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Diabetic Bone Marrow & Stem Cell Dysfunction

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Pathology

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by

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Graduate Program in Pathology

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Abstract

Defects in the proliferation, differentiation, and activity of bone marrow (BM)-derived vasculogenic/vascular stem cells (VSCs) have been observed in diabetes and contribute to the development of vascular complications. Diabetes leads to enhanced bone marrow adipogenesis, altering the composition of the BM stem cell (SC) niche and potentially disrupting the normal functioning of resident VSCs. Here, I establish that adipocytes have a negative influence on SC survival in culture. I also show that adipocytes and osteoblasts are responsible for the creation of distinct extracellular microenvironments, with unique expression patterns of several pro- and anti-angiogenic factors with known effects on VSCs, such as fibronectin, Notch ligands, stromal cell-derived factor-1, and angiopoietin-1 and -2. I conclude that alterations in marrow composition may mediate the connection between hyperglycemia, VSC dysfunction, and impaired vascular repair in diabetes.

Keywords: diabetes, bone marrow, mesenchymal progenitor cells, adipogenesis, differentiation, vascular stem cells, endothelial progenitor cells, stem cell niche

Co-Authorship Statement

Manuscript: Pathophysiological role of enhanced bone marrow adipogenesis in diabetic complications.

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Meghan A. Piccinin	Drafted the manuscript
Zia A. Khan	Supervisor; edited and finalized the manuscript

Chapters 2-4 also contain material from a manuscript in preparation which is co-authored by Meghan A. Piccinin and Zia A. Khan. Meghan Piccinin performed all experiments except generating diabetic animals. Diabetic animals were produced by Michael Ruiz (in Dr. Subrata Chakrabarti's Laboratory at Western University).

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Before starting in the lab, I attended the public lecture of a former Khan lab member, Emily Keats. She began her presentation by saying that she's learned more in four years than most will learn in a lifetime. I assumed this statement was a hyperbole, but during my short tenure under Zia Khan, I have similarly learned vastly more than I ever could of imagined. Your incredible dedication and love for science have been inspiring and I am honoured to have had you as my supervisor.

I would also like to acknowledge my advisory committee members, Drs. Cheryle Seguin and Christopher Howlett, for their direction and guidance. In our few meetings together, your questions and thoughtful advice helped me to shape this project into what it is now and I am grateful for your time and expertise.

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List of Abbreviations

ADAM	A disintengrin and metalloproteinase
AGE	Advanced glycation end-products
ALP	Alkaline phosphatase
Aminopeptidase A	Glutamyl aminopeptidase
AMPK	Adenosine monophosphate-activated protein kinase
Ang1	Angiopoietin 1
Ang2	Angiopoietin 2
ATP	Adenosine-5’-triphosphate
BH_4	Tetrahydrobiopterin
BADGE	Bisphenol A diglycidyl ether
BM	Bone marrow
BMD	Bone mineral density
BMP	Bone morphogenic protein
CBF	Core-binding factor subunit
C/EBP	CCAAT-enhancer-binding protein
CXCR4	C-X-C chemokine receptor type 4
DLL4	Delta-like ligand 4
DMEM	Dulbecco’s Modification of Eagle Medium
EBM-2	Endothelial basal media-2

EC	Endothelial cell
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
ERK	Extracellular signal-regulated kinase
FABP4	Fatty acid binding protein 4
$FADH_2$	Flavin adenine dinucleotide
FAK	Focal adhesion kinase
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FFA	Free fatty acid
FN	Fibronectin
FOXO1	Forkhead box protein O1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDM	Gestational diabetes mellitus
GLUT1	Glucose transporter 1
HDAC	Histone deacetylase
HBA1	Hemoglobin-α1
HSPG2	Heparan sulfate proteoglycan 2; also known as perlecan
HIF	Hypoxia-inducible factor
HSC	Hematopoietic stem cell

IGF-2	Insulin-like growth factor-2
IGF1R	Insulin-like growth factor-1 receptor
IR	Insulin receptor
IRS	Insulin receptor substrate
JAG1	Jagged-1
LPL	Lipoprotein lipase
MEK	Mitogen-activated protein kinase kinase
MMP	Matrix metalloproteinase
MNC	Mononuclear cell
MPC	Mesenchymal progenitor cell
MPL	Myeloproliferative leukemia protein
MSC	Mesenchymal/mesodermal stem cell
mTOR	Mammalian target of rapamycin
NADH	Nicotinamide adenine dinucleotide
N-cadherin	Neural cadherin
NO	Nitric oxide
NPH	Neutral Protamine Hagedorn
Oct4	Octamer-binding transcription factor 4; also known as POU5F1
PECAM-1	Platelet endothelial cell adhesion molecule-1; also known as CD31
PI3K	Phosphoinositide-3-kinase

PKB	Protein kinase B; also known as Akt
PKC	Protein kinase C
PKD-1	Protein kinase D-1
PPARγ	Peroxisome proliferator-activated receptor-γ
PSF	Penicillin, streptomycin, & fungizone (amphotericin)
RAGE	Receptor for advanced glycation end products
qRT-PCR	Quantitative Reverse transcription polymerase chain reaction
ROS	Reactive oxygen species
Runx2	Runt-related transcription factor 2; also known as CBFα1
RXR	Retinoid X receptor
SC	Stem cell
SCF	Stem cell factor
SCL	Stem cell leukemia
SDF-1	Stem/stromal cell-derived factor-1; also known as CXCL12
SEM	Standard error of the mean
Shh	Sonic hedgehog
SMC	Smooth muscle cell
Sox9	Sex determining region Y-box 9
SREBP1c	Sterol regulatory element-binding protein 1c; also known as ADD1
STZ	Streptozotocin

TCA cycle	Tricarboxylic acid cycle
THPO	Thrombopoietin
Tie-1	Tyrosine kinase with immunoglobulin-like and EGF-like domains-1
Tie-2	Tyrosine kinase with immunoglobulin-like and EGF-like domains-2; also known as TEK tyrosine kinase
TIMP	Tissue inhibitor of metalloproteinase
TZD	Thiazolidinedione
VCAM	Vascular cell adhesion molecule
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor-2; also known as Flk-1
VSC	Vascular stem cell; also known as an angioblast
vWF	von Willebrand factor
Wnt	Wingless-type MMTV integration site family member

Chapter 1 : Introduction[1]

1 Introduction

1.1 Diabetes

Diabetes mellitus is a chronic metabolic disease characterized by insufficient cellular responses to high blood glucose. Type 1 diabetes, comprising 5-10% of all cases, is generally considered to be a disease of the young, with most patients diagnosed before the age of twenty[1, 2]. This disorder is characterized by autoimmune destruction of the pancreatic β-cells responsible for the production of insulin in response to glycemic load[1, 2]. Destruction of β-cells results in an absolute insulin deficiency and high blood glucose levels. The cause of type 1 diabetes is considered to be polygenic and multifactorial[2]. Type 2 diabetes, also known as noninsulin-dependent diabetes mellitus, comprises the bulk of the instances of the disorder and involves a relative lack of insulin signalling stemming from peripheral insulin resistance and β-cell dysfunction[1, 3]. In this form, hyperglycemia develops slowly over the course of several years[1]. During this asymptomatic period, hyperinsulinemia may occur as the β-cells of the pancreas struggle to keep up with the ever-increasing insulin resistance of target tissues, allowing for inappropriate hepatic gluconeogenesis[1, 3]. Beta-cell function begins

[1] Portions of this chapter have been adapted from: **Piccinin MA**, Khan ZA. Pathophysiological role of enhanced bone marrow adipogenesis in diabetic complications. Adipocyte 2014; 3:4. Reproduction of portions of this article is at the permission of Taylor & Francis LLC.

to falter gradually over time, reducing insulin secretion and resulting in hyperglycemia[4]. A third form of the disease is gestational diabetes mellitus (GDM), which is initially diagnosed during pregnancy[1, 5]. GDM is characterized by insulin resistance and a considerable reduction in β-cell function by late pregnancy[5, 6]. GDM affects approximately 14% of pregnancies, and although the majority of cases resolve post-partum, those affected have a 65% chance of developing type 2 diabetes in the five years following delivery[1, 5-7].

1.1.1 Epidemiology

Diabetes is an incredibly prevalent disease, afflicting an estimated 347 million individuals worldwide as of 2008[8]. In the United States alone, there are approximately 20.9 million diabetes sufferers, with a prevalence of 6.9% in men and 5.9% in women[9]. These prevalence estimates have increased sharply since 1980, by 156% in men and 103% in women, and is predicted to continue rising, even while holding the current levels of obesity constant[9, 10]. If existing trends persist, one in three U.S. adults is anticipated to have diabetes by 2050[11]. Diabetes was responsible for 5.1 million deaths globally in 2013, with one person dying of the disease every six seconds[12]. The disease is anticipated to become the fifth leading cause of mortality worldwide by the year 2030, underlying 3.5% of all deaths[13]. This disease represents a massive burden on the global economy through significant reductions in productivity and the 2.3-fold increase in the utilization of health care resources by diabetics[14]. Twelve percent of worldwide health care expenditure is related to the care of diabetes, including $548 billion spent in the United States in 2013, primarily due to the management of diabetic sequelae[12, 15, 16].

1.1.2 Diabetic Vascular Complications

In developed countries, the morbidity associated with diabetes is primarily associated with secondary sequelae rather than the acute complications, such as ketoacidosis and diabetic coma, which are pervasive in low- and middle-income nations[17]. An estimated 72% of diabetics suffer from at least one long-term vascular complication of the disease[16]. Vascular complications can broadly be divided into two categories on the basis of the size of the blood vessel affected. Microvascular sequelae include retinopathy, neuropathy, cardiomyopathy, and nephropathy, while peripheral vascular disease and coronary artery disease are common macrovascular complications[16]. These complications are the consequence of hyperglycemia-induced damage to blood vessels and aberrant vascular repair mechanisms.

