2004).

14   Yeh, W. C., Cao, Z., Classon, M. & McKnight, S. L. Cascade regulation of
      terminal adipocyte differentiation by three members of the C/EBP family of
      leucine zipper proteins. *Genes Dev* **9**, 168-181 (1995).

15   Tanaka, T., Yoshida, N., Kishimoto, T. & Akira, S. Defective adipocyte
      differentiation in mice lacking the C/EBPbeta and/or C/EBPdelta gene. *EMBO J*
      16, 7432-7443, doi:10.1093/emboj/16.24.7432 (1997).

16   Rosen, E. D. *et al.* PPAR gamma is required for the differentiation of adipose
      tissue in vivo and in vitro. *Mol Cell* **4**, 611-617, doi:S1097-2765(00)80211-7 [pii]
      (1999).

17   Tontonoz, P., Hu, E., Graves, R. A., Budavari, A. I. & Spiegelman, B. M. mPPAR
      gamma 2: tissue-specific regulator of an adipocyte enhancer. *Genes Dev* **8**,
      1224-1234 (1994).

18   Chawla, A., Schwarz, E. J., Dimaculangan, D. D. & Lazar, M. A. Peroxisome
      proliferator-activated receptor (PPAR) gamma: adipose-predominant expression
      and induction early in adipocyte differentiation. *Endocrinology* **135**, 798-800
      (1994).

19   Mikkelsen, T. S. *et al.* Comparative epigenomic analysis of murine and human
      adipogenesis. *Cell* **143**, 156-169, doi:10.1016/j.cell.2010.09.006
      S0092-8674(10)01016-0 [pii] (2010).

20   Siersbaek, R. *et al.* Extensive chromatin remodelling and establishment of transcription factor 'hotspots' during early adipogenesis. *EMBO J* **30**, 1459-1472, doi:10.1038/emboj.2011.65 emboj201165 [pii] (2011).

21   Steger, D. J. *et al.* Propagation of adipogenic signals through an epigenomic transition state. *Genes Dev* **24**, 1035-1044, doi:10.1101/gad.1907110 24/10/1035 [pii] (2010).

22   Horner, A. *et al.* Expression and distribution of transforming growth factor-beta isoforms and their signaling receptors in growing human bone. *Bone* **23**, 95-102, doi:S8756328298000805 [pii] (1998).

23   Hogan, B. L. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev* **10**, 1580-1594 (1996).

24   Wainwright, B. J. *et al.* Isolation of a human gene with protein sequence similarity to human and murine int-1 and the Drosophila segment polarity mutant wingless. *EMBO J* **7**, 1743-1748 (1988).

25   Pinson, K. I., Brennan, J., Monkley, S., Avery, B. J. & Skarnes, W. C. An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* **407**, 535-538, doi:10.1038/35035124 (2000).

26   Tamai, K. *et al.* LDL-receptor-related proteins in Wnt signal transduction. *Nature* **407**, 530-535, doi:10.1038/35035117 (2000).

27   Mikels, A. J. & Nusse, R. Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. *PLoS Biol* **4**, e115, doi:05-PLBI-RA-1339R3 [pii]

10.1371/journal.pbio.0040115 (2006).

28   Hikasa, H., Shibata, M., Hiratani, I. & Taira, M. The Xenopus receptor tyrosine

        kinase Xror2 modulates morphogenetic movements of the axial mesoderm and

        neuroectoderm via Wnt signaling. *Development* **129**, 5227-5239 (2002).

29   Sugimura, R. *et al.* Noncanonical Wnt signaling maintains hematopoietic stem

        cells in the niche. *Cell* **150**, 351-365, doi:10.1016/j.cell.2012.05.041

        S0092-8674(12)00767-2 [pii] (2012).

30   Cadigan, K. M. & Liu, Y. I. Wnt signaling: complexity at the surface. *J Cell Sci*

        119, 395-402, doi:119/3/395 [pii]

        10.1242/jcs.02826 (2006).

31   Liu, C. *et al.* Control of beta-catenin phosphorylation/degradation by a dual-

        kinase mechanism. *Cell* **108**, 837-847, doi:S0092867402006852 [pii] (2002).

32   Ma, L. & Wang, H. Y. Suppression of cyclic GMP-dependent protein kinase is

        essential to the Wnt/cGMP/Ca2+ pathway. *J Biol Chem* **281**, 30990-31001,

        doi:M603603200 [pii]

        10.1074/jbc.M603603200 (2006).

33   Kuhl, M., Sheldahl, L. C., Malbon, C. C. & Moon, R. T. Ca(2+)/calmodulin-

        dependent protein kinase II is stimulated by Wnt and Frizzled homologs and

        promotes ventral cell fates in Xenopus. *J Biol Chem* **275**, 12701-12711 (2000).

34   Sheldahl, L. C., Park, M., Malbon, C. C. & Moon, R. T. Protein kinase C is

        differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent

        manner. *Curr Biol* **9**, 695-698, doi:S0960-9822(99)80310-8 [pii] (1999).

35   Bogoyevitch, M. A., Ngoei, K. R., Zhao, T. T., Yeap, Y. Y. & Ng, D. C. c-Jun N-terminal kinase (JNK) signaling: recent advances and challenges. *Biochim Biophys Acta* **1804**, 463-475, doi:10.1016/j.bbapap.2009.11.002 S1570-9639(09)00316-1 [pii] (2010).

36   Ross, S. E. *et al.* Inhibition of adipogenesis by Wnt signaling. *Science* **289**, 950-953, doi:8736 [pii] (2000).

37   Li, H. X., Luo, X., Liu, R. X., Yang, Y. J. & Yang, G. S. Roles of Wnt/beta-catenin signaling in adipogenic differentiation potential of adipose-derived mesenchymal stem cells. *Mol Cell Endocrinol* **291**, 116-124, doi:10.1016/j.mce.2008.05.005 S0303-7207(08)00206-2 [pii] (2008).

38   Cawthorn, W. P. *et al.* Wnt6, Wnt10a and Wnt10b inhibit adipogenesis and stimulate osteoblastogenesis through a beta-catenin-dependent mechanism. *Bone* **50**, 477-489, doi:10.1016/j.bone.2011.08.010 S8756-3282(11)01153-7 [pii] (2012).

39   Bennett, C. N. *et al.* Regulation of osteoblastogenesis and bone mass by Wnt10b. *Proc Natl Acad Sci U S A* **102**, 3324-3329, doi:0408742102 [pii] 10.1073/pnas.0408742102 (2005).

40   Kawai, M. *et al.* Wnt/Lrp/beta-catenin signaling suppresses adipogenesis by inhibiting mutual activation of PPARgamma and C/EBPalpha. *Biochem Biophys Res Commun* **363**, 276-282, doi:S0006-291X(07)01819-0 [pii] 10.1016/j.bbrc.2007.08.088 (2007).

41   Bennett, C. N. *et al.* Regulation of Wnt signaling during adipogenesis. *J Biol Chem* **277**, 30998-31004, doi:10.1074/jbc.M204527200

M204527200 [pii] (2002).

42   Park, J. R., Jung, J. W., Lee, Y. S. & Kang, K. S. The roles of Wnt antagonists Dkk1 and sFRP4 during adipogenesis of human adipose tissue-derived mesenchymal stem cells. *Cell Prolif* **41**, 859-874, doi:10.1111/j.1365-2184.2008.00565.x

CPR565 [pii] (2008).

43   Wright, W. S. *et al.* Wnt10b inhibits obesity in ob/ob and agouti mice. *Diabetes* **56**, 295-303, doi:56/2/295 [pii]

10.2337/db06-1339 (2007).

44   Christodoulides, C. *et al.* WNT10B mutations in human obesity. *Diabetologia* **49**, 678-684, doi:10.1007/s00125-006-0144-4 (2006).

45   Baron, R., Rawadi, G. & Roman-Roman, S. Wnt signaling: a key regulator of bone mass. *Curr Top Dev Biol* **76**, 103-127, doi:S0070-2153(06)76004-5 [pii] 10.1016/S0070-2153(06)76004-5 (2006).

46   Krishnan, V., Bryant, H. U. & Macdougald, O. A. Regulation of bone mass by Wnt signaling. *J Clin Invest* **116**, 1202-1209, doi:10.1172/JCI28551 (2006).

47   Bennett, C. N. *et al.* Wnt10b increases postnatal bone formation by enhancing osteoblast differentiation. *J Bone Miner Res* **22**, 1924-1932, doi:10.1359/jbmr.070810 (2007).

48   Morvan, F. *et al.* Deletion of a single allele of the Dkk1 gene leads to an increase in bone formation and bone mass. *J Bone Miner Res* **21**, 934-945, doi:10.1359/jbmr.060311 (2006).

49   Kang, S. *et al.* Wnt signaling stimulates osteoblastogenesis of mesenchymal precursors by suppressing CCAAT/enhancer-binding protein alpha and peroxisome proliferator-activated receptor gamma. *J Biol Chem* **282**, 14515-14524, doi:M700030200 [pii]

10.1074/jbc.M700030200 (2007).

50   Gaur, T. *et al.* Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. *J Biol Chem* **280**, 33132-33140, doi:M500608200 [pii]

10.1074/jbc.M500608200 (2005).

51   Holmen, S. L. *et al.* Essential role of beta-catenin in postnatal bone acquisition. *J Biol Chem* **280**, 21162-21168, doi:M501900200 [pii]

10.1074/jbc.M501900200 (2005).

52   Glass, D. A., 2nd *et al.* Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. *Dev Cell* **8**, 751-764, doi:S1534-5807(05)00097-3 [pii]

10.1016/j.devcel.2005.02.017 (2005).

53   Kanazawa, A. *et al.* Wnt5b partially inhibits canonical Wnt/beta-catenin signaling pathway and promotes adipogenesis in 3T3-L1 preadipocytes. *Biochem Biophys Res Commun* **330**, 505-510, doi:S0006-291X(05)00480-8 [pii]

10.1016/j.bbrc.2005.03.007 (2005).

54   Takada, I. *et al.* A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. *Nat Cell Biol* **9**, 1273-1285, doi:ncb1647 [pii]

10.1038/ncb1647 (2007).

55   Wakabayashi, K. *et al.* The peroxisome proliferator-activated receptor gamma/retinoid X receptor alpha heterodimer targets the histone modification enzyme PR-Set7/Setd8 gene and regulates adipogenesis through a positive feedback loop. *Mol Cell Biol* **29**, 3544-3555, doi:10.1128/MCB.01856-08 MCB.01856-08 [pii] (2009).

56   Chang, J. *et al.* Noncanonical Wnt-4 signaling enhances bone regeneration of mesenchymal stem cells in craniofacial defects through activation of p38 MAPK. *J Biol Chem* **282**, 30938-30948, doi:M702391200 [pii] 10.1074/jbc.M702391200 (2007).

57   Bilkovski, R. *et al.* Role of WNT-5a in the determination of human mesenchymal stem cells into preadipocytes. *J Biol Chem* **285**, 6170-6178, doi:10.1074/jbc.M109.054338 M109.054338 [pii] (2010).

58   Santos, A., Bakker, A. D., de Blieck-Hogervorst, J. M. & Klein-Nulend, J. WNT5A induces osteogenic differentiation of human adipose stem cells via rho-associated kinase ROCK. *Cytotherapy* **12**, 924-932, doi:10.3109/14653241003774011 (2010).

59   Arnsdorf, E. J., Tummala, P. & Jacobs, C. R. Non-canonical Wnt signaling and N-cadherin related beta-catenin signaling play a role in mechanically induced osteogenic cell fate. *PLoS One* **4**, e5388, doi:10.1371/journal.pone.0005388 (2009).

60   Quarto, N., Behr, B. & Longaker, M. T. Opposite spectrum of activity of canonical Wnt signaling in the osteogenic context of undifferentiated and differentiated mesenchymal cells: implications for tissue engineering. *Tissue Eng Part A* **16**, 3185-3197, doi:10.1089/ten.tea.2010.0133 (2010).

CHAPTER 4 – GLUCOSE ACTIVATES NON-CANONICAL WNT SIGNALING LEADING TO ENHANCED ADIPOGENESIS IN MESENCHYMAL PROGENITOR CELLS

These studies investigated the role of canonical and non-canonical Wnt signaling in MPC differentiation, and how high glucose might modify these pathways. My first aim was to establish the alterations in Wnt signaling that occurred at each level of regulation with the addition of high glucose. My second aim was to manipulate each pathway using both genetic and chemical approaches in an attempt to block the high glucose-induced increase in adipogenesis. My final aim was to corroborate these results with *in vivo* data using both rat and human bone marrow specimens. My studies consisted of the following:

1. Elucidate the high glucose-induced changes in Wnt signaling during adipogenesis.

2. Establish the precise role of β-catenin in the differentiation of MPCs to adipocytes.

3. Determine the involvement of non-canonical Wnt signaling in the skewing of MPC differentiation.

4. Examine alterations in the bone marrow diabetic stem cell niche.

4.1   Introduction

Human bone marrow MPCs are a pool of multipotent cells that give rise to adipocytes, osteoblasts, chondrocytes, and perivascular cells. Although direct associations between MPC dysfunction and diabetes have been elusive, the deregulation of MPC progeny is a likely outcome of the chronic metabolic perturbations seen in diabetes. Diabetes has been associated with fatty bone marrow[1,2], alongside moderate to severe bone loss[3-5] and increased fracture risk[6,7]. Diabetes also induces microvascular remodeling in the bone marrow[8,9], manifesting as impaired angiogenic ability, endothelial cell dysfunction, increased oxidative stress and a reduction in stem cell number[8]. Taken together, it would appear that disruption of the bone marrow microenvironment in diabetes might have detrimental consequences on stem/progenitor cell function and differentiation.

We have previously demonstrated that high levels of glucose, similar to levels seen in diabetes, cause dysfunction of MPCs[10]. MPCs showed skewed differentiation towards the adipocyte lineage, while their ability to become osteoblasts and chondrocytes was impaired. This is the first indication of glucose levels regulating MPC fate determination. Not only does this alteration provide an important link between diabetes and obesity, but it may also account for the long-term changes that are occurring in diabetic marrow. The mechanisms underlying this association, however, remain undiscovered. These mechanisms may involve Wnts, a family of secreted glycoproteins that play a role in cell fate and development[11]. In some of the early work implicating Wnt signaling in adipogenesis, Ross and colleagues showed that

preadipoctyes can be maintained in an undifferentiated state using Wnt10b, which was later shown to be mediated by blocking PPARγ and C/EBPα[12]. These, and other, findings led to the notion that Wnt signaling acts as a switch during adipogenesis; when switched off, differentiation of committed preadipoctyes is able to proceed. To date however, the role of Wnt signaling, canonical or non-canonical (i.e. β-catenin - dependent and -independent, respectively), in human MPC lineage commitment has been controversial. Previous studies have shown that high glucose levels cause Wnt activation and nuclear β-catenin accumulation in a number of human cancer cell lines[13], macrophages[14], and mesangial cells[15]. Therefore, it is crucial to understand how MPC differentiation is regulated and to decipher the role of Wnt signaling in this process.

In this study, we systematically investigate the molecular mechanisms that are responsible for the high glucose-mediated alterations in MPC differentiation. We hypothesize that high glucose is enhancing adipogenesis through selective modulation of Wnt signaling, and that this mechanism is directly responsible for the long-term phenotypic changes that are seen in the diabetic bone marrow.

4.2   Materials and methods

Experiments were approved by the Research Ethics Board at the University of Western Ontario, London, Ontario and the University of Florida, Gainesville, Florida (animal experiments).

4.2.1 Isolation and culture of mesenchymal progenitor cells

Fresh bone marrow samples (1M-125, Lonza Inc., Walkersville, MD) were obtained and mononuclear cell fraction was prepared as shown by us previously[10,16]. Bone marrow samples were cultured on fibronectin-coated (FN; 1µg/cm^2; FC010-10MG, Millipore, Temecula, CA) plates in DMEM (Life Technologies, Burlington, ON) media, supplemented with 20% FBS (Lonza Inc.), 1X PSF (antibiotic-antimycotic solution; Cellgro Mediatech Inc., Manassas, VA), and no additional growth factors. All experiments using bmMPCs were conducted on passage 2-6 cells with 3 technical and 3-5 biological replicates.