1.1.2.1 Mechanisms of Diabetic Vascular Complications

The endothelial cells (ECs) comprising the tunica intima, the innermost layer of the vasculature, are the first cells in the body to encounter chronically elevated blood glucose levels in diabetes. The most abundant glucose transporter isoform expressed by ECs is glucose transporter 1 (GLUT1), which aids in the facilitated diffusion of glucose across the plasma membrane[18-20]. The expression and function of GLUT1 is non-responsive to changes in glucose or insulin concentration, resulting in increased glucose uptake under hyperglycemic conditions and heightened intracellular glucose levels[20-23]. The cytosolic glucose is then processed via glycolysis and the tricarboxylic acid (TCA) cycle to generate electron donors nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) for use in the mitochondrial electron transport

system and provide energy to pump protons across the mitochondrial membrane[22]. A transmembrane voltage gradient is then established, which can be harnessed to drive production of adenosine-5'-triphosphate (ATP)[22]. With excess glucose being processed through the TCA cycle in hyperglycemic conditions, an increased number of electrons are transferred into the electron transport chain, raising trans-membrane hyperpolarization until a threshold limit is reached[22]. Electron movement is then halted at complex III, resulting in an accumulation of electrons at coenzyme Q[22]. Coenzyme Q dissipates this excess charge through the partial reduction of molecular oxygen to form the free radical superoxide anion[22].

Superoxide is able to exert its detrimental effects on the vasculature through a number of mechanisms. The superoxide anion is able to inactivate nitric oxide (NO) by converting the potent vasodilator into peroxynitrite[24, 25]. Peroxynitrite acts as an oxidizing agent, reacting with tetrahydrobiopterin (BH_4) which serves as a requisite cofactor for endothelial nitric oxide synthase (eNOS)[26]. This results in uncoupling of eNOS, favoring the generation of superoxide over NO production and leading to an accumulative increase in reactive oxygen species (ROS) and runaway inhibition of NO bioactivity[27, 28].

This overactive production of ROS, both directly from the electron transport chain and indirectly through uncoupled eNOS activity, leads to the induction of four mechanisms which stimulate primary biochemical changes within ECs: 1) increased activity of the polyol pathway, 2) generation of advanced glycation end-products (AGEs), 3) activation of protein kinase C (PKC), and 4) stimulation of the hexosamine pathway (reviewed in detail in Brownlee 2005)[22]. The activation of these pathways

leads to endothelial dysfunction through further induction of ROS production, the release of inflammatory cytokines, increased synthesis of extracellular matrix components, diminished EC migration and proliferation, and endothelial apoptosis (Figure 1.1)[22, 29-37].

In addition to diminishing the production of NO, hyperglycemia and hyperinsulinemia further potentiate the vasoactive effects of diabetes by stimulating the production and activation of the most powerful known endogenous vasoconstrictor, endothelin-1[38-40]. Hyperglycemia appears to enhance endothelin-1 activity through the increased activation of PKC-β and –δ, while insulin likely exerts its effect via the tyrosine kinase activity of the insulin receptor (IR)[38-40]. Interactions between endothelin-1 and its receptors on vascular smooth muscle cells results in the release of Ca^{2+} from intracellular stores and the opening on non-specific cation channels[41]. This leads to depolarization sufficient to activate L-type Ca^{2+} channels and induce perivascular cell contraction and vasoconstriction[41, 42]. Increased levels of endothelin-1 mRNA and peptide have been observed in several organs known to be susceptible to diabetic complications, such as the heart, kidneys, and retina[38, 43-45]. The dual vasoactive effects of hyperglycemia on NO and endothelin-1 lead to diminished endothelial integrity, culminating in impaired perfusion of targeted tissue and ischemia[46, 47].

In the healthy patient, vascular repair mechanisms would be employed to restore the damaged blood vessels and preserve the function and circulation of affected tissues (Figure 1.2 A)[46]. Revascularization may occur through the proliferation and migration of mature ECs adjacent to injured regions or through the chemokine-guided recruitment of

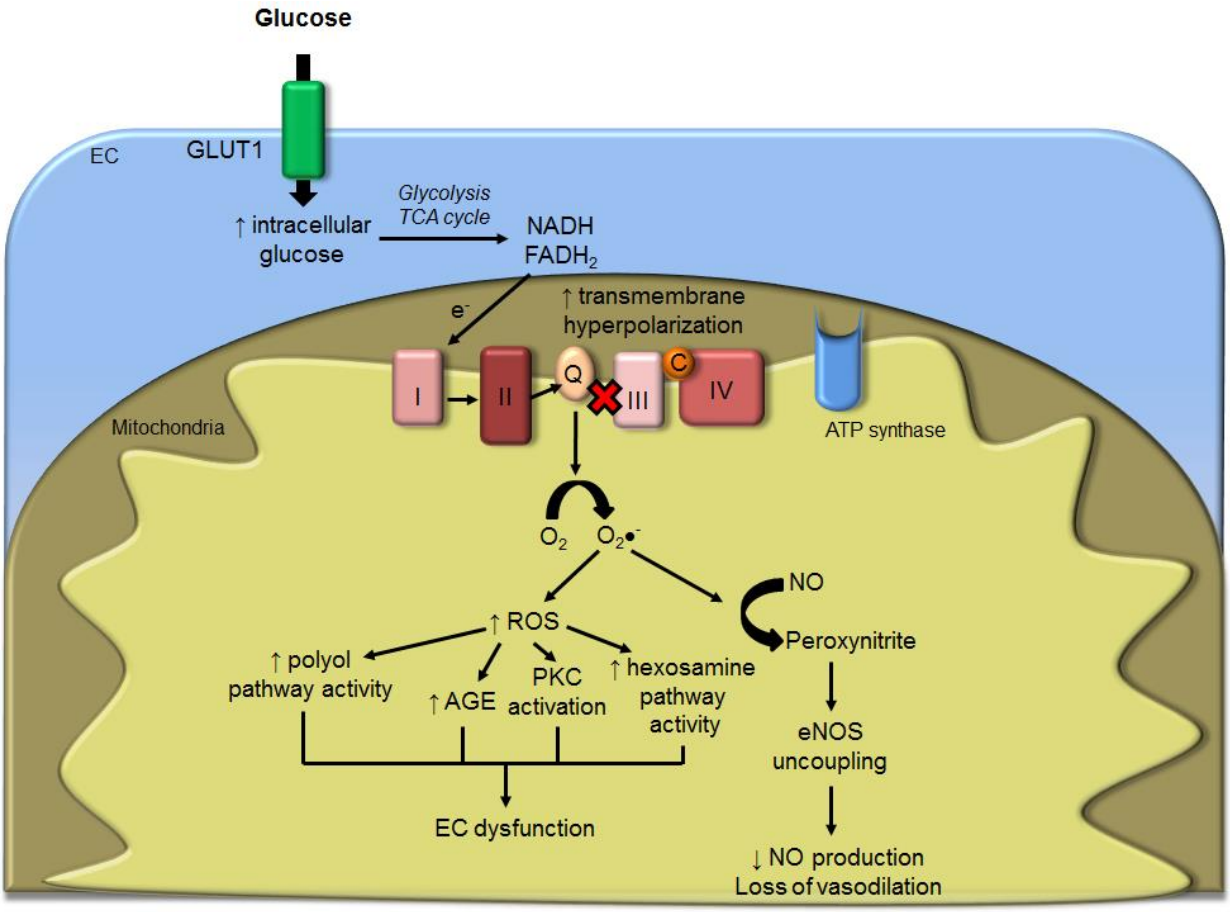


Figure 1.1. Mechanisms of hyperglycemia-induced endothelial damage in diabetes.

Glucose enters and accumulates in ECs via Glut1, which is non-responsive to changes in glucose concentration. Disproportionate glucose metabolism overwhelms the electron transport chain, causing an accumulation of electrons at coenzyme Q. This excessive charge is then dissipated through the partial reduction of molecular oxygen into the free radical superoxide. Superoxide promotes the conversion of the potent vasodilator NO into inactive peroxynitrite and uncoupling of eNOS, leading to a loss of vasoregulation. Superoxide also stimulates the generation of other ROS, which by several distinct mechanisms, mediate EC dysfunction and survival.

[AGE = advanced glycation end product; EC = endothelial cell; $FADH_2$ = flavin adenine dinucleotide; NADH = nicotinamide adenine dinucleotide; NO = nitric oxide; PKC = protein kinase C; Q = coenzyme Q; ROS = reactive oxygen species]