To induce differentiation, MPCs were seeded at a density of 40,000 cells/cm^2 on 12-well or 24-well plates (without FN coating) in specific differentiation media (StemPro® Adipogenesis; Life Technologies) with recombinant Wnt5a (50 ng/mL; 645-WN-010, R&D Systems, Minneapolis, MN), Wnt5b (50 ng/mL; 7347-WN-025, R&D Systems), and Wnt11 (50 ng/mL; 6179-WN-010, R&D Systems). IWR-1-endo (13659-10, Cayman Chemical, Ann Arbor, MI) and PNU74654 (3534/10, R&D Systems) were

both added at concentrations of 1 µM, 5 µM, and 20 µM. A Wnt signaling agonist (Calbiochem CAS 853220-52-7; EMD Millipore) was also used and added at concentrations varying from 100 nM to 5 µM. Media was changed every other day. RNA was isolated from cells after 7 days in order to perform qRT-PCR. For Oil Red O staining, cells were fixed in 10% neutral buffered formalin and placed in 100% propylene for 5 minutes before applying 0.5% Oil Red O solution (O0625-25G, Sigma-Aldrich, Oakville, ON).

4.2.2 RNA isolation and qRT-PCR

Using RNeasy Mini Plus or Micro Plus (Qiagen, Mississauga, ON), total RNA was extracted from the cells grown in culture. The quantity was determined by Qubit® Broad Range RNA assay in the Qubit® Fluorometer (Life Technologies). cDNA synthesis was performed with 200 ng of RNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). To examine the human Wnt signaling pathway, a 96-well RT^2 Profiler PCR Array (Qiagen) was used to profile the expression of 84 genes. The PCR was performed using RT^2 SYBR Green Mastermix. Data was analyzed by CFX Manager Software using normalized (ΔCT) method with β-actin normalization. For all other mRNA analyses, reactions consisted of 10 µL SsoFast EvaGreen® Supermix (Bio-Rad), 2 µL of both forward and reverse primers (at a 10 µM concentration; table 4.1), 1 µL cDNA, and 6 µL of H_2O. All reactions were performed for 40 cycles using the following temperature profiles: 95°C for 2 minutes (initial denaturation); and 55°C for 12 seconds (annealing and extension). Data was analyzed using relative quantity (ΔC_T).

Table 4.1 Primer sequence information for qRT-PCR in chapter 4.

Gene	Length (bp)	Source (catalogue number)
Adipogenesis markers		
CCAAT/enhancer binding protein alpha (C/EBPα)	88 bp	Qiagen (QT00203357)
CCAAT/enhancer binding protein beta (C/EBPβ)	121 bp	Qiagen (QT00237580)
CCAAT/enhancer binding protein delta (C/EBPδ)	90 bp	Qiagen (QT00219373)
Peroxisome proliferator-activated receptor γ2 (PPARγ2)	134 bp	$5` \rightarrow 3`$ ATTGACCCAGAAAGCGATTCC CAAAGGAGTGGGAGTGGTCT
Stem cell niche factors		
Angiopoietin-1 (Ang1)	111 bp	Qiagen (QT00046865)
Angiopoietin-2 (Ang2)	79 bp	Qiagen (QT00100947)
Nitric oxide synthase 3 (NOS3)	133 bp	SA Biosciences (PPH01298E)
Stromal cell-derived factor (CXCL12)	70 bp	Qiagen (QT00087591)
SDF receptor (CXCR4)	100 bp	Qiagen (QT02311841)
β-catenin	130 bp	Qiagen (QT00077882)
Wnt11	88 bp	Qiagen (QT00018270)
Housekeeping gene		
β-actin	104 bp	Qiagen (QT01680476)

4.2.3 Human bone marrow samples

We obtained paraffin and frozen human femoral bone marrow tissue slides of both control (US Biomax, Rockville, MD, n=3; age 63 ±2.64 yrs) and type 2 diabetic patients (US Biologicals, Salem, MA; US Biomax, and BioChain Institute, Hayward, CA; n=4, age 65.33 ± 2.33 yrs/ BMI and duration of diabetes unknown). cDNA samples from control and type 2 diabetic patients were obtained from BioChain Institute. Double staining was performed using Picture^{TM} Plus Double Staining Kit (Life Technologies). Slides were deparaffinized, hydrated through a sequential ethanol gradient and washed in PBS. Slides were then subjected to antigen retrieval in Tris-EDTA buffer (10 mM Trizma-base, 1 mM EDTA, 0.05 % Tween-20, pH 9.0) and 120 °C for 120 minutes using the Antigen Retriever^{TM} (2100 Retriever, PickCell Laboratories). Following antigen retrieval, endogenous peroxidase activity was quenched with 3% H_2O_2 diluted in methanol for ten minutes. Slides were blocked and primary antibodies (CD133 antibody, Abcam ab19898; CD45 antibody, R&D Systems MAB1430) were applied simultaneously for one hour. Slides were rinsed in PBS containing 0.05% Tween-20. Goat anti-mouse IgG-horseradish peroxidase polymer conjugate and goat anti-rabbit IgG-alkaline phosphatase polymer conjugate were then applied for 30 minutes. DAB chromogen and Fast Red were used for detection. Slides were counterstained with Mayer's hematoxylin (Sigma-Aldrich) for 30 seconds and mounted using ClearMount™ Mounting solution (Life Tehnologies). Images were taken using Olympus BX-51 microscope (Olympus Canada In., Richmond Hill, ON) equipped with a Spot Pursuit^{TM} digital camera (SPOT Imaging Solutions, Sterling Heights, MI).

4.2.4 Diabetic animal model

Streptozotocin-induced diabetic rat model was used. A single intraperitoneal injection of freshly prepared streptozotocin (65 mg/kg in citrate buffer, pH 4.5) was performed in 8-week old Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN). Diabetes was confirmed after one week following streptozotocin injection by measuring the blood glucose level (>200 mg/dL; >11 mmol/L) using the FreeStyle glucomonitor. Following two months of diabetes, rats were euthanized and femoral bones were removed, decalcified, and embedded in paraffin. Tissues were sectioned at 5 µm. All animal procedures were in compliance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The protocols for the rat studies were approved by the Institutional Animal Care and Use Committee at the University of Florida.

4.2.5 Cell transfections

MPCs were grown in culture in EBM2/20% FBS until confluent. On the day of transfection, cells were trypsinized, washed, and re-suspended in R buffer (NeonTM Transfection System, Life Technologies). Cells were then mixed with control siRNA-A (Santa Cruz Biotechnologies, Santa Cruz, CA, sc-37007), β-catenin siRNA (Santa Cruz Biotechnologies, sc-29209), GFP-RhoA Expression Vector Set (contains dominant negative and wild type; Cell BioLabs, San Diego, CA, STA-452), or GFP-Rac1 Expression Vector Set (contains dominant negative and wild type; Cell BioLabs, STA-

450) and subjected to electroporation using Neon Transfection System (Life Technologies). Transfected cells were transferred to a 24-well plate containing EBM2 media without antibiotics. The following day the media was changed to adipogenic media (StemPro® Adipogenesis Differentiation media) with or without the addition of HG.

4.2.6 PKC activity assay and β-catenin protein levels

MPCs were grown in culture in EMB2/20% FBS until confluent and then subjected to various treatments: control (DMEM/10% FBS), HG (DMEM/10% FBS + 25 mmol/L glucose), Wnt11 (DMEM/10% FBS + 50 ng/mL Wnt11), Adipo (StemPro® Adipogenesis Differentiation media), Adipo + IWR (20 µM), and Adipo + PNU (20 µM). After 7 days in culture, protein was extracted using Cell Extraction Buffer (Life Technologies, FNN0011) with Halt™ Protease and Phosphatase Inhibitor Single-Use Cocktail (Thermo Scientific, Waltham, MA) to prevent enzymatic degradation. Total protein from each sample was then quantified using Pierce™ BCA Protein Assay Kit (Thermo Scientific). Following quantification, total β-catenin (Life Technologies, KHO1211) and PKC kinase activity (Enzo Life Sciences, Farmingdale, NY, ADI-EKS-420A) was measured as per manufacturer's protocol.

4.2.7 Cell staining

Cultured cells were trypsinized and plated (15,000 cells/cm^2) on FN-coated 8-chambered slides one day prior to staining to allow for attachment of cells. Immunofluorescence staining of MPCs for β-catenin (1:100, Abcam, ab6302) and phospho-PKC T497 (1:75; Abcam, ab59411) was carried out, followed by Alexa488-conjugated secondary antibody (Life Technologies). Images were taken using Olympus BX-51 microscope. β-catenin and p-PKC staining intensity and β-catenin localization was quantified by imageJ analysis following background subtraction to account for slide to slide variability.

Table 4.2 Antibodies used for immunostaining in chapter 4.

	Antigen	Host	Source (catalogue number)	Dilution
1	β-catenin	Rabbit polyclonal	Abcam (ab6302)	1:100
2	Ang2	Rabbit polyclonal	Abcam (ab153934)	1:75
3	CD133	Rabbit polyclonal	Abcam (ab66141)	1:200
4	CD45	Rabbit polyclonal	Abcam (ab10559)	1:200
5	p-PKC	Rabbit polyclonal	Abcam (ab59411)	1:75
6	Wnt11	Rabbit polyclonal	Abcam (ab96730)	1:75

4.2.8 Statistical analysis

The data were expressed as means $\pm$ SEM. Where appropriate, analysis of variance (ANOVA) followed by two-tailed student's unpaired t-tests were performed. P values < 0.05 were considered statistically significant.

4.3   Results

4.3.1 HG primes MPCs to alter their differentiation potential

In our initial studies, we showed that exposure of MPCs to high levels of glucose (25 mmol/L; HG) enhances adipogenesis[10]. Therefore, we first wanted to assess whether the effect of HG on MPC differentiation was transient, or if HG re-programmed the cells so that an increase in adipogenesis would still be evident with removal of the stimulus. To do this, we performed a "priming" study as shown in figure 4.1. We compared three experimental groups: 1) cells pre-treated with HG for 7-days prior to the adipogenesis assay, 2) cells subjected to the adipogenesis assay in the presence of HG, and 3) cells subjected to an adipogenesis assay only. Degree of adipogenesis, as noted by the induction level of adipogenesis-specific transcription factors c/EBP α/β/δ and PPARγ2, was similar in groups 2 and 3 (pre-treatment group and adipo + HG group), and was significantly higher than group 1 (adipo alone; figure 4.2). This indicates that the effect of HG is not transitory. Interestingly, PPARγ2 (late marker of adipogenesis) levels were significantly lower in the adipo + HG group as compared to the pre-treatment group, suggesting that this priming effect may not only be increasing adipocyte number, but also accelerating the differentiation process.
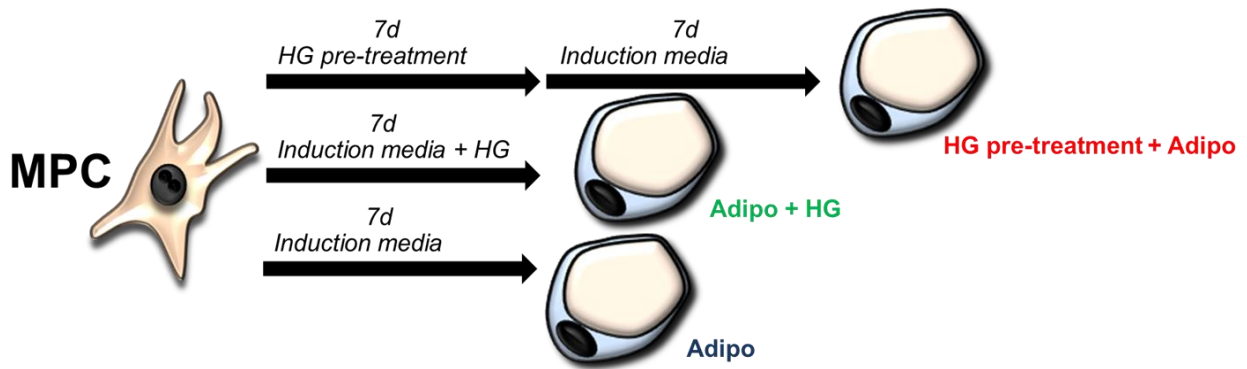
Figure 4.1: Experimental scheme for priming study. In order to assess the re-programming ability of HG, three independent experimental groups were compared: 1) cells that were pre-treated with HG (25 mmol/L) for 7 days prior to a 7 day adipogenesis assay (HG pre-treatment + Adipo), 2) cells that were subjected to a 7 day adipogenesis assay in the presence of HG (Adipo + HG), and 3) cells that were subjected to a 7 day adipogenesis assay only (Adipo).
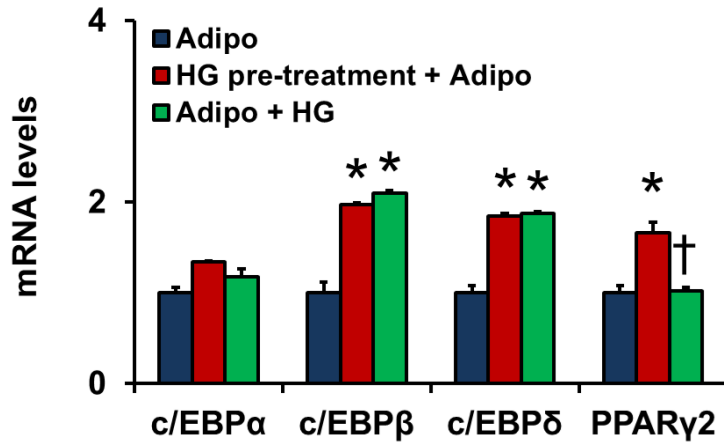
Figure 4.2: HG primes MPCs to alter their differentiation potential. Following the experimental scheme, we examined mRNA levels of adipogenesis-specific transcription factors c/EBPα, c/EBPβ, c/EBPδ, and PPARγ2 to measure the extent of differentiation. Both HG-treated groups demonstrated a significant increase in adipogenesis as compared to control ($^*p<0.05$ as compared to Adipo group). PPARγ2 (late marker of adipogenesis) was significantly lower in the Adipo + HG group as compared to the pre-treated group ($^†p<0.05$), which remained elevated when compared to control ($^*p<0.05$) [c/EBP=CCAAT/enhancer binding protein; PPARγ2=peroxisome proliferator-activated receptor γ2].

4.3.2 HG selectively modulates Wnt signaling during adipogenesis

We used a real time PCR-based array approach to profile the Wnt signaling pathway during the processes of adipogenesis. Presence of HG in the induction media depressed the expression of most Wnt ligands (figure 4.3A). Interestingly, we saw an increase in autogenous Wnt11 expression by HG. Treatment of MPCs with HG alone (no differentiation media) also upregulated Wnt11 expression (figure 4.3B), indicating that HG, specifically, was responsible for this increase. In contrast to the Wnt ligands, all Fzd receptors were upregulated in HG exposure (Figure 4.4A). Analysis of β-catenin, β-catenin inhibitors (TLE1/2 and β-catenin interacting protein-1/CTNNBIP1), and downstream target genes demonstrated that HG does not induce β-catenin-mediated transcription (figure 4.4B). When MPCs were treated with HG alone, β-catenin was absent from the nucleus, although cytoplasmic and membrane β-catenin protein levels showed an increase (figures 4.5A-C). This, again, illustrated that canonical Wnt signaling is not transcriptionally active in HG-treated cells. However, the intriguing behaviour of Wnt11 transcript levels in HG indicated a potential connection to non-canonical Wnt signaling.
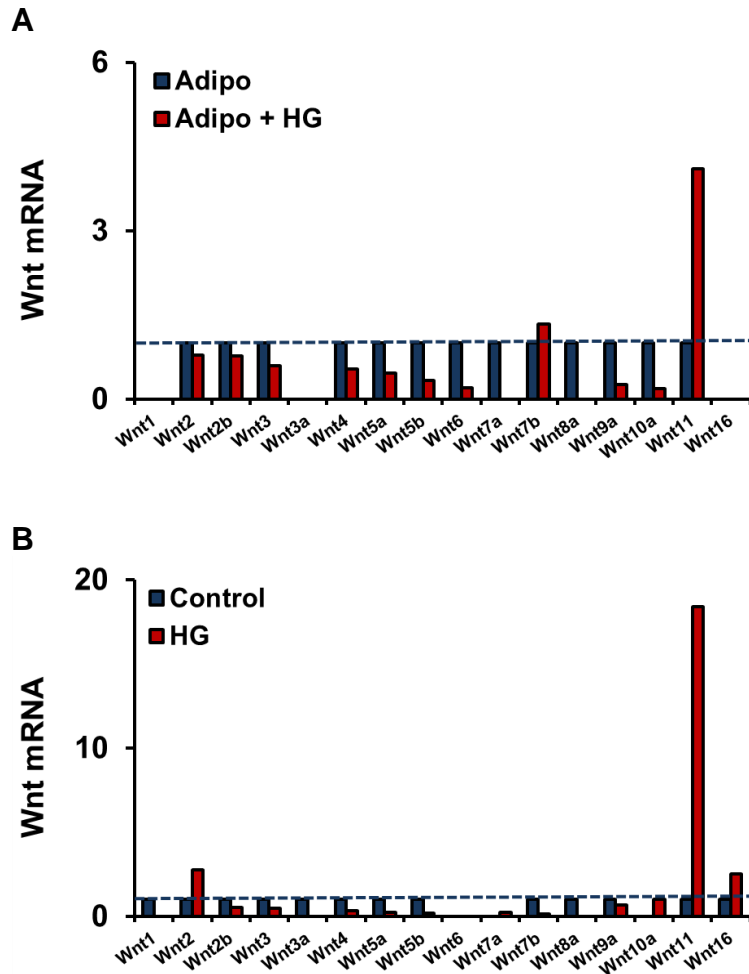
A

B

Figure 4.3: HG induces Wnt11. (A**)** MPCs were differentiated into adipocytes in the presence or absence of HG (25 mmol/L) and mRNA expression of Wnt ligands was examined. Wnt ligands displayed a varied pattern with the addition of HG to the adipogenic induction media. While most were suppressed, there was an upregulation of Wnt11. (B) To confirm that HG was responsible for this increase, we profiled the ligands in control (DMEM/10% FBS) and HG-treated MPCs, and noted a similar upregulation of Wnt11 by HG [HG=high glucose].
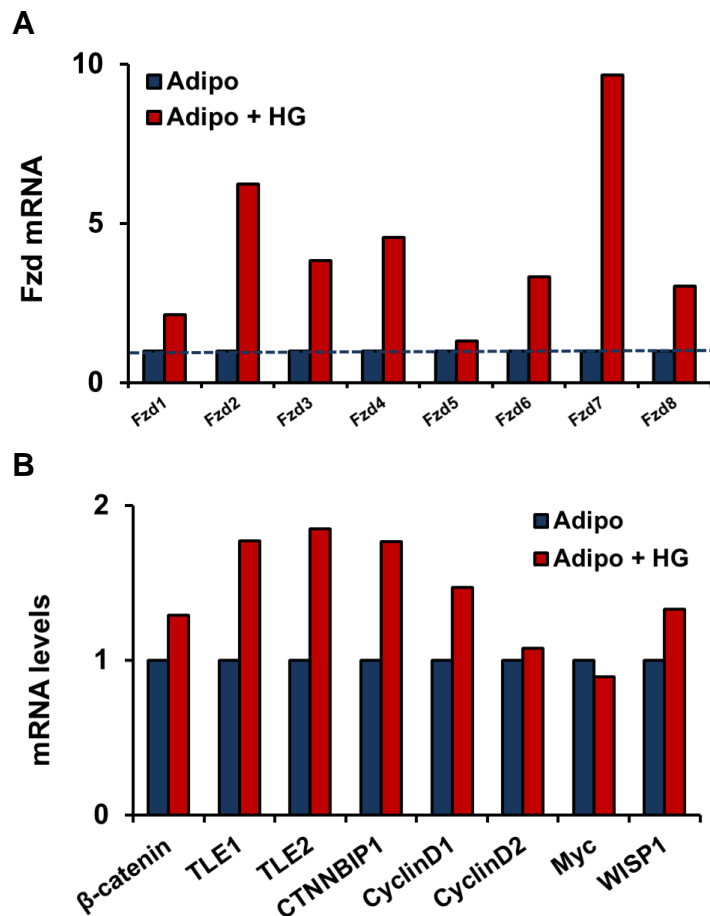
Figure 4.4: HG selectively modulates Wnt signaling in adipocytes. MPCs were differentiated into adipocytes in the presence or absence of HG (25 mmol/L) and mRNA expression of specific Wnt signaling genes was examined. (A) Each FZD receptor (Fzd1-8) was upregulated in HG-treated adipocytes. (B) Though β-catenin was increased in HG, analysis of downstream target genes (CyclinD1/2, Myc, WISP1) and negative transcriptional regulators of Wnt signaling (TLE1/2, CTNNBIP1) revealed that β-catenin was not active within the nucleus [HG=high glucose; Fzd=frizzled receptor; TLE1/2=transducin-like enhancer protein 1/2; CTNNBIP1=β-catenin interacting protein-1; WIPS1=Wnt1-inducible-signaling pathway protein 1].
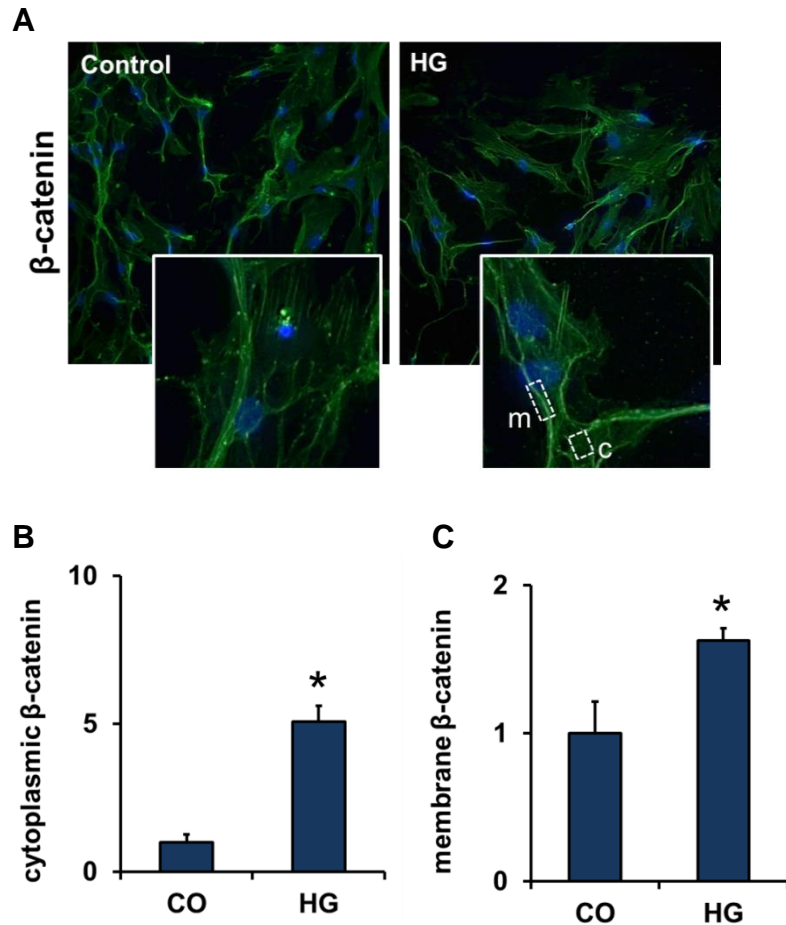
Figure 4.5: Absence of nuclear β-catenin in MPCs. (A) β-catenin protein localization in MPCs after treatment with HG (25 mmol/L) for 24 hours in normal growth media (DMEM/10% FBS) [Green = Alexa Fluorochrome 488; β-catenin, blue = DAPI for nuclear stain; 20x magnification; insert at 40x; c = cytoplasmic, m = membrane]. Quantification of cytoplasmic (B) and membrane (C) β-catenin staining was done using ImageJ software (data are represented as mean ± SEM; *p<0.05 compared to control) [HG=high glucose].

4.3.3 Wnt11 mediates the effects of HG to stimulate adipogenesis

To determine whether Wnt11 mediates the effects of HG in enhancing adipogenesis, we performed MPC differentiation in the presence of exogenous Wnt proteins and used Oil Red O staining to highlight resulting lipid droplets. Exogenous Wnt5a and Wnt5b, both downregulated by HG (figures 4.3A and B), showed no significant effect on adipogenesis (figure 4.6). However, with the addition of non-canonical Wnt11, the intensity of the Oil red staining was dramatically increased, mimicking the effects of HG (figure 4.6).
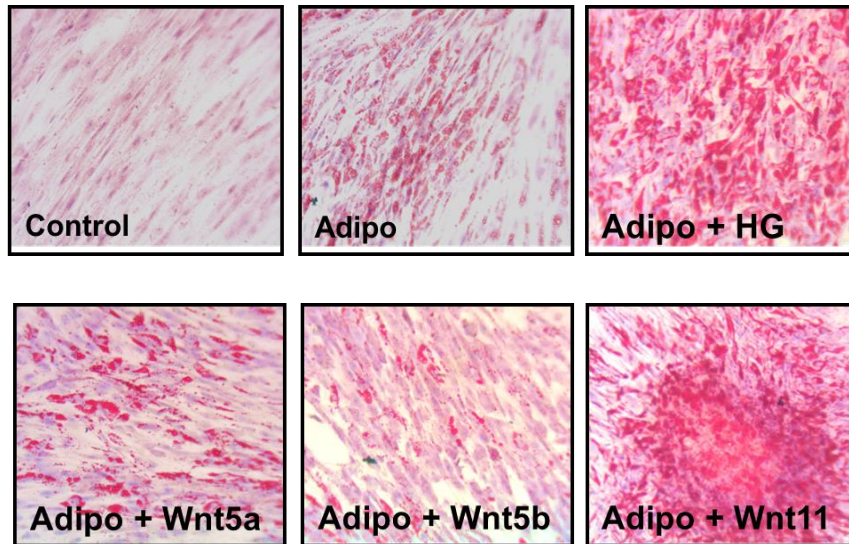
Figure 4.6: Wnt11 enhances adipogenesis. We performed various adipogenesis assays with specific modifications to the culture medium and used Oil Red O to stain the resulting lipid droplets. MPCs in normal growth media (DMEM/10% FBS; control) and those that were subjected to a 7 day adipogenesis assay (Adipo) were used as controls. With the addition of HG (25 mmol/L), adipogenesis was enhanced. Wnt5a (50 ng/mL) and Wnt5b (50 ng/mL) displayed no significant effect. With the addition of Wnt11 (50 ng/mL), both the intensity and number of cells stained was enhanced, mimicking the effects of HG [HG=high glucose].

4.3.4 β-catenin positively regulates adipogenesis in MPCs

We further examined the role of Wnt/β-catenin signaling through the use of small molecule inhibitors. IWR-1-endo (IWR-1) functions in the intracellular compartment where it increases Axin levels, thereby increasing β-catenin degradation (figure 4.7A). PNU74654 (PNU), on the other hand, blocks the interaction of β-catenin and TCF/LEF (figure 4.7B). To confirm whether these inhibitors are functional, we examined mRNA levels of β-catenin and found that β-catenin was highly suppressed with the addition of IWR-1 but not in the PNU-treated group (figure 4.7C). We also measured β-catenin protein levels and, though results did not reach statistical significance, the pattern was reflective of the mRNA results (figure 4.7D). This was expected as IWR-1 only degrades "free" β-catenin which is available to participate in the canonical Wnt pathway[17]. The use of IWR-1, which inhibits canonical β-catenin-dependent signaling, showed reduced adipogenesis (figure 4.8A,C). On the other hand, PNU treatment showed no change (Figure 4.8B). However, mRNA levels of adipogenesis-specific transcription factors after PNU treatment revealed inhibition of early C/EBPβ and δ but not the late C/EBPα or PPARγ2 (figure 4.8C). This indicates that β-catenin is involved in adipogenesis, but not through nuclear accumulation and transcription-dependent mechanisms.
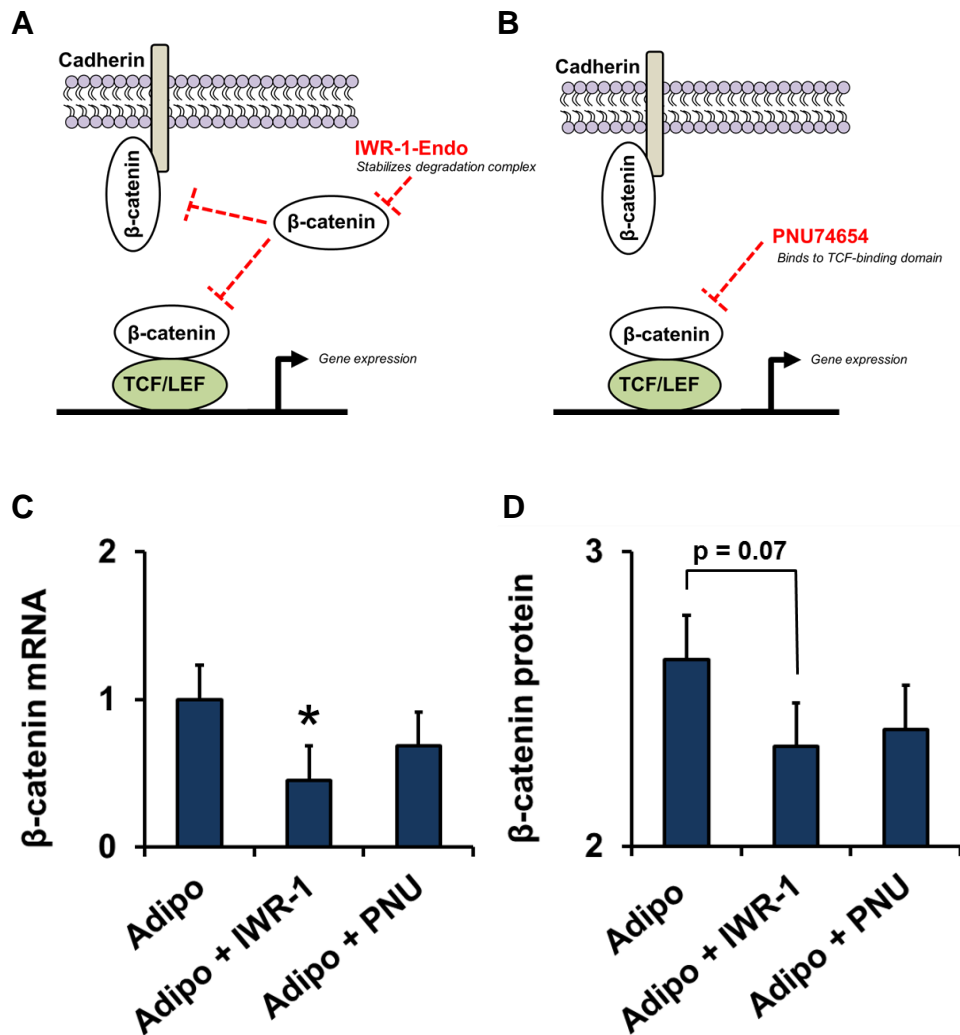
Figure 4.7: IWR-1 suppresses β-catenin. Two canonical Wnt pathway antagonists were used to assess the role of β-catenin in adipogenesis. (A) IWR-1 works to stabilize the destruction complex, thus increasing β-catenin degradation. (B) PNU functions within the nucleus to block the binding of β-catenin to the TCF/LEF complex. We looked at the effect of IWR-1 and PNU on β-catenin mRNA (C) and protein (D) levels after 7 days and found that IWR significantly suppresses β-catenin mRNA (data are represented as mean ± SEM; *p<0.05 as compared to Adipo) [IWR-1=IWR-1-Endo; PNU=PNU74654].
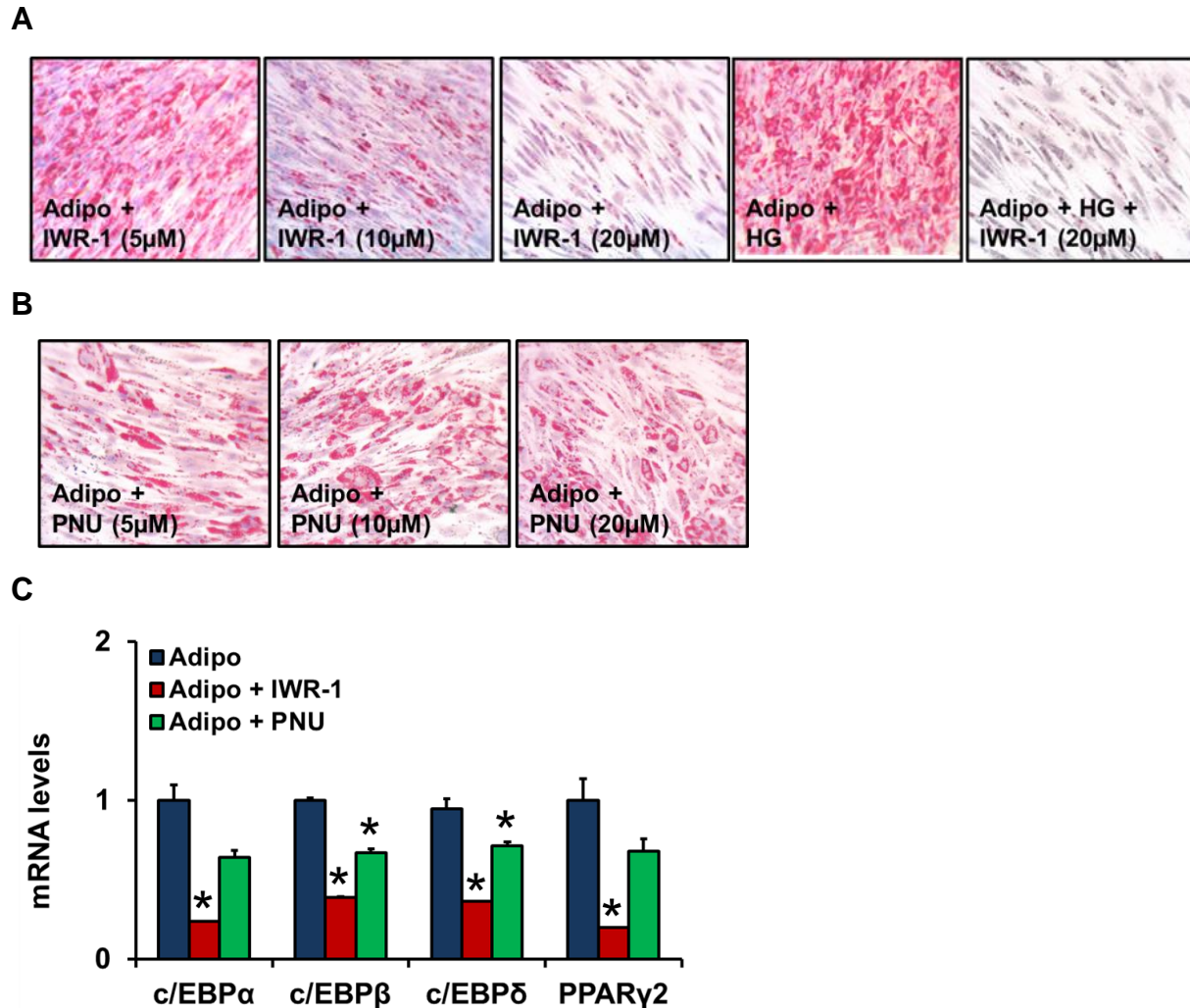
Figure 4.8: IWR-1, but not PNU, inhibits adipogenesis. (A) Adding the intracellular inhibitor IWR-1 to an adipogenesis assay at increasing concentrations (1 μM, 5 μM, and 20 μM) caused a dose-dependent decrease in Oil Red O staining after 7 days. IWR-1 (20 μM) was added alongside HG (25 mmol/L; far right panel) and normalized the increase in adipogenesis noted by HG alone (fourth panel). (B) PNU at any concentration (1 μM, 5 μM, or 20 μM) had no effect on adipogenesis. (C) Using qRT-PCR, we found that IWR-1 significantly suppressed adipogenesis-specific transcription factors at day 7, while PNU suppressed only c/EBPβ and c/EBPδ (data are represented as mean ± SEM; *$p < 0.05$ as compared to Adipo) [IWR-1=IWR-1-Endo; HG=high glucose; PNU=PNU74654; c/EBP=CCAAT/enhancer binding protein; PPARγ2=peroxisome proliferator-activated receptor γ2].