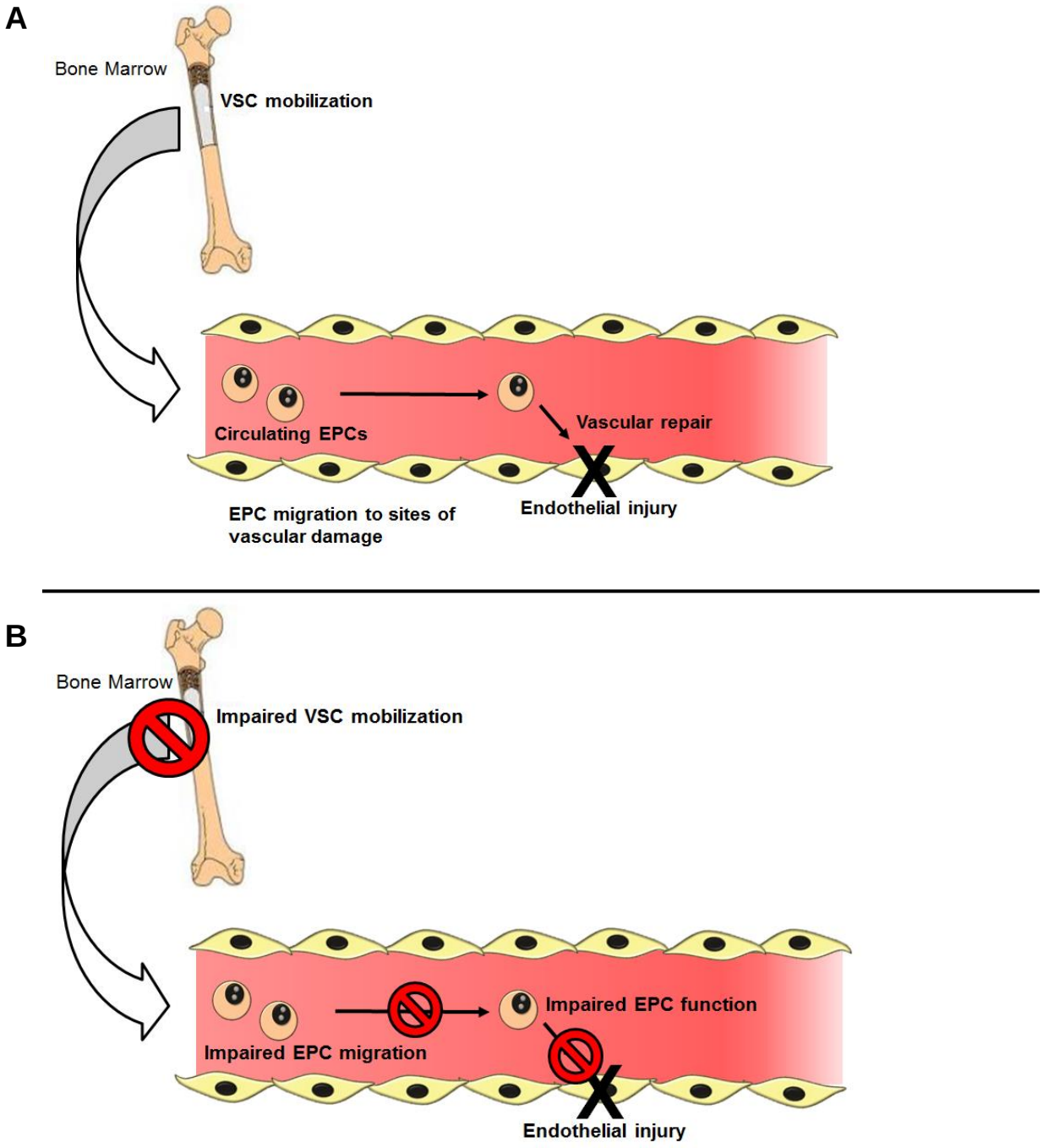

A
Bone Marrow
VSC mobilization
Circulating EPCs
Vascular repair
EPC migration to sites of vascular damage
Endothelial injury
B
Bone Marrow
Impaired VSC mobilization
Impaired EPC migration
Impaired EPC function
Endothelial injury

Figure 1.2. Impaired vascular repair in diabetes.

(A) In healthy individuals, endothelial injury would signal for the mobilization of stem cells from the BM into circulation and contribute to vascular regeneration. Injury may cause some vascular stem cells to produce lineage-restricted EPCs which home to the site of injury and repair damaged vasculature, though the identity of these cells is not fully clear. (B) In diabetics, this response to endothelial damage is disrupted, through a combination of reduced VSCs in the marrow and impaired EPC migration and vasculogenic function.

[BM = bone marrow; EPC = endothelial progenitor cell; VSC = vascular stem cell]

bone marrow (BM)-derived endothelial progenitor cells (EPCs) to the site of injury[48-50]. This response is disrupted and occurs unevenly in type 1 diabetics with poor glycemic control and nearly all type 2 diabetic subjects (Figure 1.2 B)[51]. The non-uniform distribution of vascular repair leads to divergent, tissue-specific complications, with heightened retinal and renal vessel formation and a lack of revascularization in the lower limbs and heart[52-56]. These pathological changes may be partially resultant from deficits in EPC development, proliferation, migration, and/or function. Analyses of EPCs from most diabetic subjects reveal a reduced number in both the circulation and the bone marrow, as well as impaired proliferation, mobilization, and capacity for vessel formation[56-66]. The mechanisms underlying these cellular changes have not yet been fully elucidated, but may relate to the diabetes-induced alterations to the BM microenvironment from which EPCs originate.

1.2 Stem Cells in the Bone Marrow

The primary role of the BM is to support the maintenance and differentiation of hematopoietic stem cells (HSCs). In addition to blood cell precursors, the marrow is also an abundant source of other precursors including mesenchymal and vascular cells. Each of these progenitor cell classes reside within a hierarchy of progressively more differentiated cell types (Figure 1.3). HSCs are responsible for the formation of novel blood cells, generating leukocytes, erythrocytes, and thrombocytes. Mesenchymal precursors regulate the creation of the marrow stroma that supports the HSC population, while vascular stem cells (VSCs) serve as a pool of progenitors for blood vessel formation.

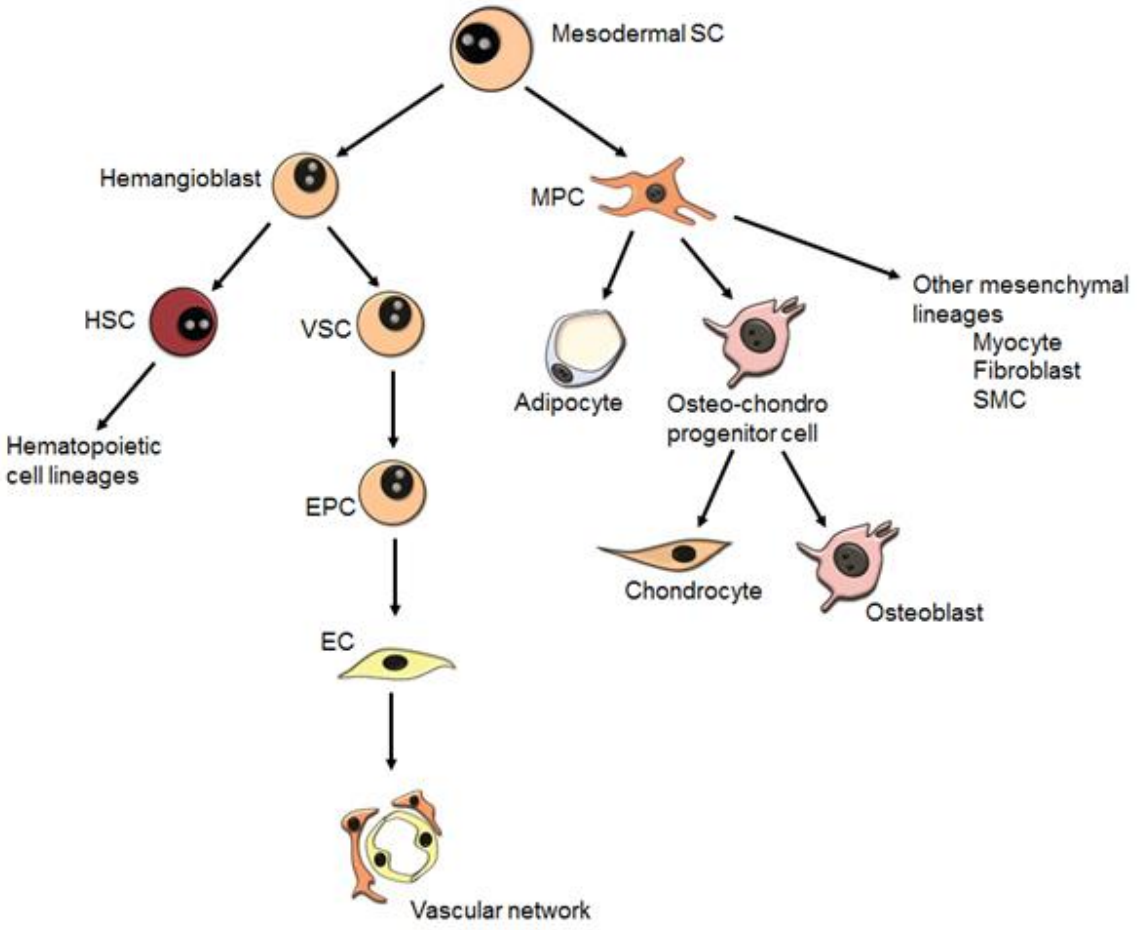


Figure 1.3. Developmental origin and potential of marrow-resident SCs.

Hypothesized model of stem cell hierarchy in the marrow showing mesodermal SCs giving rise to embryonic hemangioblasts and mesenchymal progenitor cells (MPCs, also known as mesenchymal stem/stromal cells (MSCs)). In the developing embryo, hemangioblasts serve as precursors for hematopoietic and vascular lineages, though the postnatal existence of hemangioblasts is disputed. MPCs have a tri-lineage differentiation potential and able to develop into adipocytes, osteoblasts, and chondrocytes.

[EC = endothelial cell; EPC = endothelial progenitor cell; HSC = hematopoietic stem cell; MPC = mesenchymal progenitor cell; SC = stem cell; SMC = smooth muscle cell; VSC = vascular stem cell]

1.2.1 Mesenchymal Precursors

The first description of mesenchymal precursors in the BM came from Friedenstein, Chailakhjan, and Lalykina in 1968, who observed a monolayer of colony-forming fibroblastic cells developing from guinea pig marrow aspirates[67]. These mesenchymal precursors are possibly the most abundant precursor type in the marrow yet their identity and true differentiation potential is obscure and controversial. These cells are often referred to as marrow/mesenchymal stem/stromal cells (MSCs) in the literature. I have elected to refer to these cells as mesenchymal precursor/progenitor cells (MPCs) due to the lack of experimental evidence for the 'stem' cell phenotype and hematopoietic differentiation ability. Furthermore, stromal cell definition may also be misleading depending on the context. For example, a well-known function of these mesenchymal precursors is tissue repair following injury and thus, not solely a supportive framework for other functional cell types within the BM. Therefore, I believe a proper term for these cells is mesenchymal progenitor/precursor cell.

MPCs have typically been isolated from the BM mononuclear cell fraction on the basis of their adherence to plastic surfaces relative to hematopoietic cells, although this imprecise method invariably results in a contaminated heterogeneic cell population[68-70]. As the characterization of MPCs improved and selective surface markers were identified, monoclonal antibodies such as Stro-1 have been employed in order to better isolate a homogenous subset of cells for experimentation and analysis[68, 69]. Other surface antigens of mesenchymal precursors include CD73, CD90, and CD105, though the cells must be devoid from the expression of CD11b, CD14, CD19, CD34, CD45, and

CD79α in order to exclude cells with a hematopoietic phenotype[71, 72]. Additionally, the cells must be capable of *in vitro* differentiation into osteoblasts, adipocytes, and chondroblasts (Figure 1.4), though *in vivo*, chondrogenesis is typically localized to the osteochondral environment of joint cavities as opposed to our primary area of interest, the BM[71, 73, 74].