4.3.5 Role of β-catenin in adipogenesis

To confirm the notion that β-catenin is important for adipogenesis, though not through transcription-mediated mechanisms, we employed two approaches. First, we treated MPCs with a Wnt agonist (a cell-permeable pyrimidine that increases intracellular β-catenin levels without altering the destruction complex). We observed enhanced adipogenesis after treatment with this agonist (figure 4.9). Then, we used small interfering-RNA (siRNA) to knockdown β-catenin. Contrary to what we expected, a near complete knockdown of β-catenin dramatically increased adipogenesis (figure 4.10A, B).
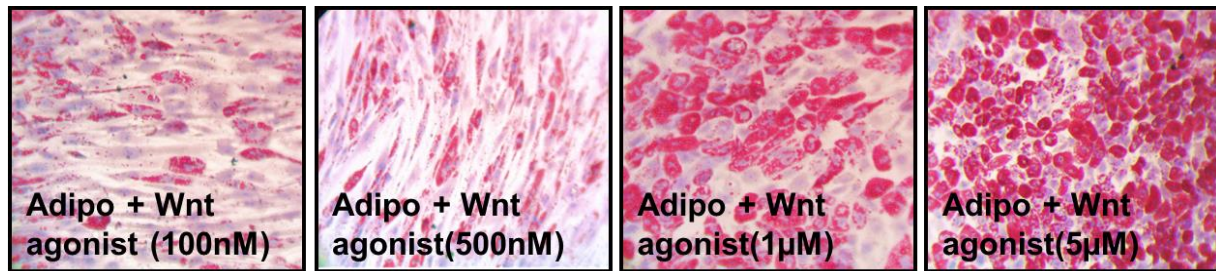
Figure 4.9: Wnt agonist enhances adipogenesis. The addition of Wnt agonist to the adipogenesis-specific induction media at increasing concentrations (100 nM, 500 nM, 1μM, and 5 μM) enhanced Oil Red O staining in a parallel manner, indicating an increase in adipogenesis with Wnt agonist treatment.
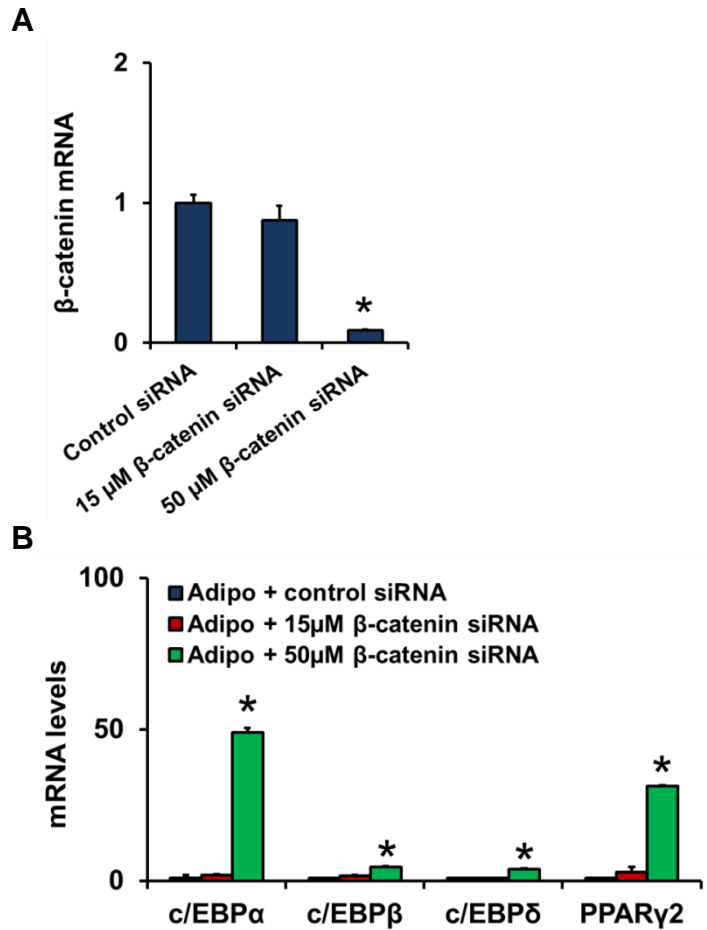
Figure 4.10: β-catenin protein plays a role in adipogenesis. Following transfection of MPCs with siRNA (15 µM or 50 µM) to silence the β-catenin gene, we performed a 7 day adipogenesis assay. (A) mRNA levels of β-catenin indicated a knockdown efficiency of greater than 90% when 50 µM siRNA was used. 15 µM β-catenin had no effect (data are represented as mean ± SEM; *$p<0.05$ as compared to control siRNA). (B) qRT-PCR analysis of adipogenesis-specific transcription factors revealed that siRNA-induced suppression of β-catenin caused a significant increase in adipogenesis (*$p<0.05$ as compared to Adipo + control siRNA) [siRNA=small interfering-RNA; c/EBP=CCAAT/enhancer binding protein; PPARγ2=peroxisome proliferator-activated receptor γ2].

4.3.6 β-catenin depletion induces a switch from canonical to non-canonical Wnt signaling

Our previous observations led us to hypothesize that there is a switch from canonical to non-canonical Wnt signaling upon β-catenin gene knockdown. Therefore, we examined the expression of the likely candidate, non-canonical Wnt11, in MPCs that were transfected with β-catenin siRNA and had undergone adipogenesis. We noted drastically enhanced Wnt11 levels (figure 4.11) in these cells, pointing to non-canonical signaling, and more specifically Wnt11, as the potential mediator of the HG-induced increase in adipogenesis.
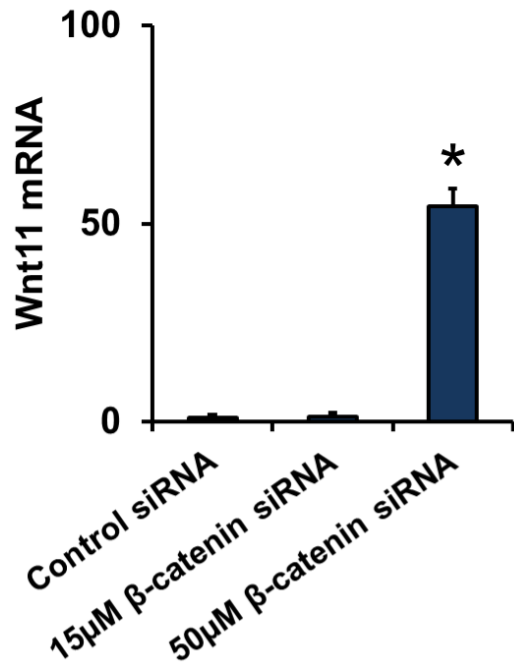
Figure 4.11: Complete β-catenin knockdown activates non-canonical Wnt signaling. Wnt11 mRNA was examined in MPCs that were transfected with 15 µM and 50 µM β-catenin siRNA and had undergone a 7 day adipogenesis assay. Wnt11 mRNA levels were drastically elevated in cells treated with 50 µM β-catenin siRNA, indicating activation of non-canonical Wnt signaling (data is represented as mean ± SEM; *p<0.05 as compared to control siRNA) [siRNA=small interfering-RNA].

4.3.7 Adipogenesis is not activated through the Wnt/PCP pathway

Our next objective was to dissect out the non-canonical Wnt pathway and determine whether the Wnt/PCP pathway or the Wnt/Ca^{2+} pathway is involved in adipogenesis. To achieve this, we used genetic and chemical modulation of the key players in each segment of the non-canonical pathway. First, we transfected MPCs with dominant negative (DN) and wild-type (WT) Rac1. DN Rac1 enhanced adipogenesis as compared to WT transfected cells (figure 4.12A), suggesting that basal Rac1 activity may be inhibiting differentiation. Similar results were obtained when we inhibited Rac1 using a specific pharmacological Rac1 inhibitor (Z62954982; figure 4.12B). Furthermore, RhoA modulation by dominant negative transfection and Rho Kinase Inhibitor VII confirmed that both Rac1 and RhoA have overlapping functions in MPCs (data not shown). Rac and Rho are believed to have opposing functions[18,19]. This suggests that their role during adipogenesis may be related to cell shape change, which would result in overlapping readout.
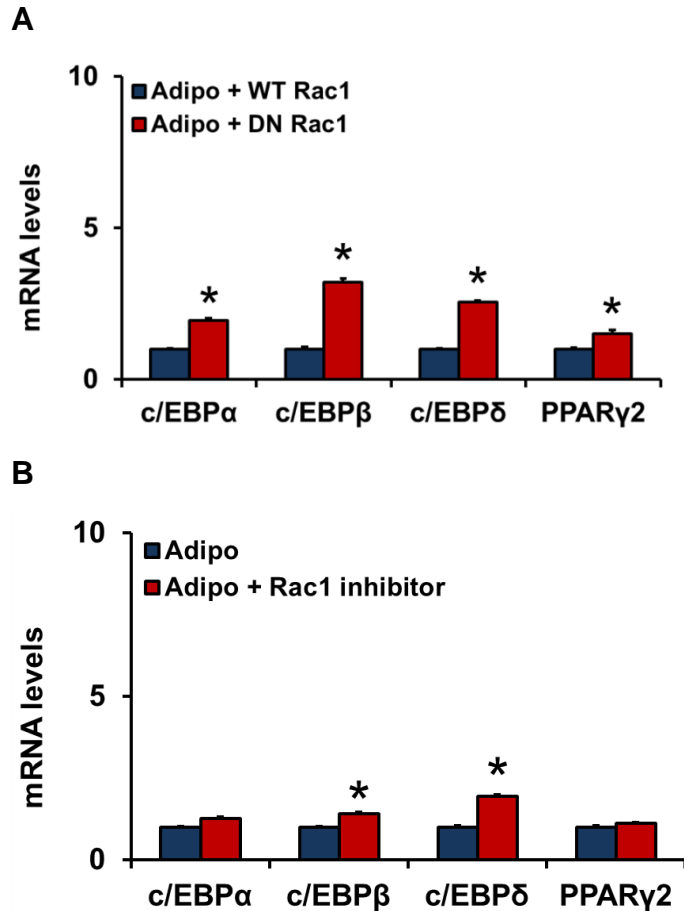
Figure 4.12: Adipogenesis is not stimulated through the Wnt/PCP pathway. (A) We transfected MPCs with DN and WT Rac1 and performed a 7 day adipogenesis assay. DN Rac1 enhanced adipogenesis as compared to WT Rac1 transfected cells (data is represented as mean ± SEM; *p<0.05 as compared to Adipo + WT Rac1). (B) We confirmed our results using a chemical inhibitor of Rac1. Mirroring the DN results, adipogenesis was enhanced after treatment with the Rac1 antagonist, with early transcription factors c/EBPβ and c/EBPδ being significantly increased as compared to control (*p<0.05 as compared to Adipo) [WT=wild-type; DN=dominant negative; c/EBP=CCAAT/enhancer binding protein; PPARγ2=peroxisome proliferator-activated receptor γ2].

4.3.8 Non-canonical Wnt/Ca^{2+} signaling stimulates adipogenesis

Building on these studies, we examined the role of the Wnt/Ca^{2+} pathway in adipogenesis. Inhibition of PKC through Cheletherine chloride (Che) or PCK-ε V1-2 [20], which specifically blocks the epsilon isoform of PKC, reduced adipogenesis in MPCs (figure 4.13A). To prevent signaling on the alternate side of the same pathway, we used KN93 to inhibit CAMKII. CAMKII paralleled PKC activity during MPC differentiation (figure 4.13A). These results demonstrated a positive role for the Wnt/Ca^{2+} pathway in the regulation of adipogenesis. Our previous results point to Wnt11 as mediating the effects of HG, therefore we tested whether Wnt11 would normalize the effects of PKC inhibition. Interestingly, we found that Wnt11 does significantly dampen the effects of both PKC inhibitors (figure 4.13B). Next, we examined phospho-PKC (p-PKC) levels in human MPCs that had been treated with HG (25 mmol/L) or Wnt11 (50 ng/mL) for 24 hours in normal media. We found increased p-PKC in MPCs treated with Wnt11, but not with HG (figures 4.14A and B). This may be due to the short nature of HG exposure. Increased p-PKC levels upon Wnt11 treatment were found to co-localize to the nucleus (figure 4.14A). This was an unusual and unexpected finding. Interestingly, PKC-epsilon (PKCε) has been shown to accumulate in the nuclei of 3T3-F442A cells[20]. We confirmed the role of PKC by measuring activity and noted the same outcome as with the p-PKC finding (figure 4.14C). Once again, we demonstrated a significant increase of PKC activity in the Wnt11-treated group, confirming the important relationship between this non-canonical ligand and the PKC-mediated Wnt/Ca^{2+} pathway.
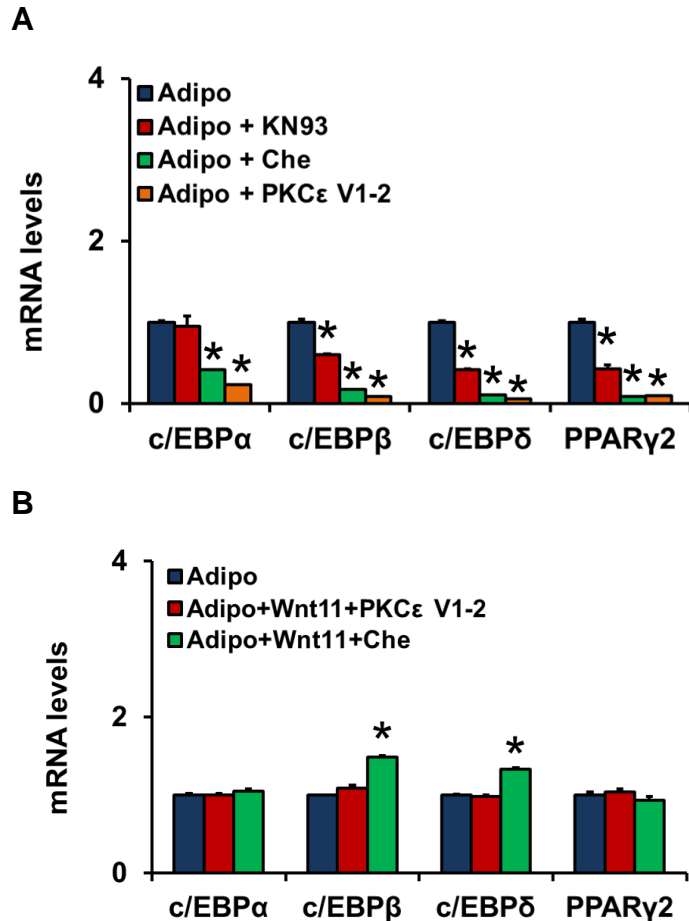
Figure 4.13: Adipogenesis is activated through non-canonical signaling. (A) To examine the Wnt/Ca^{2+} pathway, we performed a 7 day adipogenesis assay with the addition of three chemical inhibitors: Che (general PKC inhibitor), PKCε V1-2 (inhibits PKCε), and KN93 (inhibits CaMKII). Each of these three inhibitors functioned to block adipogenesis (data is represented as mean ± SEM; *p<0.05 as compared to Adipo). (B) The presence of Wnt11 (50 ng/mL) in the differentiation media normalized the inhibiting function of the PKC blockers (*p<0.05 as compared to Adipo) [Che=cheletherine chloride; PKCε V1-2=PKC epsilon inhibitor; c/EBP=CCAAT/enhancer binding protein; PPARγ2=peroxisome proliferator-activated receptor γ2].
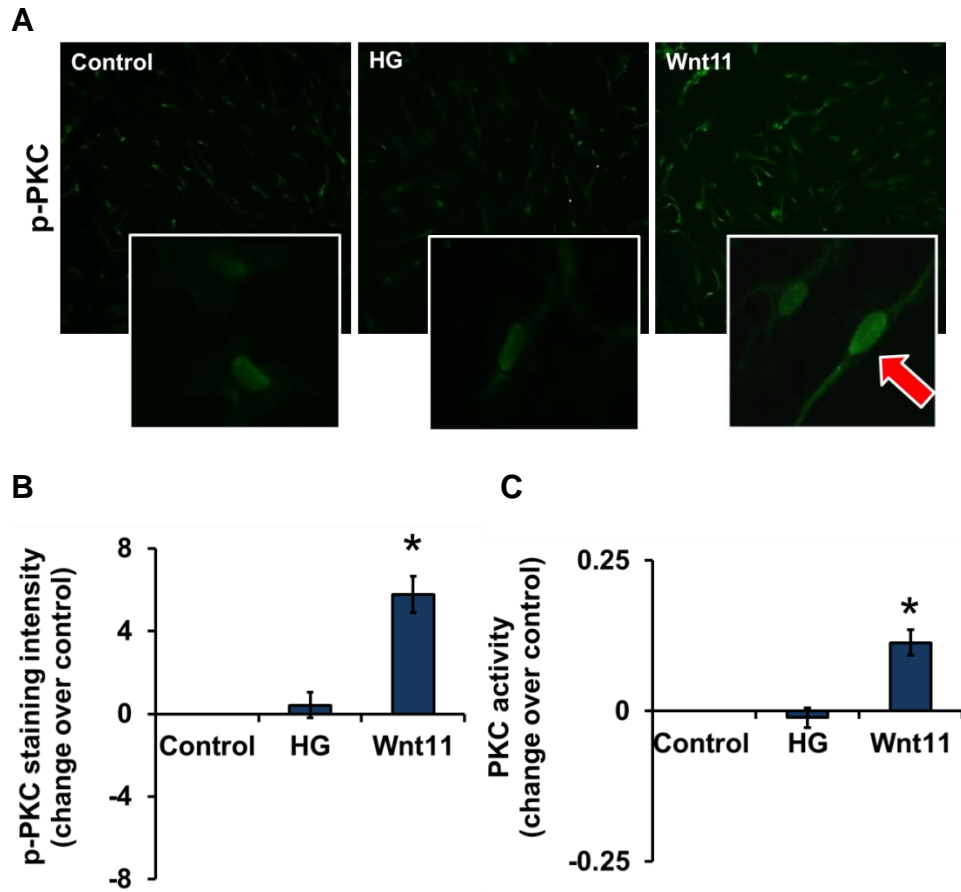
Figure 4.14: Wnt11 stimulates PKC. (A) We carried out immunostaining for p-PKC in human MPCs that had been treated with HG (25 mmol/L) or Wnt11 (50 ng/mL) for 24 hours in normal DMEM/10% FBS media. [Green = Alexa Fluorochrome 488; p-PKC; immunostaining at 10x magnification; insert at 40x; red arrow showing specific nuclear localization]. (B) We then compared the nuclear staining intensity using ImageJ and found a significant increase in the Wnt11 group as compared to control (data is represented as mean ± SEM; *p<0.05 compared to control). (C) We also measured PKC activity using an ELISA-based assay after treatment with HG or Wnt11 for 24 hours and showed significantly increased activity by Wnt11 only (*p<0.05 compared to control) [p-PKC=phospho-PKC].

4.3.9 Reduced CD133+ stem cells in diabetic bone marrow

In order to investigate whether the enhanced differentiation of MPCs to adipocytes is a direct reflection of alterations occurring in the stem cell niche, we examined CD133-expressing cell number in human control and type 2 diabetic bone marrow samples. Human samples are quite difficult to procure, and we could only obtain type 2 diabetic patient samples. However, this afforded us the ability to determine stem cell number unambiguously, as CD133 is specific to stem/progenitor cells in humans but not rodents. We used immunohistochemistry to stain for CD133+ cells that were devoid of CD45 reactivity, as well as CD133+/CD45+ HSCs. The rationale behind this was to determine alterations in cells with vasculogenic ability[16,21,22]. These subsets may comprise MPCs as well as cells that give rise to EPCs, as we have shown previously[16,21,23,24]. The qualitative analysis demonstrated a lower number of CD133+CD45- cells in the diabetic bone marrow (figure 4.15A and B). These findings highlight the potential functional consequence of increased bone marrow adiposity. The reduction in CD133-expressing cells may be due to depletion (i.e. MPCs differentiating into adipocytes, thereby reducing the number of CD133-expressing cells) or a non-permissive change in the stem cell microenvironment.
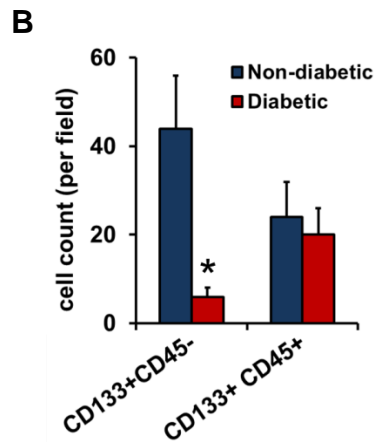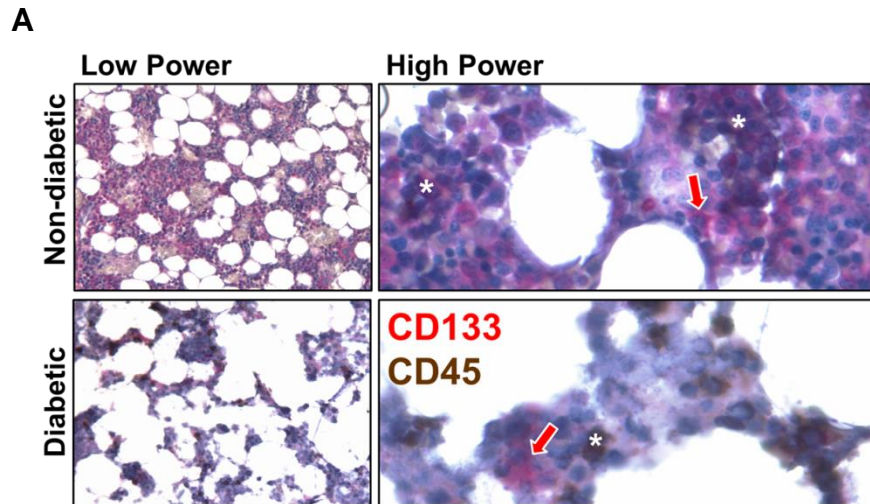
Figure 4.15: Vasculogenic stem cells are depleted in diabetic bone marrow. (A) Control (n = 3) and type 2 diabetic (n = 4) bone marrow samples were stained for vasculogenic stem/progenitor cells (CD133+CD45-) and HSCs (CD133+CD45+). (B) Staining was quantified by examining cell count per field, and confirmed a significantly lower number of CD133-expressing cells (negative for CD45) in diabetic bone marrow as compared to non-diabetic controls (data is represented as mean ± SEM; *p<0.05 compared to non-diabetic).

4.3.10 HG modulates Ang2 in the bone marrow

We looked at various factors that may be responsible for maintaining stem cells in a quiescent/undifferentiated state (figure 4.16A), including NO, stromal cell-derived factor-1 (SDF-1), Ang1 and Ang2[25]. Although we found no changes in NO, CXCL12 (SDF-1) or CXCR4 (SDF-1 receptor) (data not shown), we did note the dramatic elevation of Ang2 mRNA expression in diabetic bone marrow samples (figure 4.16B). Because Ang1 and Ang2 bind to the same Tie2 receptor but antagonize each other's actions[26,27], we examined the ratio of Ang1 to Ang2 in our samples and saw a significant reduction in diabetic bone marrow (figure 4.16C), indicating a possible role for Ang2 in altering homeostasis and disrupting the stem cell niche. Interestingly, diabetes is associated with vascular remodeling in the marrow[8] as well as inflammation[28], both reminiscent of Ang2 actions. Because of the predominant number of adipocytes in the marrow samples, we reasoned that adipocytes are the source of Ang2. In support of this concept, we induced the differentiation of MPCs into adipocytes in the presence or absence of high glucose. As expected, cells treated with HG exhibited significantly higher Ang2 levels (figure 4.16D). We also noted an increase in Tie2 mRNA in diabetic bone marrow (figure 4.16E), indicating a potential up-regulation of the antagonistic Ang-Tie signaling system in a high glucose setting.

Since type 2 diabetes is associated with increased insulin levels and dyslipidemia alongside hyperglycemia, we used a type 1 diabetic rat model to corroborate HG effect and Ang2 expression. We obtained femur bone tissues from 2-month-old diabetic and control rats (figure 4.17A) and stained for Ang2. A very distinct staining pattern was

noted in the diabetic samples, with Ang2 being localized to the marrow adipocytes

specifically (figure 4.17B). This staining pattern confirms the deregulation of Ang1/2

upon MPC differentiation, and indicates that the adipocytes themselves are upregulating

these proteins in response to HG. In addition, diabetic rat tissues displayed more

positivity for Ang2 than control tissues, highlighting the effect of hyperglycemia. Lastly,

we stained for Wnt11 to confirm a seminal role for this protein in HG-mediated

adipogenesis (figure 4.17C). We noted Wnt11 staining in stem/progenitor cells to be

clustered around the adipocytes. This localization pattern points to a cell autogenous

role of Wnt11 in initiating differentiation.
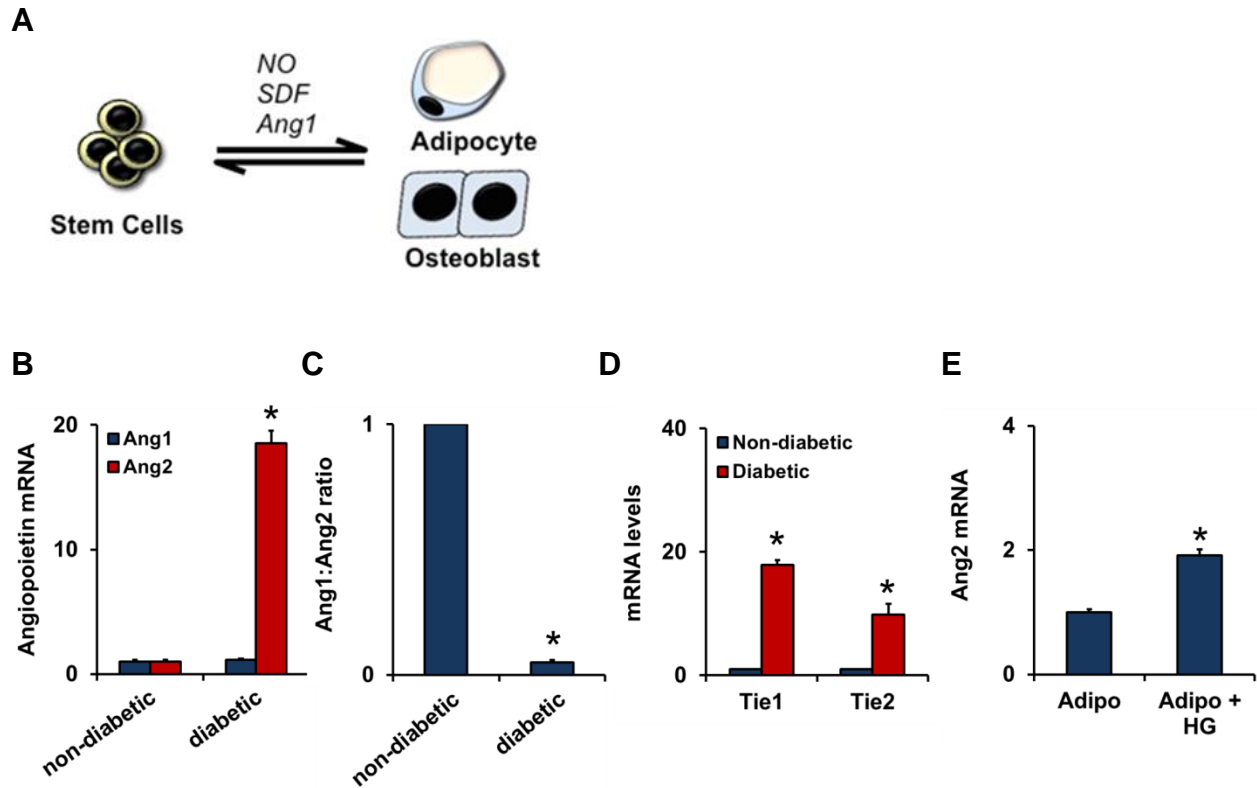
Figure 4.16: Diabetes modulates Ang2 to initiate disruption of the stem cell niche.
(A) Factors known to be necessary for maintaining stem/progenitor cells in a quiescent/undifferentiated state in the bone marrow stem cell niche. (B) Examining mRNA levels of these factors in human control (n = 3) and diabetic (n = 4) bone marrow samples revealed a significant elevation of Ang2 in diabetes (data is represented as mean ± SEM; *p<0.05 compared to non-diabetic). (C) Ang1:Ang2 ratio in control and diabetic samples indicated a substantially lower amount of Ang1 as compared to Ang2 (*p<0.05). (D) Tie1 and Tie2 mRNA was elevated in human diabetic bone marrow (*p<0.05). (E) To confirm the role of Ang2 in adipocytes specifically, we differentiated MPCs to the adipocyte lineage in the presence or absence of high glucose and examined mRNA levels of Ang2 after 7 days. Again, we noted the significant increase of Ang2 in the HG-treated samples (*p<0.05 compared to Adipo) [NO=nitric oxide; SDF=stromal cell-derived factor; Ang1/2=angiopoietin-1/2; Tie 1/2=receptor for Ang].
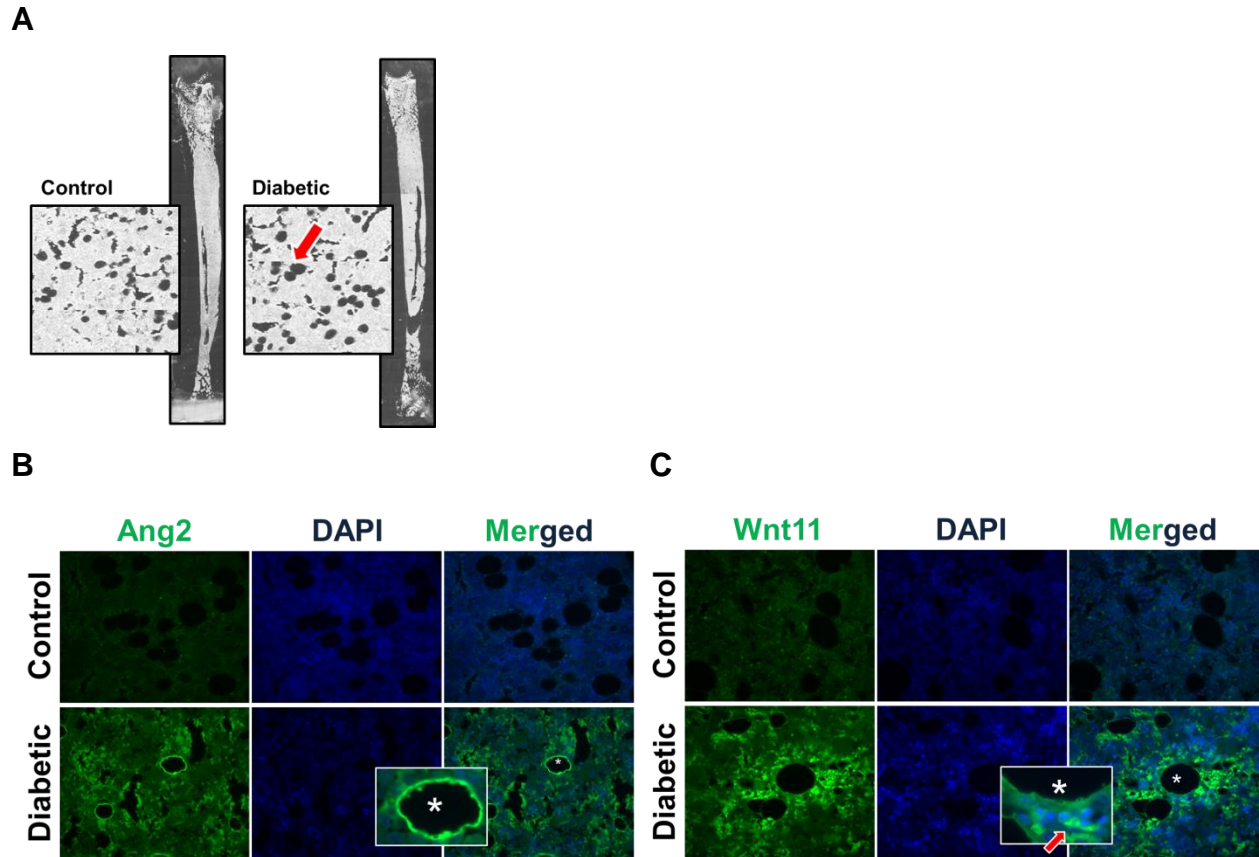
Figure 4.17: Localization of Ang2 and Wnt11 in diabetic rat marrow. (A) Phase contrast image of femur bone tissue from two month old type 1 diabetic (n = 5) and control (n = 6) rats (red arrow highlighting adipocytes). (B) Staining bone marrow for Ang2 revealed positivity on the membrane of adipocytes in the diabetic samples only [Green = Alexa Fluorochrome 488; Ang2, blue = DAPI for nuclear stain; 20x magnification; insert at 40x]. (C) In contrast, immunostaining for Wnt11 revealed localization to the stem/progenitor cells surrounding the adipocytes [Green = Alexa Fluorochrome 488; Wnt11, blue = DAPI for nuclear stain; 20x magnification; insert at 40x; red arrow highlighting Wnt11-positive progenitor cells] [Ang2=angiopoietin 2].