1.2.1.1 Osteoblastogenesis & Chondrogenesis

The predominant factor involved in regulating osteoblastic differentiation is Runt-related transcription factor 2 (Runx2; also known as core-binding factor subunit α 1 (CBFα1)[75]. Once induced by bone morphogenic protein-7 (BMP7), Runx2 and its heterodimeric subunit CBFβ bind the Runx consensus sequence present in the promoter regions of key osteoblastic genes, leading to the development of MPCs into osteochondro-progenitor cells[76-78]. Runx2-induced expression of the transcription factor Sp7 (also known as osterix) guides these bi-potential cells towards the osteoblastic lineage, while repressing Sex determining region-Y box 9 (Sox9) that directs chondrogenic development[77, 79]. Together, Runx2 and Sp7 drive the expression of the major osteoblastic genes that contribute to the bone cell phenotype, including bone gamma-carboxyglutamic acid-containing protein (BGLAP; also known as osteocalcin), collagen 1A1, and osteopontin[76, 80]. Additionally, Runx2 also plays a major role in suppressing the cell division of differentiating progenitor cells, entering osteoblasts into a post-proliferative state[81].

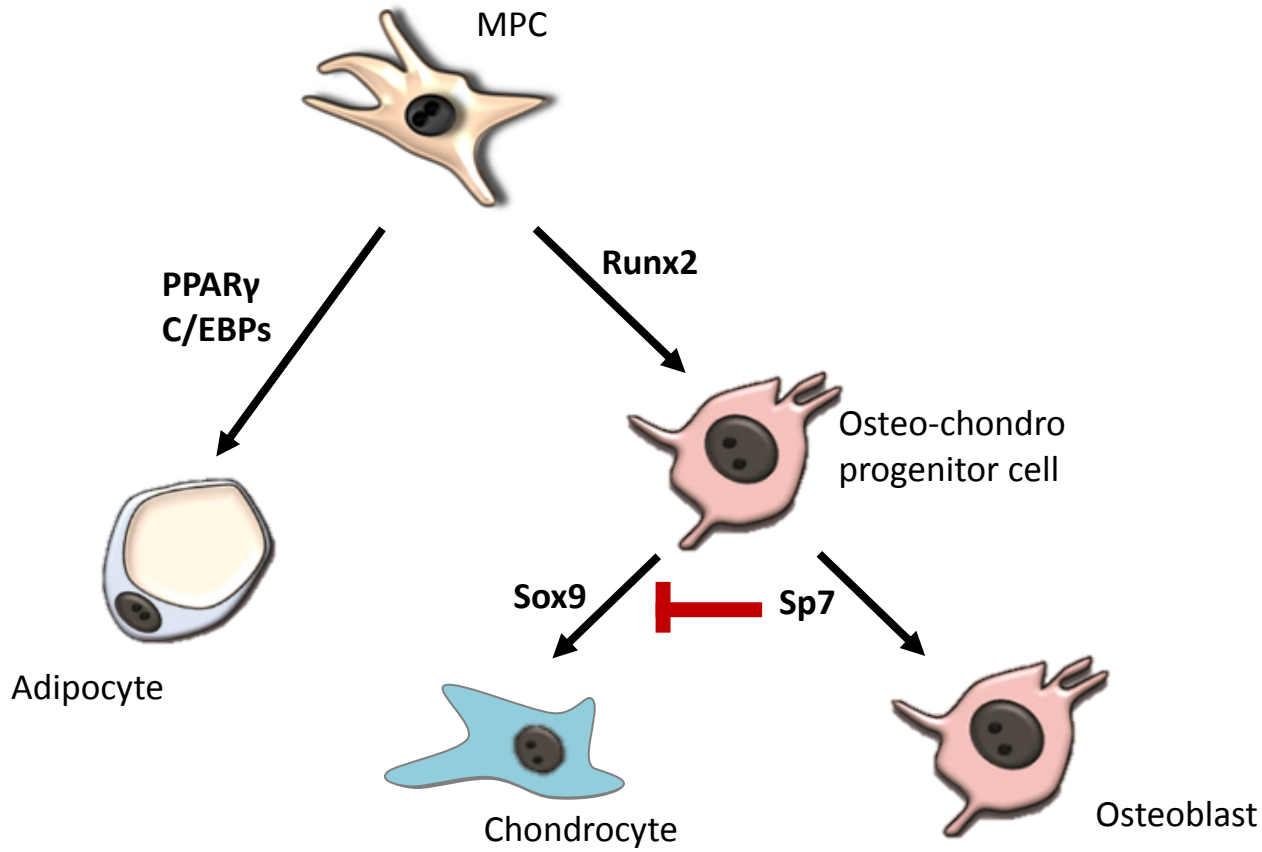


Figure 1.4. Key transcription factors regulating MPC differentiation.

MPCs differentiate into adipocytes upon induction of PPARγ and the C/EBP family of transcription factors. Activation of Runx2 stimulates MPC commitment to osteoblastic or chondrogenic lineages. Subsequent expression of Sox9 directs differentiation towards chondrocytes, while Sp7 inhibits this pathway to facilitate osteoblastogenesis.

[C/EBP = CCAAT-enhancer-binding protein; MPC = mesenchymal progenitor cell; PPARγ = peroxisome proliferator-activated receptor γ; Sox9 = sex determining region Y-box 9]

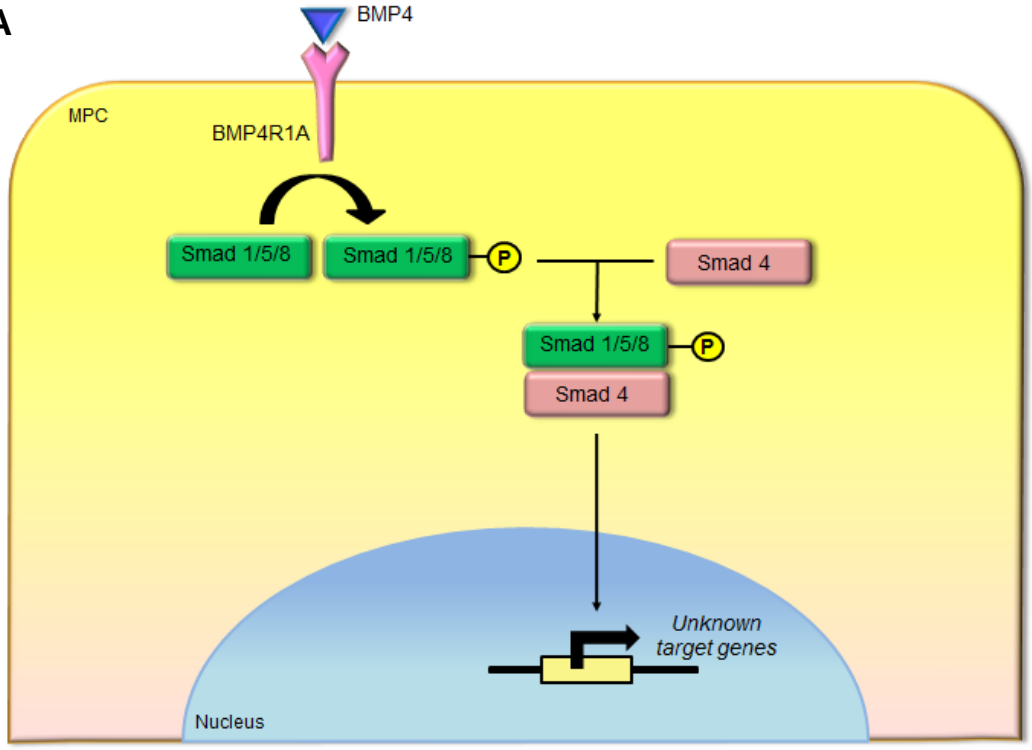
1.2.1.2 Adipogenesis

Adipogenic differentiation of MPCs is a biphasic process, broadly divided into two stages: determination and terminal differentiation. In culture, the process requires approximately seven days from the initiation of adipogenic stimulation and is tightly regulated by an intricate cascade of transcription factors and ligand-receptor interactions[82].

1.2.1.2.1 Adipogenic Determination

Determination involves commitment of precursor cells to development along the adipogenic lineage as a pre-adipocyte (Figure 1.5A). At this stage, pre-adipocytes remain morphologically identical to their precursor, although these cells have lost the ability to differentiate along any other developmental pathway[82, 83]. The molecular mechanisms and interactions underlying adipogenic commitment are not well understood. BMP4 is believed to play an important role in determination, having repeatedly been shown capable of committing murine C3H10T1/2 cells to adipocyte development[84-87]. Hypomethylation of the BMP4 locus is suspected to be involved, as treatment with 5-azacytidine, a potent DNA methyltransferase inhibitor, is able to induce commitment of precursor cells to the adipogenic lineage by increasing the accessibility of the BMP4 transcriptional start site[88, 89]. Interactions between BMP4 and its cell surface receptor BMP4R1A result in the rapid phosphorylation of Smad1/5/8, which complexes with Smad4[86]. This protein complex undergoes nuclear translocation, where it controls gene expression, though the specific targets are not yet known[86, 90]. In many

A


BMP4
MPC
BMP4R1A
Smad 1/5/8
Smad 1/5/8
P
Smad 4
Smad 1/5/8
P
Smad 4
Unknown
target genes
Nucleus

B

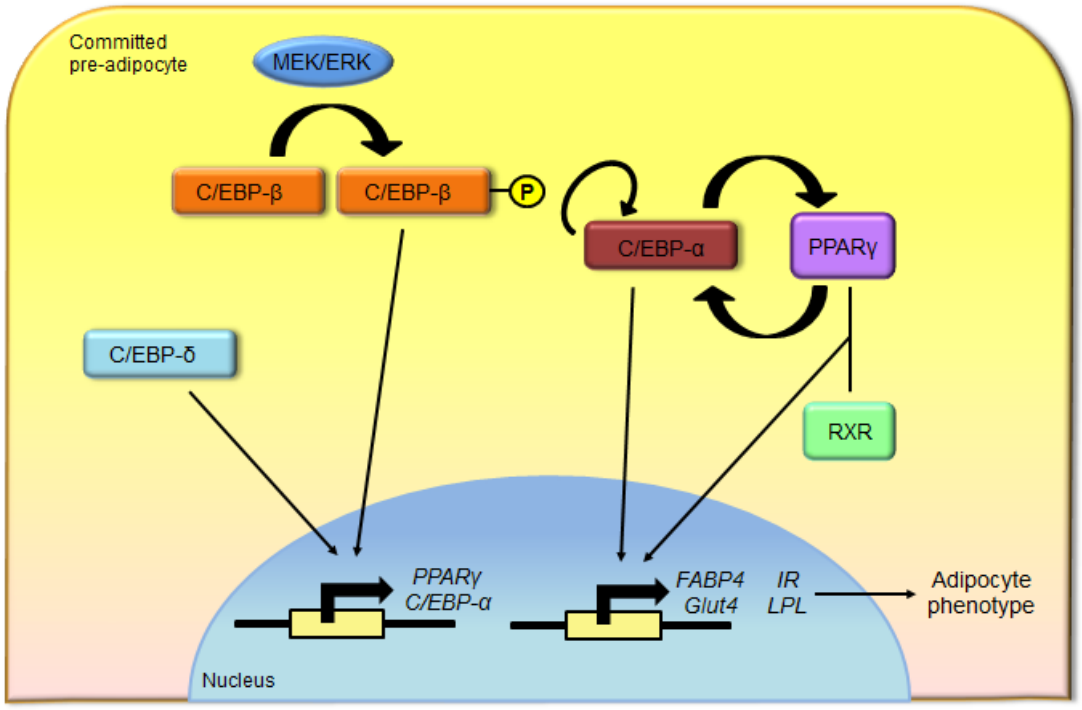

Committed
pre-adipocyte
MEK/ERK
C/EBP-β
C/EBP-β
P
C/EBP-α
PPARγ
C/EBP-δ
RXR
PPARγ
C/EBP-α
FABP4
Glut4
IR
LPL
Adipocyte
phenotype
Nucleus

Figure 1.5. Schematic illustrating adipogenic determination and terminal differentiation.