4.4   Discussion

The present study establishes the molecular mechanisms behind the skewing of MPC differentiation potential by high glucose levels and examines, in a broader sense, how this may participate in diabetic bone pathology. The salient findings of our study include: 1) HG "primes" MPCs, re-programming autocrine Wnt signaling, 2) there is a switch from canonical to non-canonical Wnt signaling in adipogenesis, and 3) the non-canonical Wnt11/PKC pathway is responsible for the skewing of lineage potential that is seen in MPCs in high glucose (figure 4.18).

Much of what we know about the process of adipogenesis comes from studies in rodent cell lines (e.g. 3T3-L1 mouse embryo-derived 'pre-adipocytes') and rodent multipotential cells. The process involves two phases: the initial commitment phase and the later terminal differentiation phase[29]. Wnt signaling has been linked to the initial commitment phase where canonical and non-canonical ligands have been shown to regulate adipogenesis-specific transcription factors. Addition of Wnt10b inhibits PPARγ and reduces adipogenesis, whereas knockout of LRP6 enhances adipogenesis[30,31]. Interestingly, this signaling pathway tightly regulates osteogenesis as well. Addition of Wnt10b has been shown to enhance osteogenesis in rodent bone marrow-derived ST2 (stromal cell line) cells[32]. Reports of both positive[33] and negative[34,35] regulation of adipogenesis by non-canonical Wnts are also ample. Early studies indicated a positive role for non-canonical Wnts in the regulation of bone mass[34,36,37].
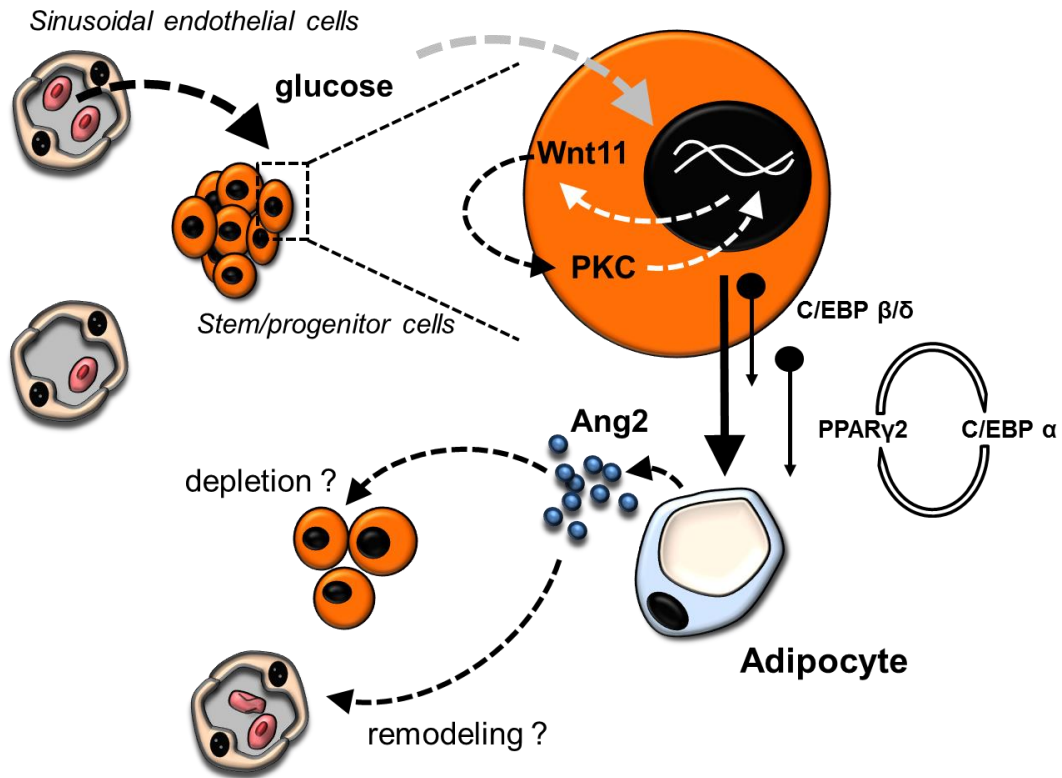
Figure 4.18: Schematic of seminal findings. HG causes upregulation of autogenous Wnt11 in MPCs to increase adipogenesis through the non-canonical Wnt/PKC pathway. Adipocytes themselves upregulate Ang2, which may be disrupting the bone marrow microenvironment through remodeling or directly causing depletion of CD133-expressing stem cells.

However, a more recent study has convincingly demonstrated enhanced bone resorption by non-canonical signaling[38].

We have provided experimental evidence that β-catenin protein is required for adipogenesis in human bone marrow-derived MPCs. This role of β-catenin is independent of transcription-mediated mechanisms. Interestingly, complete suppression of β-catenin induced an unexpected increase in adipogenesis and represented a switch from canonical to non-canonical Wnt signaling, evidenced by the dramatic elevation of Wnt11. Not only does this demonstrate a dose-dependent effect of β-catenin *in vitro*, but it was the first indication of non-canonical Wnt signaling as being a major player in adipogenesis. We also set out to examine the dual non-canonical pathways in order to establish a comprehensive understanding of Wnt signaling in the process of adipogenesis. While the Wnt/PCP pathway seems to inhibit adipogenesis, the Wnt/PKC pathway positively regulated this process. With the use of both a general PKC and PKCε-specific inhibitor, we were able to suppress adipogenesis, even in the presence of high glucose. Further studies must be done in order to elucidate whether essential adipogenic transcription factors PPARγ2 and c/EBPα are a direct target of PKC.

Since MPCs are an important cellular constituent of bone marrow and give rise to the mesenchymal lineage cells including osteoblasts, chondrocytes, and adipocytes, chronic hyperglycemia in diabetes may be altering the cellular makeup of the bone marrow through disrupting this cell type specifically. Bone loss and increased marrow adiposity have become hallmarks of the diabetic bone phenotype[1,2], though not much is known about the mechanisms behind these changes. Based on our findings, we can postulate that hyperglycemia induces Wnt11 in marrow MPCs leading to increased

143

adipogenesis and impaired osteogenesis. Enhanced adipogenesis is associated with elevated Ang2 expression. We know that Ang1-Tie2 signaling is involved in the long-term repopulation of bone marrow HSCs, crucial for the maintenance of the stem cell niche[39]. Elevated Ang2 in diabetes may disrupt Ang1-Tie2 signaling, reducing stem/progenitor cells in diabetic bone marrow. Elevated Ang2 may also play a role in microvascular remodeling and inflammation in the bone marrow of diabetic patients. These observations certainly warrant further studies on the functional significance of marrow Ang2 in diabetes.

4.5 References

1   Botolin, S. *et al.* Increased bone adiposity and peroxisomal proliferator-activated receptor-gamma2 expression in type I diabetic mice. *Endocrinology* **146**, 3622-3631, doi:en.2004-1677 [pii]10.1210/en.2004-1677 (2005).

2   Botolin, S. & McCabe, L. R. Bone loss and increased bone adiposity in spontaneous and pharmacologically induced diabetic mice. *Endocrinology* **148**, 198-205, doi:en.2006-1006 [pii]10.1210/en.2006-1006 (2007).

3   Bouillon, R. Diabetic bone disease. *Calcif Tissue Int* **49**, 155-160 (1991).

4   Hough, S. *et al.* Correction of abnormal bone and mineral metabolism in chronic streptozotocin-induced diabetes mellitus in the rat by insulin therapy. *Endocrinology* **108**, 2228-2234 (1981).

5   Glajchen, N. *et al.* Bone mineral metabolism in experimental diabetes mellitus: osteocalcin as a measure of bone remodeling. *Endocrinology* **123**, 290-295 (1988).

6   Schwartz, A. V. *et al.* Older women with diabetes have an increased risk of fracture: a prospective study. *J Clin Endocrinol Metab* **86**, 32-38 (2001).

7   de, L., II *et al.* Bone mineral density and fracture risk in type-2 diabetes mellitus: the Rotterdam Study. *Osteoporos Int* **16**, 1713-1720, doi:10.1007/s00198-005-1909-1 (2005).

8   Oikawa, A. *et al.* Diabetes mellitus induces bone marrow microangiopathy. *Arterioscler Thromb Vasc Biol* **30**, 498-508, doi:10.1161/ATVBAHA.109.200154ATVBAHA.109.200154 [pii] (2010).

145

9   Spinetti, G. *et al.* Global remodeling of the vascular stem cell niche in bone marrow of diabetic patients: implication of the microRNA-155/FOXO3a signaling pathway. *Circ Res* **112**, 510-522, doi:10.1161/CIRCRESAHA.112.300598CIRCRESAHA.112.300598 [pii] (2013).

10   Keats, E. & Khan, Z. A. Unique responses of stem cell-derived vascular endothelial and mesenchymal cells to high levels of glucose. *PLoS One* **7**, e38752, doi:10.1371/journal.pone.0038752PONE-D-12-03648 [pii] (2012).

11   Kestler, H. A. & Kuhl, M. From individual Wnt pathways towards a Wnt signalling network. *Philos Trans R Soc Lond B Biol Sci* **363**, 1333-1347, doi:10.1098/rstb.2007.2251U500443625H992N6 [pii] (2008).

12   Ross, S. E. *et al.* Inhibition of adipogenesis by Wnt signaling. *Science* **289**, 950-953, doi:8736 [pii] (2000).

13   Chocarro-Calvo, A., Garcia-Martinez, J. M., Ardila-Gonzalez, S., De la Vieja, A. & Garcia-Jimenez, C. Glucose-induced beta-catenin acetylation enhances Wnt signaling in cancer. *Mol Cell* **49**, 474-486, doi:10.1016/j.molcel.2012.11.022S1097-2765(12)00979-3 [pii] (2013).

14   Anagnostou, S. H. & Shepherd, P. R. Glucose induces an autocrine activation of the Wnt/beta-catenin pathway in macrophage cell lines. *Biochem J* **416**, 211-218, doi:10.1042/BJ20081426BJ20081426 [pii] (2008).

15   Lin, C. L. *et al.* Wnt/beta-catenin signaling modulates survival of high glucose-stressed mesangial cells. *J Am Soc Nephrol* **17**, 2812-2820, doi:ASN.2005121355 [pii]10.1681/ASN.2005121355 (2006).

16   Khan, Z. A. *et al.* Endothelial progenitor cells from infantile hemangioma and umbilical cord blood display unique cellular responses to endostatin. *Blood* **108**, 915-921, doi:108/3/915 [pii]10.1182/blood-2006-03-006478 (2006).

17   Chen, B. *et al.* Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nat Chem Biol* **5**, 100-107, doi:10.1038/nchembio.137nchembio.137 [pii] (2009).

18   Koh, C. G. Rho GTPases and their regulators in neuronal functions and development. *Neurosignals* **15**, 228-237, doi:000101527 [pii]10.1159/000101527 (2006).

19   Wojciak-Stothard, B. & Ridley, A. J. Rho GTPases and the regulation of endothelial permeability. *Vascul Pharmacol* **39**, 187-199, doi:S1537189103000089 [pii] (2002).

20   Webb, P. R., Doyle, C. & Anderson, N. G. Protein kinase C-epsilon promotes adipogenic commitment and is essential for terminal differentiation of 3T3-F442A preadipocytes. *Cell Mol Life Sci* **60**, 1504-1512, doi:10.1007/s00018-003-2337-z (2003).

21   Melero-Martin, J. M. *et al.* In vivo vasculogenic potential of human blood-derived endothelial progenitor cells. *Blood* **109**, 4761-4768, doi:blood-2006-12-062471 [pii]10.1182/blood-2006-12-062471 (2007).

22   Khan, Z. A. *et al.* Multipotential stem cells recapitulate human infantile hemangioma in immunodeficient mice. *J Clin Invest* **118**, 2592-2599, doi:10.1172/JCI33493 (2008).

23   Keats, E. C. & Khan, Z. A. Vascular stem cells in diabetic complications:

evidence for a role in the pathogenesis and the therapeutic promise. *Cardiovasc*

Diabetol **11**, 37, doi:10.1186/1475-2840-11-371475-2840-11-37 [pii] (2012).

24   Melero-Martin, J. M. *et al.* Engineering robust and functional vascular networks in

vivo with human adult and cord blood-derived progenitor cells. *Circ Res* **103**,

194-202, doi:10.1161/CIRCRESAHA.108.178590CIRCRESAHA.108.178590 [pii]

(2008).

25   Frenette, P. S., Pinho, S., Lucas, D. & Scheiermann, C. Mesenchymal stem cell:

keystone of the hematopoietic stem cell niche and a stepping-stone for

regenerative medicine. *Annu Rev Immunol* **31**, 285-316, doi:10.1146/annurev-

immunol-032712-095919 (2013).

26   Gomei, Y. *et al.* Functional differences between two Tie2 ligands, angiopoietin-1

and -2, in regulation of adult bone marrow hematopoietic stem cells. *Exp*

Hematol **38**, 82-89, doi:10.1016/j.exphem.2009.11.007S0301-472X(09)00451-2

[pii] (2010).

27   Maisonpierre, P. C. *et al.* Angiopoietin-2, a natural antagonist for Tie2 that

disrupts in vivo angiogenesis. *Science* **277**, 55-60 (1997).

28   Yellowlees Douglas, J. *et al.* Bone marrow-CNS connections: implications in the

pathogenesis of diabetic retinopathy. *Prog Retin Eye Res* **31**, 481-494,

doi:10.1016/j.preteyeres.2012.04.005S1350-9462(12)00030-4 [pii] (2012).