Differentiation of MPCs into adipocytes is governed by a tightly-regulated transcriptional cascade. (A) The first stage of adipogenesis involves commitment of MPCs to the adipogenic lineage in a process known as determination. Determination is primarily regulated by a BMP and Smad signalling pathway. (B) Terminal differentiation of committed preadipocytes into mature adipocytes begins with C/EBP-β and C/EBP-δ inducing the transcription of PPARγ and C/EBPα, which are then able to facilitate the transcription of genes responsible for producing the adipocytic phenotype.

[BMP4 = bone morphogenic protein 4; BMP4R1A = bone morphogenic protein 4 receptor 1A; C/EBP = CCAAT-enhancer-binding protein; ERK = extracellular signal-regilated kinase; FABP4 = fatty acid binding protein 4; Glut4 = glucose transporter 4; IR = insulin receptor; LPL = lipoprotein lipase; MEK = mitogen-activated protein kinase kinase; MPC = mesenchymal progenitor cell; PPARγ = peroxisome proliferator-activated receptor γ]

well-studied murine pre-adipocyte cell lines, determination and terminal differentiation are separated by a brief period of proliferation, known as mitotic clonal expansion, though it has previously been demonstrated that this stage is not a requirement for human BM-MPC development[91, 92].

1.2.1.2.3 Terminal Differentiation

The terminal differentiation of committed progenitor cells into mature adipocytes is largely controlled by peroxisome proliferator-activated receptor-γ (PPARγ), which is known as 'the master regulator of adipogenesis', and the CCAAT-enhancer-binding protein (C/EBP) family of transcription factors. Pro-adipogenic stimuli promote the hyper-phosphorylation and activation of C/EBP-β by mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signalling[93, 94]. C/EBP-β acts synergistically with C/EBP-δ to enhance the expression of PPARγ and C/EBP-α through direct binding to potential C/EBP sites located within the PPARγ and C/EBP-α promoter regions[83, 95]. PPARγ and C/EBP-α form a positive feedback loop, in which each factor is capable of promoting the expression of the other in order to maintain the differentiated state[96, 97]. C/EBP-α is also able to bind its own C/EBP-regulatory element to reinforce its own expression independently of PPARγ-mediated regulation[98]. PPARγ, in tandem with its heterodimeric partner retinoid X receptor (RXR), and C/EBP-α act in concert to stimulate the expression of a number of adipocyte-specific genes, including fatty acid binding protein-4 (FABP4), Glut4, lipoprotein lipase, glycerophosphate dehydrogenase, and acetyl CoA carboxylase, among others (Figure 1.5B)[97]. C/EBP-α

also becomes phosphorylated by cyclin D3, resulting in the formation of growth-inhibitory complexes in order to enter adipocytes into a non-proliferative state[99].

1.2.2 Vascular Stem Cells

VSCs are defined as "self-renewing multipotent stem cell[s] that [give] rise to vascular lineages"[100]. VSCs, which may also be known as angioblasts, share a common developmental origin with HSCs as hemangioblasts residing within embryonic blood islands, though some evidence suggests that hemangioblasts may persist into adulthood in small numbers within the BM[101-103]. The development of a hemangioblast into either a VSC or an HSC is contingent on the expression of vascular endothelial growth factor (VEGF) receptor-2 (VEGFR-2; also commonly known as fetal liver kinase-1 or Flk-1) and the stem cell leukemia (SCL) transcription factor, respectively[104, 105]. SCL appears to be the predominant director of hemangioblast development and hematopoietic fate via Runx1 signalling, while VEGFR-2 is necessary for vascular lineage progression[104-107]. Hemangioblasts have been characterized by the expression of VEGFR-2 and SCL, along with CD133 (a stem and progenitor cell marker; also known as AC133 or prominin-1) and CD34 (a cell adhesion factor mediating stem cell (SC) attachment to the BM)[101, 103]. Stimulation of VSCs with VEGF induces their development into lineage-restricted EPCs, which can then enter into circulation, differentiate into mature ECs, and contribute to the formation or repair of the arteriovenous network[108, 109].

1.2.2.1 Endothelial Progenitor Cells

EPCs are committed progenitor cells that will eventually give rise to the ECs that participate in the repair of damaged vasculature[110]. BM-derived EPCs enter into circulation and migrate to the site of vascular injury, where they restore impaired blood flow[111, 112]. Vessel repair may be comprised of angiogenesis and vasculogenesis. In angiogenesis, blood vessels are formed by sprouting or intussusception (splitting) of pre-existing vascular networks[113]. Vasculogenesis denotes the *de novo* formation of blood vessels from precursor cells. In the embryo, vasculogenesis is employed in the formation of the earliest vascular plexus, after which angiogenesis was originally believed to take over as the predominant mechanism of vessel formation[113-115]. Only relatively recently has vasculogenesis been accepted as a complementary method of postnatal neovascularization, with circulating EPCs now serving as a novel biomarker for vascular health[116-120].

Asahara and colleagues were the first to identify and isolate EPCs from adult circulation in 1997[121]. EPCs were obtained through magnetic bead selection for CD34-positive cells in the leukocyte fraction of peripheral blood. After seven days of culture, these progenitor cells adopted an endothelial-like phenotype and gene expression pattern. Labelled CD34-positive cells were then injected into an athymic mouse model of hind limb ischemia to evaluate vasculogenic capacity. After 6 weeks, there was significant incorporation of CD34-positive cells into capillaries of the ischemic limb relative to an injection of control CD34-depleted cells, suggesting a great potential of these cells to contribute to neovascularization. Following this initial discovery of EPCs

in postnatal circulation, these cells have since been shown to significantly contribute to vessel formation in both physiological and pathological capacities[46, 49, 122, 123].

EPCs that have entered into circulation can be obtained through culture of peripheral blood mononuclear cells in a VEGF-containing medium[108, 124]. Characterizing EPCs has proven difficult, largely due to the co-occurrence of mature circulating ECs, likely shed from the vessel walls, within peripheral circulation[46]. Additionally, there appear to be two distinct categories of EPCs that are able to contribute to blood vessel repair and development[125]. These two cell types can be distinguished primarily on the basis of their morphology and ability to proliferate[124, 126]. Early-outgrowth EPCs are spindle-shaped CD14- and CD45-positive cells that appear after about 10-14 days of culture and exhibit a low propensity for mitogenesis[125-131]. This population is believed to arise from the reprogramming of myeloid progenitors or monocytes into cells with an endothelial-like phenotype[130-134]. Also contained within the peripheral blood mononuclear fraction is a small subset of cobblestone-shaped, highly proliferative cells, known as late-outgrowth colonies or endothelial colony-forming cells that appear after three or more weeks of culture[126, 128, 129, 135-137]. Late-outgrowth cells are free from monocytic and hematopoietic markers and correspond to BM-derived EPCs[138]. Both early- and late-outgrowth cell types appear to have a similar capacity for angiogenesis *in vivo*, though early EPCs were shown to be incapable of forming the capillary-like structures characteristic of vasculogenesis[126, 129, 139, 140]. From this point forward, discussion of EPCs will centralize solely on the BM-derived late-outgrowth EPC cell type.

As an intermediate between SCs and mature ECs, EPCs express markers of both stem and fully differentiated cells[141]. In the BM, VSC precursors able to give rise to EPCs are characterized by the expression of VEGFR-2, CD34, and CD133[142-144]. CD133 is the only one of these markers not expressed by mature endothelial cells, which allows EPCs or cells capable of giving rise to EPCs to be distinguished from the circulating EC population[50, 115]. A single surface marker specific to EPCs has yet to be described, although CD133 is currently considered to be the putative marker for the identification of EPCs[145-147]. Functional characteristics of EPCs include the ability to uptake acetylated low density lipoprotein and adhere to the fucose-binding lectin Ulex europaeus agglutinin-1, which are features also shared by mature ECs[127, 135]. Upon activation, BM-EPCs enter peripheral circulation and increase their expression of endothelial markers, such as vascular endothelial-cadherin (VE-cadherin), von Willebrand factor (vWF), tyrosine kinse with immunoglobulin-like and EGF-like domains 2 (Tie-2), eNOS, and CD31 (also known as platelet endothelial cell adhesion molecule-1 or PECAM-1), and reduce expression of the SC marker CD133[108, 113, 148]. Once fully differentiated and incorporated into the endothelium, the expression of CD133 is abolished[148-150]. In culture, CD133 is lost upon adherence of cells to culture plates. While the expression of VEGFR-2 persists following maturation, CD34 may or may not be expressed by differentiated ECs depending on the size of the blood vessel[113, 148].

Though the specific cellular and non-cellular interactions in the marrow that govern EPC derivation and release into circulation are poorly understood, a number of studies have unanimously reported that the proliferation, differentiation, and migration of EPCs are stimulated by tissue ischemia. A lack of oxygen within a microenvironment

prevents the proteosomal degradation of heterodimeric hypoxia-inducible factor (HIF) within resident cells by a pair of mechanisms[151]. Under normoxic conditions, hydroxylation of two prolyl residues of the HIF-α subunit facilitates the protein's interaction with a ubiquitin ligase and targets the protein for destruction[151-155]. Additionally, a HIF-α carboxyl-terminal asparginyl residue may undergo β-hydroxylation in order to prevent the binding of the transcriptional co-activator p300[151, 153, 156]. Both of these hydroxylation reactions require the use of molecular oxygen as a requisite cofactor[157, 158]. Under ischemic conditions, the failings of these regulatory measures are unable to target HIF to the proteosome or prevent the transcriptional activity of the protein. HIF can then up-regulate the expression and secretion of VEGF and stem/stromal cell-derived factor-1 (SDF-1), which are believed to serve as principal signalling molecules in EPC activation and mobilization, respectively[159-165]. VEGF and SDF-1, along with other angiogenic factors such as insulin-like growth factor-2 (IGF-2), enhance the expression and activity of matrix metalloproteinase (MMP)-9 in the BM, which cleaves the membrane-bound Kit-ligand, known as stem cell factor (SCF) to release its soluble form[166-168]. Soluble SCF interacts with its receptor, c-Kit, expressed by various stromal cells to augment the BM microenvironment in favor of SC proliferation and mobilization into circulation[147, 166, 169]. Several signalling pathways have been implicated in directing the proliferation and migration of EPCs, including Sonic hedgehog (Shh) acting through phosphoinositide-3-kinase (PI3K) and protein kinase B (PKB)-mediated phosphorylation of NOS, as well as VEGF-induced signalling via a protein kinase D-1 (PKD-1)-histone deacetylase (HDAC) 7 axis[170-172]. These signalling cascades result in an influx of EPCs from the BM into circulation, which use

an SDF-1- and/or IGF2-mediated chemotactic gradient in order to home to sites in need to neovascularization[164, 168, 173-175].

In diabetes, the EPC-recruiting response to hyperglycemia-induced vascular injury appears to be impaired. In cultured samples of peripheral blood obtained from type 1 diabetics, the number of EPCs in circulation was reduced by 44% relative to control subjects, suggesting that the disease impairs mobilization of precursor cells from the BM[62]. The same study also found that the EPCs of diabetic patients exhibited a significantly diminished capacity for angiogenesis in an *in vitro* assay[62]. Analysis of CD34-positive EPCs derived from type 1 diabetics showed reduced *in vitro* differentiation of the progenitors into mature ECs[176]. Similar results demonstrating impaired differentiation, migration, and function have also been obtained through the study of type 2 diabetic and mixed diabetic population EPCs, as well as animal models[57-59, 61, 118, 177-182]. Additionally, our laboratory has shown that hyperglycemia significantly increases caspase-3 activity in EPCs[183]. The number of EPCs in circulation has been identified as an important biomarker for vascular function and overall cardiovascular risk and thus, understanding the mechanisms underlying diabetic EPC dysfunction is crucial[117-120]. Furthermore, the quantities of EPCs from subjects with long-duration type 1 diabetes free from vascular complications were equivalent to non-diabetic controls and demonstrated an enhanced migratory ability[184]. This finding suggests that preservation of the EPC population and its function are critical in preventing or ameliorating diabetic damage to the vasculature.

The underlying cause of the EPC dysfunction that occurs in diabetes is not presently known[178]. A number of potential mechanisms have been suggested, including

impairment of circulating EPC survival under hyperglycemic conditions and a reduction in the hypoxia-induced expression of pro-angiogenic factors VEGF and SDF-1[185-188]. I propose that a major contributor to this EPC dysfunction may be disrupted signalling resulting from diabetes-induced alterations to the BM SC niche.

1.2.3 Stem Cell Niche

As with other SCs, the fates of VSCs *in vivo* are governed by intracellular gene regulation, though this intrinsic program is subject to influence by external elements in order to maintain an appropriate balance between self-renewal and differentiation[189]. These extrinsic cues come from SCs' interactions with the surrounding microenvironment. This microenvironment is comprised of soluble paracrine signalling molecules, interactions with nearby stromal cells, and the extracellular matrix, which are collectively referred to as the *SC niche*[189-191]. The specific composition of these niches are vital in regulating SC quiescence, self-renewal, and differentiation in nearly every progenitor cell type[166, 191, 192]. The BM SC niche is comprised of a number of cell types, as well as their secretory products, cell-cell interactions, and extracellular matrix (ECM) (Figure 1.6). A number of SC niches have been identified and well-characterized, including the epithelial, intestinal, and hematopoietic niches. Recent work has broadened our understanding of the BM HSC niche, including changes to the niche in disease states such as diabetes and obesity[193, 194]. Although EPC dysfunction is associated with the development of pathological vascular complications, comparatively little is known about the role of the diabetic BM SC niche in mediating this process.

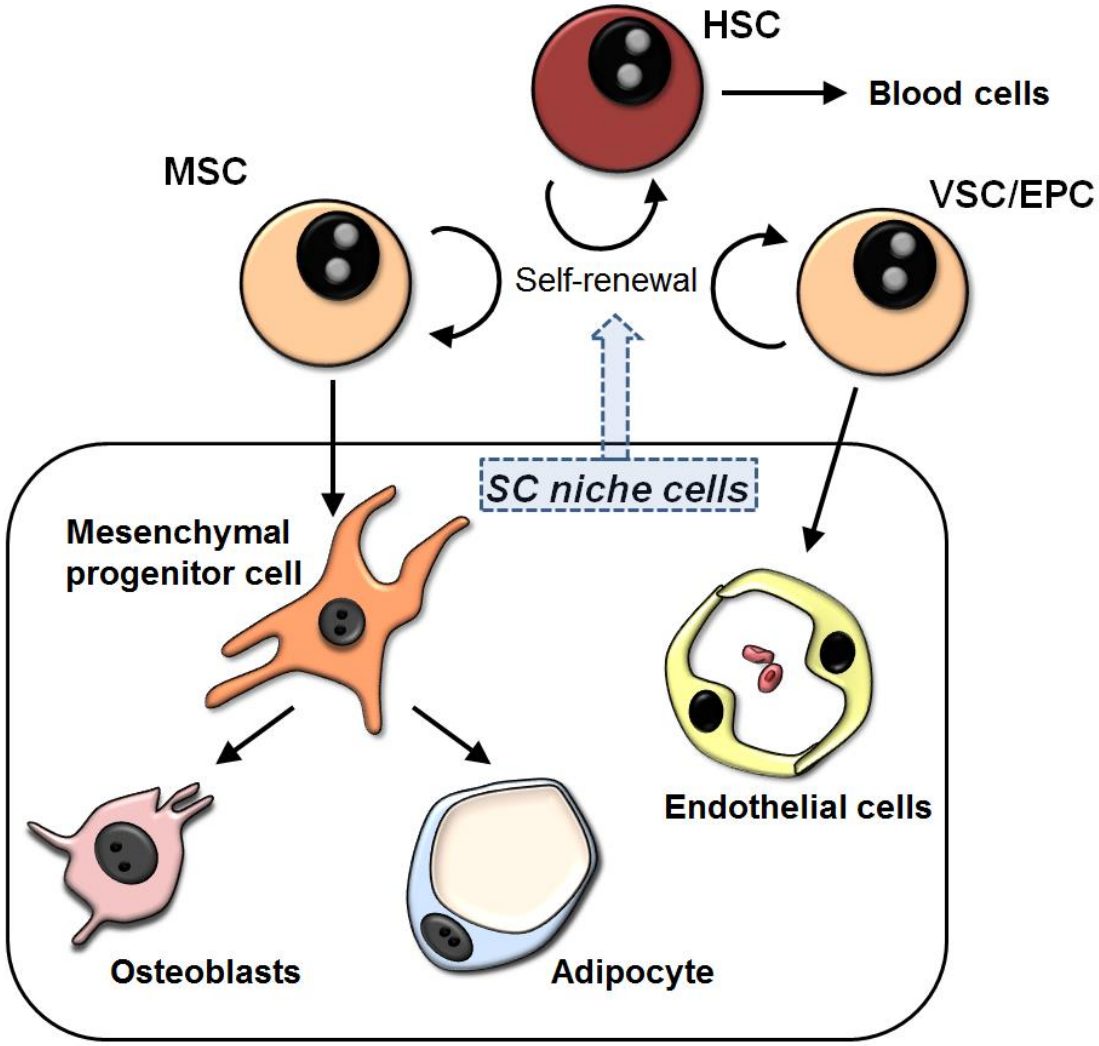


Figure 1.6. Schematic of interactions between BM SC niche components.

Within the BM reside three SC types: HSCs, MSCs, and VSCs. The self-renewal and differentiation of these SCs is controlled by the surrounding microenvironment, which includes MPCs, adipocytes, osteoblasts, and endothelial cells.

[EPC = endothelial progenitor cell; HSC = hematopoietic stem cell; MSC = mesenchymal stem cell; SC = stem cell; VSC = vascular stem cell]

1.3 Bone and Marrow Complications of Diabetes

One of the extravascular complications of diabetes involves changes to the composition and structural integrity of the skeletal system. This phenomenon was first described by Morrison and Bogan in 1927, who observed impaired bone development and skeletal atrophy in children with long-standing diabetes[195]. Clinically, it has been well-documented that diabetes induces a significant increase in fracture risk among both type 1 and type 2 diabetics, as described by a 2007 systematic review by Janghorbani et al. and a 2007 meta-analysis by Vestergaard[196, 197]. These fractures are particularly common in the radius, femur, and hip and may partially be the result of an increased propensity for falls due to retinopathy and lower limb neuropathy[198].

Interestingly, type 1 and type 2 diabetes have distinct effects on skeletal composition. Type 1 diabetics suffer from a substantially increased risk of fragility fractures – approximately 6-fold higher than the general population – and as to be expected, display osteopenia and significant reductions in bone mineral density (BMD)[196, 197, 199-203]. One study has reported the prevalence of osteoporosis (defined as BMD at least 2.5 standard deviations below the mean BMD of an average 30-year old white woman) as 19.1% among type 1 diabetics (with mean age of 30 years), with osteopenia (defined as BMD 1-2.5 standard deviations below the same standard) being found in 34-67% of patients[201, 204-206]. Low BMD has also been found to correlate with an increased severity and incidence of chronic vascular complications, underscoring the interrelatedness between the BM and endothelial damage[201, 203, 206]. As type 1 diabetes

typically presents during childhood or adolescence, low bone mass may be the result of impaired bone formation during critical skeletal growth periods[195, 207].