29   Cristancho, A. G. & Lazar, M. A. Forming functional fat: a growing understanding

of adipocyte differentiation. *Nat Rev Mol Cell Biol* **12**, 722-734,

doi:10.1038/nrm3198nrm3198 [pii] (2011).

148

30   Kawai, M. *et al.* Wnt/Lrp/beta-catenin signaling suppresses adipogenesis by inhibiting mutual activation of PPARgamma and C/EBPalpha. *Biochem Biophys Res Commun* **363**, 276-282, doi:S0006-291X(07)01819-0 [pii]10.1016/j.bbrc.2007.08.088 (2007).

31   Longo, K. A. *et al.* Wnt10b inhibits development of white and brown adipose tissues. *J Biol Chem* **279**, 35503-35509, doi:10.1074/jbc.M402937200M402937200 [pii] (2004).

32   Kang, S. *et al.* Wnt signaling stimulates osteoblastogenesis of mesenchymal precursors by suppressing CCAAT/enhancer-binding protein alpha and peroxisome proliferator-activated receptor gamma. *J Biol Chem* **282**, 14515-14524, doi:M700030200 [pii]10.1074/jbc.M700030200 (2007).

33   Kanazawa, A. *et al.* Wnt5b partially inhibits canonical Wnt/beta-catenin signaling pathway and promotes adipogenesis in 3T3-L1 preadipocytes. *Biochem Biophys Res Commun* **330**, 505-510, doi:S0006-291X(05)00480-8 [pii]10.1016/j.bbrc.2005.03.007 (2005).

34   Takada, I. *et al.* A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. *Nat Cell Biol* **9**, 1273-1285, doi:ncb1647 [pii]10.1038/ncb1647 (2007).

35   Wakabayashi, K. *et al.* The peroxisome proliferator-activated receptor gamma/retinoid X receptor alpha heterodimer targets the histone modification enzyme PR-Set7/Setd8 gene and regulates adipogenesis through a positive feedback loop. *Mol Cell Biol* **29**, 3544-3555, doi:10.1128/MCB.01856-08MCB.01856-08 [pii] (2009).

36   Chang, J. *et al.* Noncanonical Wnt-4 signaling enhances bone regeneration of mesenchymal stem cells in craniofacial defects through activation of p38 MAPK. *J Biol Chem* **282**, 30938-30948, doi:M702391200 [pii]10.1074/jbc.M702391200 (2007).

37   Tu, X. *et al.* Noncanonical Wnt signaling through G protein-linked PKCdelta activation promotes bone formation. *Dev Cell* **12**, 113-127, doi:S1534-5807(06)00508-9 [pii]10.1016/j.devcel.2006.11.003 (2007).

38   Maeda, K. *et al.* Wnt5a-Ror2 signaling between osteoblast-lineage cells and osteoclast precursors enhances osteoclastogenesis. *Nat Med* **18**, 405-412, doi:10.1038/nm.2653nm.2653 [pii] (2012).

39   Arai, F. *et al.* Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* **118**, 149-161, doi:10.1016/j.cell.2004.07.004S0092867404006622 [pii] (2004).

CHAPTER 5 – SUMMARY AND FUTURE DIRECTIONS

5.1 Overall findings and implications

Long-term diabetes manifests in a vast array of secondary complications, and throughout my studies I have touched on a number of these. Complications arise due to micro- and macro-angiopathies in the target organs. It would then be the most clinically relevant to target the vasculature in order to prevent these complications. For this reason, my initial studies focused on vascular stem cells – cells that have the capacity to differentiate into mature, functional cells of the vascular wall. I investigated the effects of HG on derivatives of VSCs- EPCs and MPCs. While I found that late-outgrowth EPCs are able to evade the negative effects of HG, MPCs displayed some functional impairment. This included skewing of their differentiation potential to the adipocyte lineage, the basis of the second portion of my studies. Recently, Wnt signaling has been deemed a major regulator of MPC differentiation. Then, to investigate the mechanism behind the HG-induced changes in MPCs, I employed a comprehensive approach to studying Wnt signaling, examining both the canonical (β-catenin dependent) and non-canonical (β-catenin independent) pathways. I have challenged the conventional perception of canonical/β-catenin-mediated signaling on MPC differentiation. Importantly, I have also established the seminal role of Wnt11 in mediating the effects of HG by signaling through the Wnt/Ca^{2+}/PKC pathway. Not only is this mechanism relevant in regards to MPC fate, but it provides an important link between the HG-effect and phenotypic alterations occurring in the diabetic bone marrow.

Overall, the implications of these studies are numerous and warrant further investigation in order to become clinically feasible. Importantly, we did not report any major dysfunctions in our HG-treated EPCs. This progenitor population, then, should retain the ability to create vascular networks *in vivo*, even in the diabetic setting. However, blood vessels are composed of two interacting cell types: ECs and perivascular cells. Both are necessary for proper functioning of vascular networks and maintenance of tissue dynamics. Following our intensive characterization of both EPCs and MPCs in a high glucose setting, it appears that MPCs may be responsible for the inept vascular repair mechanisms that are noted in long-term diabetes. Their inability to migrate and altered differentiation potential indicates that in a situation of neovascularization, the supportive role of the perivascular cell is likely missing. Despite these alterations, vascular dysfunctions could be corrected by the *ex vivo* expansion of autologous EPCs and MPCs with subsequent implantation of both cell types into the site of injury. Direct, versus systemic, injection of the progenitor populations would evade problems associated with MPC migration in high glucose. Neovascularization using a single cell suspension nestled in a gel scaffold (fibronectin//collagen/matrigel) has proven to be successful in the nude mouse model[1,2]. The presence of red blood cells within the lumens of the new vessels indicated that functional anastomoses had formed between the implanted cells and host vasculature. The overall impact of creating functional and stable vascular networks *in vivo* would have far-reaching consequences. The prospect of cell therapy and tissue engineering would become a much more accessible goal; one that is currently unattainable due to our lack of consensus on how to immediately perfuse these tissues. Additionally, we would retain the ability to target

individual sites of vascular dysfunction, such as in chronic diabetic complications. While this field has made many leaps in recent years, additional effort must be focused on the preservation of implants and the long-term effects of these types of procedures.

The notion of MPC dysfunction in diabetes is a novel concept; because of their immunomodulatory properties and multi-lineage potential, many groups have focused solely on the therapeutic potential of this progenitor cell population. MPCs in diabetic animals have been shown to improve wound healing[3], cardiac function[4], nephropathy[5] and neuropathy[6,7]. However, these beneficial effects are mostly owing to the paracrine role of MPCs, such as their ability to secrete the angiogenic cytokines VEGF and bFGF. Although MPCs are certainly useful in various clinical situations, the long-term *in vivo* effects of high glucose on this cell type have been largely overlooked. While we previously demonstrated that growth is not altered after 12 days in HG, we also indicated their enhanced differentiation to adipocytes. Increased adipogenesis correlated with a decrease in both osteogenesis and chondrogenesis, bringing into question the possible concurrent regulation of MPC progeny in HG.

As MPCs are derived from bone marrow, it is not surprising that these observations support the typical bone phenotype of diabetic patients. There is hope that with the arrest of excessive adipogenesis, there will be a natural correction of the various osteogenic dysfunctions that present with chronic diabetes. Many attempts have been made to reverse these disorders by targeting the primary transcription factor involved in adipogenesis, PPARγ. While there has been some success in this area – as well as some evidence to dispute its relevance – in a long-term clinical situation, blocking the actions of PPARγ will have negative consequences on lipid metabolism.

The gamma isoform of PPAR is found predominantly in adipose tissues and is activated by fatty acids or their derivatives to regulate insulin sensitivity. It is therefore not surprising that PPARγ is the molecular target for the Thiazolidinedione class of anti-diabetic drugs. Inhibiting this receptor in diabetic patients, then, may help evade some bone disorders, but at the considerable cost of initiating or accelerating severe, insulin-resistant type 2 diabetes and the metabolic syndrome. Thus, targets upstream to PPARγ should be investigated in order to successfully block adipogenesis without affecting the lipid metabolism properties of this receptor. We have presented a novel mechanism, designating the non-canonical Wnt/Ca^{2+}/PKC pathway as a major regulator of adipogenesis. We have also provided evidence that HG enhances adipogenesis by activating Wnt11 to initiate this signaling cascade. If we could disrupt the actions of Wnt11, this will not only inhibit the HG-induced increase in adipogensis, but may correct for associated osteogenic dysfunctions without altering PPARγ. To date, Wnt11 has been under-studied. There have been some reports of its involvement in cardiomyocyte differentiation[8-10], as well as retinoic acid-induced neuronal differentiation[11]. Others have indicated a role for Wnt11 in cancer cell migration[12,13]. However, no one has studied the effects of Wnt11 knockdown on adipocyte differentiation. While it is overly generous to conclude that modulation of the Wnt/PKC pathway will solve all diabetic complications, it is certainly an avenue of research that is worth exploring. There is hope that with an understanding of the complete picture of MPC differentiation, we will one day have the capability to tailor it specifically to a patient's needs.

At the epicentre of all of my studies lies the VSC. Regarding VSCs, two important concepts should be noted. Firstly, this cell type is functionally distinct from the

hematopoietic population in the bone marrow. Secondly, VSCs are responsible for postnatal vasculogenesis and, as such, differentiate into both EPCs and MPCs. Taken together, my studies focus on the bone marrow stem cell niche and suggest the premature differentiation of VSCs into adipoctyes in the diabetic condition. An excessive increase in adipocytes may modify the microenvironment, further destabilizing stem/progenitor cells and causing their activation from a quiescent state. In a slightly different scenario, HG is directly altering the niche environment through increased adipocyte-derived Ang2. Ang2 itself may play a role in vascular remodeling and inflammation. As a result, cellular homeostasis will be disrupted and vasculogenic stem/progenitor cells will become severely depleted. MPCs have previously been implicated in maintaining the hematopoietic stem cell niche through their structural support and elaboration of growth factors[14]. It seems, then, that there is an intricate balance that exists to support this niche, and it is one that may easily tip with the disruption of a single constituent. The decrease in VSC number accounts for the bone phenotype that is seen with chronic diabetes, and may also explain why vascular repair mechanisms are consistently falling short.

5.2   Limitations

Although we have successfully revealed several novel findings regarding diabetic complications, no study is without limitations. Because we work with human-derived stem cells, they must first be isolated from blood/bone marrow and expanded in culture. We are limited in our ability to recapitulate the complexity of an *in vivo* environment, meaning that we may be altering characteristics of our isolated cells through chemical and physical growth conditions that are necessary for their survival in culture. This concept is illustrated in chapter 2, where the outcomes of our EPC functional assays were quite varied from many other published results. The likely culprit for these differences was our use of 20% fetal bovine serum, when many other groups choose to use 1% - indicating how even one component of growth medium can alter a cell's behaviour. The extracellular matrix has been shown to control stem cell fate through variations in microenvironment elasticity or stiffness[15]. Because our MPCs were seeded on plastic culture plates, we cannot rule out the possibility that crosstalk between stem cells and matrix *in vivo* may allow for a different outcome when considering differentiation in HG. Further studies will require the incorporation of matrix events to obtain a clear picture of stem cell properties. It would be interesting to examine whether pre-commitment of MPCs to the osteogenic lineage through matrix stiffness could partially overcome the effects of HG.

Another limitation to our studies is the lack of extensive *in vivo* experimentation. Although we included some immunostaining data, it would be beneficial to provide evidence of a similar situation (increased marrow adipogenesis and decreased

osteogenesis) and mechanism occurring in a human type 1 diabetic marrow sample.

We would also like to increase our sample size in order to draw stronger conclusions.

For future manipulation studies of the Wnt/PKC pathway, the streptozotocin-treated type

1 diabetic mouse would be useful due to its ability to replicate the chronic complications

of human diabetes. Using this model, we could effectively establish the impact of

blocking adipogenesis on ostoegenesis, and vice versa. However, we have to keep in

mind the variation that can present across species and even within a species (i.e. some

murine MPCs have been shown to differ in terms of behaviour and marker

expression[16,17]). Certainly, *in vivo* studies are a valuable tool for furthering your studies,

but do not deduct from the importance of utilizing human-derived cells as a model

system.

5.3 Future Directions

In order to efficiently combat the outlined diabetic dysfunctions, it would be most beneficial to target the conversion of stem/progenitor cells to adipocytes (figure 5.1). Hypothetically, this should resolve alterations occurring in the stem cell niche, increasing the number of vasculogenic stem cells available for vascular repair while rectifying the osteoporotic disorders associated with chronic diabetes.
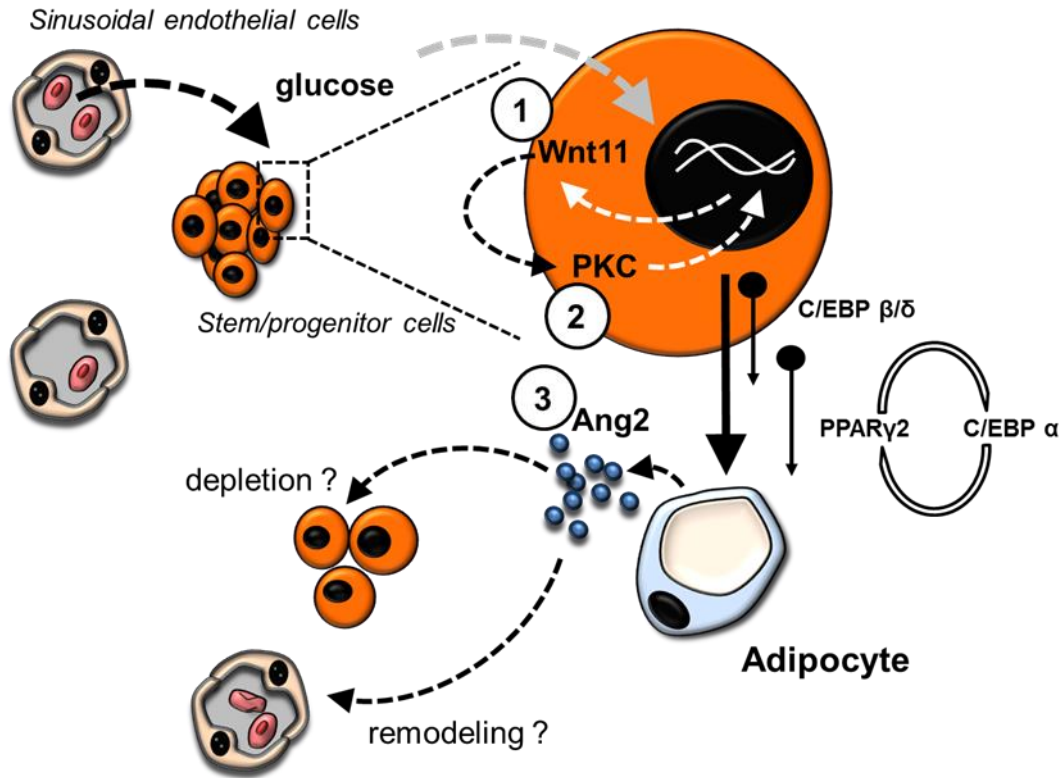
Figure 5.1: Potential molecular targets for diabetic complications. We would like to inhibit the conversion of CD133+ stem cells to adipocytes in an attempt to rectify the altered homeostasis in diabetic bone marrow. Potential targets for therapy are highlighted: 1) Wnt11 normalization, 2) PKC activity inhibition, and 3) reversal of the Ang1/Ang2 ratio.

5.3.1 Ang/Tie signaling

The next step in this research project should focus on our intriguing results surrounding the Ang-Tie signaling system. We have provided evidence to suggest that Ang2 is a direct target of HG, and is up-regulated by marrow adipocytes. This factor may be responsible for disrupting cellular homeostasis, causing the differentiation of undifferentiated stem cells in the bone marrow niche. Ang2, then, could be a promising candidate molecule for effectively blocking this activation.

5.3.2 PKC profiling

The PKC family consists of at least 12 isoforms that differ in terms of expression, localization, and functionality[18]. While we have indicated a potential role for PKCε in adipogenesis, it will be important to profile PKC isoforms to establish a clear understanding of the Wnt/PKC pathway in this process. In addition, more work must be done to determine the downstream targets of PKC. We have shown its nuclear localization after Wnt11 treatment, but it would be valuable to uncover its binding partners and gene targets.