Type 2 diabetes however, despite being characterized by normal or heightened BMD (up to 8% higher than non-diabetic subjects after controlling for confounding factors), is also associated with an increase in fracture risk due to inferior bone quality[196, 197, 199, 208-210]. The impaired bone quality is associated with altered collagen crosslinking, build-up of AGEs, and reduced bone turnover[211]. Interestingly, the risk of fracture in type 2 diabetes appears to have a biphasic distribution, with a reduced fracture risk in newly diagnosed diabetics and significantly greater risk with increasing duration of the disease[212].

1.3.1 Mechanisms of Skeletal Involvement in Diabetes

The involvement of the skeleton in diabetes may arise as the combined consequence of several mechanisms, including altered regulation of vitamin D, reduced calcium absorption in the intestine, and accumulation of AGEs within the bone. The changes in blood chemistry associated with diabetes have also been shown to alter the development of marrow MPCs, preventing their proliferation and skewing lineage potential in favor of adipogenic development over osteoblastogenesis[183, 213, 214]. The distinct effects of type 1 and 2 diabetes on bone composition may be explained by a disrupted balance between BM-MPC self-renewal and differentiation, with promotion of adipogenesis over osteoblastogenesis in type 1 diabetes and at the expense of self-renewal in type 2 diabetes. Both diseases result in an altered cellular composition of the BM and the microenvironment surrounding VSCs and EPCs, which may mediate

EPC dysfunction. There appear to be several mechanisms underlying the shift in MPC developmental potential in diabetes, including hyperinsulinemia, hyperlipidemia, hyperglycemia, and the use of certain diabetic medications.

1.3.1.1 Hyperinsulinemia

Insulin is responsible for the regulation of a number of different processes in adipocytes: the accumulation of triglycerides, increasing glucose transport, enhancing the rate of lipogenesis, inhibiting lipolysis, and promoting adipogenic differentiation[215]. *In vitro*, insulin signalling is requisite to induce adipogenesis in cell culture systems and *in vivo* IR-knockout models are subject to compromised adipogenic differentiation[216-220]. Hyperinsulinemia is often present in the initial stages of type 2 diabetes as pancreatic β-cell insulin production surges in an attempt to combat increasing peripheral insulin resistance[221, 222]. Insulin binding activates IR or IGF-1 receptor (IGF1R), which leads to the tyrosine phosphorylation of the insulin receptor substrate (IRS) and stimulation of a tyrosine signalling pathway involving PI3K and PKB (Figure 1.7)[215]. The specific effects of insulin on adipocytes occur via two mechanisms. Firstly, PKB activates mammalian target of rapamycin (mTOR), which in turn stimulates sterol regulatory element-binding protein 1c (SREBP1c; also known as adipocyte determination and differentiation-dependent factor 1 or ADD1) to regulate the transcription of adipogenic genes, such as fatty acid synthase (FAS) and lipoprotein lipase (LPL)[223-226]. PKB also phosphorylates forkhead box protein O1 (FOXO1), precluding its entry into the nucleus where it serves as a transcriptional repressor of PPARγ[227, 228].

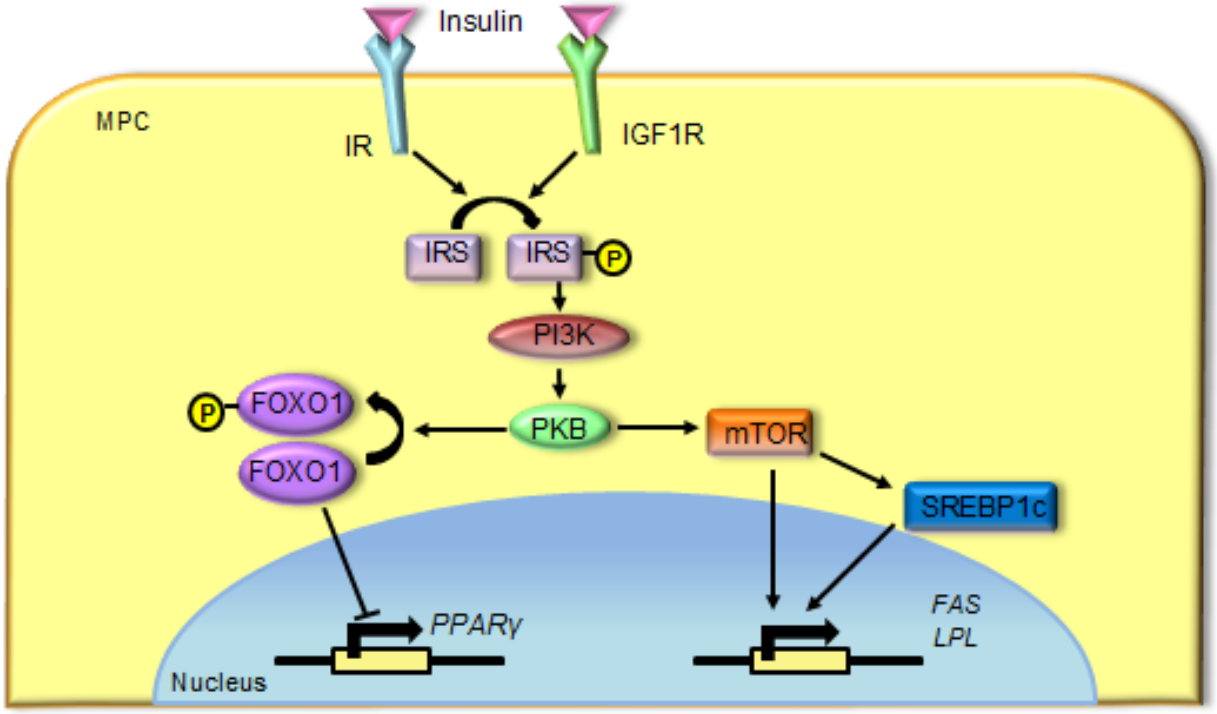


Figure 1.7. Hyperinsulinemia promotes adipogenesis in MPCs.

Binding of insulin to IR or IGF1R stimulates the phosphorylation of IRS, leading to the activation of a PI3K/PKB signalling pathway. This pathway induces the transcription of PPARγ and adipocyte-specific genes, such as FAS and LPL.

[FAS = fatty acid synthase; FOXO1 = forkhead box protein O1; IGF1R = insulin-like growth factor-1 receptor; IR = insulin receptor; IRS = insulin receptor substrate; LPL = lipoprotein lipase; MPC = mesenchymal progenitor cell; mTOR = mammalian target of rapamycin; PI3K = phosphoinositide-3-kinase; PKB = protein kinase B; PPARγ = peroxisome proliferator activated receptor γ]

Insulin has also been suggested to have anabolic actions on bone[229]. Osteoblasts possess high affinity receptors capable of binding insulin at physiological concentrations, and insulin signalling has been shown to be critical for osteogenic progenitor cell proliferation, bone mineralization, and bone turnover[230-235]. Insulin has also been shown to positively influence the survival of osteoblasts in a dose-dependent manner[236]. As hyperinsulinemia characterizes the early stages of type 2 diabetes, the unique effects insulin exerts on osteoblasts may contribute to the increased BMD and reduced fracture risk seen in newly-diagnosed diabetic subjects[212, 237, 238]. Following β-cell failure (or in type 1 diabetics), patients devolve into a hypoinsulinemic state, preventing the occurrence of these osteo-anabolic effects and potentially contributing to the enhanced fracture risk in the type 2 diabetic population with increasing disease duration[239].

1.3.1.2 Hyperlipidemia

Hyperlipidemia is nearly a universal hallmark of type 2 diabetes and is also a frequent comorbidity in type 1 diabetes, particularly when poorly controlled[240-242]. Plasma levels of free fatty acids (FFAs) are elevated in many diabetics and have been shown to contribute to the development of insulin resistance and cardiovascular disease[242-246]. Significant elevations in the relative quantities of di- and tri-unsaturated fatty acids relative to saturated fats have also been found in the plasma of non-obese diabetic mice[247]. PPARγ is capable of serving as a physiological sensor of lipid levels, with both mono- and poly-unsaturated fatty acids binding to and activating the transcription factor to promote adipogenesis[248, 249]. In addition to the effects of fatty

acids on undifferentiated cells, treatment of osteoblasts with serum high in fatty acids was found to induce their cross-differentiation into adipocytes, as characterized by Oil Red O staining and up-regulation of FABP4[250]. Diabetes has also been associated with increases in the endogenous production of prostaglandins[251-253]. Prostaglandins have similarly been shown to bind PPARγ to promote adipogenic differentiation, while also inhibiting osteoblastogenesis of MPCs[254-256].

1.3.1.3 Hyperglycemia

1.3.1.3.1 PI3K-PKB Pathway

High levels of blood glucose have been demonstrated to increase adipocyte formation, lipid accumulation, and the expression of PPARγ in mouse BM-derived MSCs[257]. It has been suspected that hyperglycemia partially mediates its effects through changes to post-receptor insulin signalling, which may be implicated in the development of insulin resistance[257, 258]. High levels of glucose increases the activity of PI3K and the subsequent phosphorylation of PKB, both of which are involved in the insulin signalling cascade[257, 258]. PKB-facilitated de-repression of the *pparγ* gene though FOXO1 nuclear export leads to the induction of PPARγ and C/EBPα expression, along with increased adiposity of the bone marrow[257, 259-261].

1.3.1.3.2 Reactive Oxygen Species

As described earlier, diabetes is characterized by overproduction of ROS. Excessive movement of electrons through the TCA cycle eventually overwhelms the electron transport system and results in the generation of superoxide from molecular

oxygen. Superoxide is then able to inhibit the action of glycolytic enzyme glyceraldehyde-3 phosphate (GAPDH), which leads to the stimulation of the AGE pathway that has been shown to be increasingly activated in diabetes[22, 262, 263]. AGEs are proteins or lipids that become glycosylated following exposure to sugars and accelerate cellular oxidative damage and have been implicated in both micro- and macro-vascular diabetic complications[264-267]. Binding of AGEs and their receptors, known as RAGE, have been associated with reduced bone formation by osteoblasts and diminished matrix mineralization, in addition to impaired osteoblastogenesis[268, 269]. AGE-RAGE interactions have also been identified as promoters of the MPC and osteoblast apoptosis, contributing to the depletion of the BM SC niche[270, 271]. Additionally, AGEs in collagen lead to heightened cross-linking and increased stiffness of the collagen network, possibly resulting in bone fragility[263, 272].