5.3.3 Targeting Wnt11

We have uncovered a novel and seminal role for Wnt11 in mediating the effects of HG on MPC differentiation. We have also demonstrated its dramatic upregulation in

160

the case of decreased β-catenin signaling, linking the canonical and non-canonical Wnt pathways in this process. We would like to establish whether Wnt11 plays a role in adipogenesis in the absence of high glucose, or if its upregulation is a direct effect of diabetes. The next step in Wnt11 studies should utilize siRNA/shRNA to block its expression. We can then study the effect of knockdown on MPC differentiation to adipocytes, as well as attempt to normalize the HG-mediated increase in adipocytes.

5.3.4 Concurrent regulation of adipo/osteogenesis in HG

It would also be valuable to investigate further the effects of HG on osteogenesis. Our preliminary results demonstrated the inhibition of canonical Wnt signaling in HG-treated osteoblasts (data not shown), resulting in a lower number when compared to control. We would like to conclusively state whether this was due to an increase in adipogenic cues in these cells, or if HG was acting independently to inhibit the process of osteogenesis. It would be relevant to study mRNA levels of *both* adipogenic and osteogenic transcription factors in cells that have been differentiated along each lineage in the presence of HG. When considering treatment options, these results will have various implications (i.e. if regulation proves to be interdependent and we were attempting to inhibit HG-mediated adipogenesis, the result could be a drastic, and unwanted, osteogenic response). For this reason, it may be more relevant to target VSCs directly in order to avoid having to correct for the response of MPC progeny.

5.3.5 Crosstalk in the stem cell niche

To further examine alterations that are occurring in the diabetic stem cell niche, it would be of interest to replicate the microenvironment in an *in vitro* setting. To date, the consequence of matrix proteins on the outlined process of HG-induced adipogenesis is unknown. Discerning the spatial organization of the various cellular components would allow us a better understanding of crosstalk mechanisms, and may provide insight into new targets for inhibiting the activation of VSCs.

5.3.6 Time-course in diabetic mouse model

Lastly, we would like to test whether our results are mirrored in an *in vivo* model. A time course experiment in diabetic mice over the several months would allow us to pinpoint when changes in the bone marrow are taking place, and in which order they occur. To establish this novel mechanism of increased adipogenesis (Wnt11/PKC signaling) in a rodent model would be an important next step for the eventual translation of science to clinical practice.

5.4   References

1   Melero-Martin, J. M. *et al.* Engineering robust and functional vascular networks in vivo with human adult and cord blood-derived progenitor cells. *Circ Res* **103**, 194-202, doi:10.1161/CIRCRESAHA.108.178590 CIRCRESAHA.108.178590 [pii] (2008).

2   Lin, R. Z. & Melero-Martin, J. M. Bioengineering human microvascular networks in immunodeficient mice. *J Vis Exp*, e3065, doi:10.3791/3065 3065 [pii] (2011).

3   Kwon, D. S. *et al.* Treatment with bone marrow-derived stromal cells accelerates wound healing in diabetic rats. *Int Wound J* **5**, 453-463, doi:10.1111/j.1742-481X.2007.00408.x IWJ408 [pii] (2008).

4   Abdel Aziz, M. T. *et al.* Effect of bone marrow-derived mesenchymal stem cells on cardiovascular complications in diabetic rats. *Med Sci Monit* **14**, BR249-255, doi:869439 [pii] (2008).

5   Ezquer, F. E. *et al.* Systemic administration of multipotent mesenchymal stromal cells reverts hyperglycemia and prevents nephropathy in type 1 diabetic mice. *Biol Blood Marrow Transplant* **14**, 631-640, doi:10.1016/j.bbmt.2008.01.006 S1083-8791(08)00050-5 [pii] (2008).

6   Shibata, T. *et al.* Transplantation of bone marrow-derived mesenchymal stem cells improves diabetic polyneuropathy in rats. *Diabetes* **57**, 3099-3107, doi:10.2337/db08-0031

db08-0031 [pii] (2008).

7   Kinnaird, T. *et al.* Local delivery of marrow-derived stromal cells augments

collateral perfusion through paracrine mechanisms. *Circulation* **109**, 1543-1549,

doi:10.1161/01.CIR.0000124062.31102.57

01.CIR.0000124062.31102.57 [pii] (2004).

8   He, Z. *et al.* Transduction of Wnt11 promotes mesenchymal stem cell

transdifferentiation into cardiac phenotypes. *Stem Cells Dev* **20**, 1771-1778,

doi:10.1089/scd.2010.0380 (2011).

9   Abdul-Ghani, M. *et al.* Wnt11 promotes cardiomyocyte development by caspase-

mediated suppression of canonical Wnt signals. *Mol Cell Biol* **31**, 163-178,

doi:10.1128/MCB.01539-09

MCB.01539-09 [pii] (2011).

10   Nagy, II *et al.* Wnt-11 signalling controls ventricular myocardium development by

patterning N-cadherin and beta-catenin expression. *Cardiovasc Res* **85**, 100-109,

doi:10.1093/cvr/cvp254

cvp254 [pii] (2010).

11   Elizalde, C. *et al.* Distinct roles for Wnt-4 and Wnt-11 during retinoic acid-induced

neuronal differentiation. *Stem Cells* **29**, 141-153, doi:10.1002/stem.562 (2011).

12   Nishioka, M. *et al.* Possible involvement of Wnt11 in colorectal cancer

progression. *Mol Carcinog* **52**, 207-217, doi:10.1002/mc.21845 (2013).

13   Dwyer, M. A. *et al.* WNT11 expression is induced by estrogen-related receptor

alpha and beta-catenin and acts in an autocrine manner to increase cancer cell

migration. *Cancer Res* **70**, 9298-9308, doi:10.1158/0008-5472.CAN-10-0226

0008-5472.CAN-10-0226 [pii] (2010).

14   Yin, T. & Li, L. The stem cell niches in bone. *J Clin Invest* **116**, 1195-1201,

doi:10.1172/JCI28568 (2006).

15   Engler, A. J., Sen, S., Sweeney, H. L. & Discher, D. E. Matrix elasticity directs

stem cell lineage specification. *Cell* **126**, 677-689, doi:S0092-8674(06)00961-5

[pii]

10.1016/j.cell.2006.06.044 (2006).

16   Sung, J. H. *et al.* Isolation and characterization of mouse mesenchymal stem

cells. *Transplant Proc* **40**, 2649-2654, doi:10.1016/j.transproceed.2008.08.009

S0041-1345(08)01059-2 [pii] (2008).

17   Fiorina, P. *et al.* Immunomodulatory function of bone marrow-derived

mesenchymal stem cells in experimental autoimmune type 1 diabetes. *J Immunol*

183, 993-1004, doi:10.4049/jimmunol.0900803

jimmunol.0900803 [pii] (2009).

18   Nishizuka, Y. Intracellular signaling by hydrolysis of phospholipids and activation

of protein kinase C. *Science* **258**, 607-614 (1992).

<div align="center">**Curriculum Vitae**</div>

PERSONAL INFORMATION

Name: Emily C. Keats

Revised: July, 2013

EDUCATION

A) Universities Attended:
2009-   Ph.D.   Western University, London ON
                       Field/Discipline: Pathology
                       Thesis title: Vascular stem cells in diabetic complications
                       Thesis advisor: Zia A. Khan, PhD

2004-2008   B.Sc.   McGill University, Montreal, QC
                       Field/Discipline: Anatomy and Cell Biology

DISTINCTIONS, HONOURS, FELLOWSHIPS, SCHOLARSHIPS

06-2013   Till & McCulloch Trainee Award, Banff AB
04-2013   Dr. M Daria Haust Award, Western University, London ON
11-2012   1st Prize for Best Poster Presentation at the 3rd Annual Diabetes Research Day 2012, London ON
2012-2013   Queen Elizabeth II Graduate Scholarships in Science and Technology, Ministry of Training, Colleges, and Universities, Ontario
09-2012   Dutkevich Memorial Foundation Travel Award, Western University, London ON
05-2012   Dr. Cameron Wallace Award, Western University, London ON
05-2012   Dr. M Daria Haust Award, Western University, London ON
04-2012   Till & McCulloch Meetings Trainee Award 2012, Montreal QC
03-2012   Best "Clinical Investigation and Metabolic Disease" presentation, 2012 London Heath Research Day, London ON
11-2011   Nominated, Canadian Institutes of Health Research Vanier Award 2011
11-2011   Best Basic Science Presentation (2nd Prize) at the 2nd Annual Diabetes Research Day 2011, London ON
06-2011   Dutkevich Memorial Foundation Travel Award, Western University, London ON
2011-2012   Lawson Research Fund, Graduate Student Award. Lawson Health Research Institute, London ON
2010-2013   Western Graduate Research Scholarship, Western University, London ON
2010-2013   Schulich Graduate Scholarship, Western University, London ON

UNIVERSITY and HOSPITAL COMMITTEE MEMBERSHIPS

A) University Committee Memberships:
2011-2013   Vice Chair, Western Pathology Association, Western University, London ON

<div align="center">166</div>

2011-2013	Communications Committee, Department of Pathology, Western University, London ON
2007-2009	Chair, Awareness Committee, WaterCan Charity Organization, McGill Chapter, Montreal QC
2007-2009	Canadian Foundation for AIDS Research (CANFAR), McGill University, Montreal QC
2007-2008	Science Undergraduate Society, Environmental Policy Commissioner Committee, McGill University, Montreal QC
2005-2007	Peer Mentoring Program, McGill University, Montreal QC

B) Hospital Committee Memberships:

N/A

INVITED LECTURES/PRESENTATIONS

2011-10-12	"Critical review of journal articles", presented to PathTox 4980E, Western University, London ON
2010-10-06	"Journal Critique", PathTox 4980E, Western University, London ON

OTHER PROFESSIONAL ACTIVITIES

2012-2013	Teaching Assistant, Pathology 2420A, Western University, London ON
2011-2012	Teaching Assistant, Pathology 2420A, Western University, London ON
2010-2011	Teaching Assistant, Pathology 2420A, Western University, London ON
2008-2009	Teaching Assistant, Histology, McGill University, Montreal QC

PUBLICATIONS

Publication Summary:

	Published/ In press	Submitted/ (in preparation)	Career Totals
Book Chapters	-	-	-
Peer-reviewed Articles	7	- (1)	7
Abstracts (National/International)	9	-	9
Abstracts (Regional)	12	-	12
			28

ARTICLES IN PEER-REVIEWED JOURNALS

1. Kleiman A, **Keats EC**, Chan NG, and Khan ZA. Elevated IGF2 prevents leptin induction and terminal adipocyte differentiation in hemangioma stem cells. Exp Mol Pathol. 94(1):126-36, 2013.
2. Chen S, Chakrabarti R, **Keats EC**, Chen M, Chakrabarti S, Khan ZA. Regulation of vascular endothelial growth factor expression by extra domain B segment of fibronectin in endothelial cells. Invest Ophthalmol Vis Sci. 53(13):8333-43, 2012.

3. Welten CM, **Keats EC**, Ang LC, and Khan ZA. Hemangioblastoma stromal cells show committed stem cell phenotype. Can J Neurol Sci. 39, 821-827, 2012.
4. **Keats E** and Khan ZA. Unique responses of stem cell-derived vascular endothelial and mesenchymal cells to high levels of glucose. PLoS ONE 7: e38752, 2012.
5. Roach EE, Chakrabarti R, Park NI, **Keats EC**, Yip J, Chan NG, and Khan ZA. Intrinsic regulation of hemangioma involution by platelet-derived growth factor. Cell Death Dis. 3: e328, 2012.
6. Kleiman A, **Keats EC**, Chan NG, and Khan ZA. Evolution of hemangioma endothelium. Exp Mol Pathol. 93:264-272, 2012.
7. **Keats EC** and Khan ZA. Vascular stem cells in diabetic complications: evidence for a role in the pathogenesis and the therapeutic promise. Cardiovasc Diabetol 11(1): 37, 2012. *******"Highly accessed" within 2 weeks of online publication.*

ARTICLES SUBMITTED
1. **Keats EC**, Dominguez JM, Grant MB, and Khan ZA. Glucose activates non-canonical Wnt signaling leading to enhanced adipogenesis in mesenchymal progenitor cells**.** ***Submitted to:*** Cell Metabolism, July 2013 [CELL-METABOLISM-D-13-00514]

ARTICLES IN PREPARATION
1. Wilkins E, Park NI, **Keats EC**, Darling MR, and Khan ZA. Increased expression of kallikrein 7 in mucoepidermoid carcinoma. Manuscript in preparation. ***Planned submission:*** August 2013

ABSTRACTS and PROFESSIONAL PRESENTATIONS

a) National/International Meetings

1. **Keats EC** and Khan ZA. Essential role of Wnt signaling in mesenchymal progenitor cell differentiation to adipocytes in high glucose. Accepted for presentation at the Till & McCulloch Meetings, October 2013.
2. **Keats EC** and Khan ZA. Essential and unexpected role of Wnt signaling pathway in high glucose-induced mesenchymal progenitor cell differentiation. Accepted for presentation at the 2013 CDA/CSEM Professional Conference and Annual Meetings.
3. **Keats EC** and Khan ZA. High levels of glucose cause cell cycle arrest and modify Wnt signalling to skew the differentiation of mesenchymal progenitor cells. Presented at the CDA/CSEM Professional Conference and Annual Meetings, October 2012.
4. **Keats EC** and Khan ZA. Unique cellular responses of stem cell-derived vascular cells to high levels of glucose. Presented at the Till & McCulloch Meetings, April 2012.
5. Kleiman A, **Keats EC**, and Khan ZA. Insulin-like growth factor-2 provides autocrine signals for stem cell expansion and prevents full cellular differentiation in infantile hemangioma. Presented at the Till & McCulloch Meetings, April 2012.
6. **Keats EC** and Khan ZA. High glucose enhances adipogenesis in human mesenchymal stem cells without altering their growth, proliferation, and migratory capacity. *Can J Diabetes* 35, A127, 2011.
7. **Keats EC** and Khan ZA. High glucose causes impairment of mature endothelial cells but not adult endothelial progenitor cells. *Can J Diabetes* 35, A126, 2011.
8. **Keats EC** and Khan ZA. Unique cellular responses of adult blood-derived endothelial progenitor cells and mature endothelial cells to high glucose. *Diabetes* 60 (supp 1),

1624, 2011
9. **Keats EC** and Khan ZA. High levels of glucose cause increased matrix protein production by human mesenchymal stem cells without altering cell growth, proliferation, and differentiation. *Diabetes* 60 (supp 1), 1621, 2011

B) Regional Meetings (workshops, symposia, research days)

1. **Keats EC** and Khan ZA. Mesenchymal progenitor cell differentiation by selective modulation of the Wnt pathway in diabetes. Presented at the London Health Research Day, March 2013.
2. **Keats EC** and Khan ZA. High levels of glucose alter mesenchymal progenitor cell differentiation by selective modulation of the Wnt pathway. Presented at the 3rd Annual Diabetes Research Day, November 2012.
3. **Keats EC** and Khan ZA. Changes in activity and differentiation of mesenchymal progenitor cells contribute to the pathogenesis of diabetes. Presented at the London Health Research Day, March 2012.
4. Kleiman A, **Keats EC**, and Khan ZA. Insulin-like growth factor 2 increases hemangioma stem cell growth without altering differentiation. Presented at the 2011 J Allyn Taylor International Prize in Medicine Symposium, London ON, November 2011.
5. **Keats EC** and Khan ZA. Unique cellular responses of stem cell-derived endothelial and mesenchymal cells to high levels of glucose. Presented at the 2011 J Allyn Taylor International Prize in Medicine Symposium, London ON, November 2011.
6. **Keats EC** and Khan ZA. Potential role of mesenchymal stem cells in increased adipogenesis in diabetes. Presented at the 2nd Annual Diabetes Research Day, London ON, November 2011.
7. **Keats EC** and Khan ZA. Stem cell-derived endothelial cells are resistant to the adverse effects of glucose: implication for therapeutic vasculogenesis. Presented at the 2nd Annual Diabetes Research Day, London ON, November 2011.
8. **Keats EC** and Khan ZA. Adult blood-derived endothelial progenitor cells and mature endothelial cells exhibit differential response to high levels of glucose. Presented at the Margaret Moffat Research Day, London ON, 2011.
9. **Keats EC** and Khan ZA. Matrix protein switching by human mesenchymal stem cells by high glucose: implications for therapeutic use. Presented at the Margaret Moffat Research Day, London ON, 2011.
10. **Keats EC** and Khan ZA. Differential cellular responses of vascular cells to hyperglycemia. Presented at the Lawson Research Day, London ON, 2011.
11. **Keats EC** and Khan ZA. Vascular stem cells as a therapeutic target for chronic diabetic complications. Presented at the 1st Annual Diabetes Research Day, London ON, 2010.
12. **Keats EC** and Khan ZA. Vascular stem cells as therapeutic target for chronic diabetic complications. Presented at the Annual Pathology Research Day. London ON, 2010.