Oxidative stress induced by hyperglycemia has also been found to activate the PI3K/PKB pathway, which acts to inhibit osteoblastic maturation and stimulate adipogenesis[273]. Osteoblasts exposed to ROS resultant from high glucose demonstrate decreased expression of Runx2 and osteocalcin, with a concomitant increase in the abundance of the adipogenesis-related factors PPARγ, adipsin, and aP2[273]. ROS is also able to prevent matrix mineralization and enhance the accumulation of lipid droplets in osteoblasts[273].

1.3.1.3.3 Non-Canonical Wnt-PKC Pathway

While most of the wingless-type MMTV integration site family member (Wnt) family of genes are responsible for the negative regulation of adipogenesis, we have

shown that Wnt11 may be induced by hyperglycemia to enhance the adipocytic differentiation of marrow cells[274]. While the mechanism remains to be fully elucidated, our current working model is that, through a non-canonical pathway, hyperglycemia induces a switch in Wnt11 signalling that differentially activates the various isoforms of PKC, specifically inducing the phosphorylation and consequent activation of PKC-γ or -ε[274, 275]. PKC-ε is trans-located from the cytoplasm to the nucleus where it is expressed in spatiotemporal symmetry with C/EBPβ, suggesting a potential interaction[274, 276]. Through a currently unknown process likely involving the phosphorylation and regulation of key nuclear adipogenic factors, PKC-ε activation results in the acceleration of adipocytic differentiation[276].

1.3.1.3.4 Hyperglycemia on Osteoblasts

Understanding the effects of excessive glucose on pre-osteoblasts has been complicated by the inconsistent definitions of hyperglycemia used by researchers, although this also allows for the characterization of a dose-response effect to increasing glucose concentrations. Moderate elevations in glucose level (15 mmol/L) have been shown to promote the proliferation and differentiation of pre-osteoblasts via activation of the PI3K/PKB signalling pathway, while also significantly reducing calcium uptake and deposition[277, 278]. As glucose concentration increased to 20-35.5 mM, differentiation of pre-osteoblasts and the expression of osteogenic genes progressively decreased[278, 279]. Escalating glucose levels have also been associated with significant increases in ROS, as well as apoptosis in mature osteoblasts[280]. Hyperglycemia has been shown to down-regulate pro-osteoblastic genes Runx2, BGLAP, and osteonectin with simultaneous up-

regulation of adipogenic genes PPARγ, adipsin, and adipocyte protein 2 (aP2), as well as lipid accumulation, suggesting the potential for cross-differentiation[281, 282].

Diabetes is characterized by the accelerated formation of AGEs due to the greater availability of glucose[283]. AGEs are capable of inhibiting the osteoblastic differentiation of precursor cell lines via repression of Sp7 independently of hyperglycemia[284]. Additionally, long-term interactions between AGEs and its receptor, RAGE, stimulates the apoptosis of osteoblastic cells both *in vitro* and *in vivo*, particularly in more mature cell types[270]. In addition, the formation of AGEs has been shown to increase collagen network stiffness, leading to a reduction in its ductility and contributing to the increased susceptibility to fractures in diabetics[211, 285].

1.3.1.4 Effects of Diabetic Medications

1.3.1.4.1 Thiazolidinediones

Thiazolidinediones (TZDs or glitazones) are a class of oral anti-diabetic medications once commonly prescribed to improve insulin responsiveness that have since fallen from favour over concerns regarding their cardiovascular and hepatic safety[286, 287]. TZDs exert their beneficial effect on insulin resistance through high affinity binding to and activation of PPARγ[288]. Within adipose deposits, this interaction skews the differentiation potential of resident progenitor cells towards the fat cell lineage, leading to weight gain[289]. In the BM, TZDs skew the development of MPCs by driving adipogenesis directly through the induction of PPARγ, while simultaneously suppressing osteoblastic development[290-293]. Treatment of osteo-adipo precursor cells with TZD

results in up-regulation of fat cell-specific factors, such as adipsin and FABP4[293]. BM-MPCs treated with rosiglitazone demonstrate suppression of early markers of osteoblastic differentiation, such as Runx2, as well as biochemical indicators of bone formation, such as osteocalcin, alkaline phosphatase, and collagen 1[293-295]. Others have attributed the loss of bone density to TZD-induced apoptosis of osteoblasts and mature osteocytes in concomitance with inhibition of bone formation[296-298].

TZDs have been variously reported as having both positive and negative effects on the differentiation and function of osteoclasts. TZDs have been shown to be potent inhibitors of osteoclast formation and minimizers of bone resorption[299, 300]. Alternatively, osteoclast-specific deletion of PPARγ has also been found to result in osteopetrosis and increased bone mass, while TZD-mediated stimulation of PPARγ can increase osteoclastic differentiation and lead to excessive bone resorption[301, 302]. This effect appears to be the result of a PPARγ-stimulated increase in the transcription of c-Fos, which plays a critical role in the differentiation of osteoclasts[301, 303]. Additionally, rosiglitazone has been shown to increase the abundance of C-terminal telopeptide in some diabetic populations, which serves as a biomarker of osteoclast function, though it has also been reported to inhibit osteoclastogenesis and bone resorption[295].

1.3.1.4.2 Insulin Analogues

As insulin is a well-known inducer of adipogenesis, it is of little surprise that starting diabetic patients on exogenous insulin therapy is often associated with a significant increase in adiposity. Type 2 diabetic subjects treated solely with Neutral Protamine Hagedorn (NPH) insulin reported an average increase in body weight of 7.5

kg over 12 months of therapy[304]. This weight gain appears to be a consequence of increased lipid accumulation from fully developed fat cells and promotion of the adipogenic differentiation of progenitor cells[305]. Interestingly, not all insulin analogues have an identical effect on progenitor cells, with lower mRNA expression of adipogenic markers PPARγ and leptin among preadipocytes treated with insulin detemir[305-307]. Insulin detemir has been shown to have a minimal effect on MPC differentiation or weight gain relative to other insulin formulations[305, 308-310]. This may be a function of the medication's reduced affinity for the insulin receptor, though treatment of preadipocytes with both equimolar and equipotent concentrations of insulin detemir resulted in significantly less differentiation than did treatment with human insulin[305, 306, 311]. Specifically in regards to bone, treatment of type 2 diabetics with insulin therapy has been associated with an increased risk of fractures[312, 313].

1.3.1.4.3 Metformin

Metformin is the often the first-line pharmacological therapy of choice in the management of type 2 diabetes[314]. Metformin is an orally administered biguanide that moderates plasma glucose levels by suppressing excessive hepatic gluconeogenesis[315]. Unlike many other anti-diabetic medications, metformin is not associated with increased enhanced adipogenesis or weight gain, and in many cases, actually leads to a reduction in weight[316, 317]. A retrospective chart review of type 2 diabetics treated with metformin alone identified a significant increase in BMD, while joint therapy with metformin and insulin failed to produce this increase[318]. Other studies have similarly confirmed a decrease in the risk of fractures among diabetics treated with

metformin[319, 320]. *In vivo* animal models have shown increased BMD and bone formation when treated with metformin, while the administration of metformin to *in vitro* culture systems appears to promote osteoblastic differentiation of MPCs and inhibit adipogenesis[321-326]. Metformin increases the expression of several biochemical markers of bone formation among osteoblasts, such as alkaline phosphatase, collagen 1, and BGLAP[323]. Metformin also enhances the production of osteoprotegerin, an osteoclast-inhibitory factor, and decreases the expression of the osteoclast-stimulating factor RANKL by osteoblastic cells to reduce bone turnover[321].

Metformin has been shown to increase bone density by promoting the osteogenic differentiation of MPCs both *in vitro* and *in vivo*, with up-regulation of osteoblastic transcription factor Runx2 and no discernable effect on PPARγ, potentially leading to a slight reduction in fracture risk[319, 322-324]. This enhanced osteogenesis appears to be the result of increased activation of AMP-activated protein kinase (AMPK) leading to up-regulation of eNOS and BMP2[324, 327, 328]. Metformin may also reduce levels of sclerostin, an osteocyte-produced glycoprotein with anti-anabolic effects on bone through Wnt and β-catenin signalling to enhance bone density[318].

1.4 Rationale

Previously, the primary role of adipocytes was believed to be energy storage, though as of late, an important regulatory function for adipocytes has been realized. Adipocytes are now recognized as acting systemically through the production of hormones such as adiponectin, resistin, and leptin, as well as acting locally via the actions of an assortment of cytokines. We believe that diabetes-induced shift in the developmental potential of BM-MPCs leads to the formation of a unique cellular composition within the BM, skewed in favor of adipocytes. Studies of numerous other SC types have established the importance of the extracellular environment surrounding SCs in guiding cellular differentiation. Many studies have investigated the role of the BM hematopoietic SC niche on HSC development, including variations of the niche within the context of disease states such as diabetes[193, 329-331]. Interestingly, relatively little has been written about the interactions between the BM microenvironment and resident VSCs and EPCs, even though the cell types are significant contributors to vascular health. The interactions between VSCs/EPCs, BM stromal cells, and other BM components that govern the differentiation and release of EPCs into circulation are poorly understood[46]. Elucidating the mechanism by which diabetes leads to impairments in EPC survival, migration, and proliferation may provide a novel therapeutic approach for the prevention and management of diabetic complications.

1.5 Hypothesis

I hypothesize that the diabetes-induced changes in the differentiation potential of BM-MPCs alters the composition of the BM SC niche in a way which significantly impairs resident VSC survival.

1.6 Specific Aims

In order to test out hypothesis, I established three primary objectives:

1. To construct an *in vitro* niche modelling system to assess the impact that co-culture with MPCs, adipocytes, and osteoblasts have on the survival and adherence of CD133-expressing VSCs
2. To identify genes that are differentially regulated by adipocytes and osteoblasts relative to MPCs that may affect the properties of VSCs
3. To utilize a rat model of diabetes to correlate our *in vitro* gene expression data to an *in vivo* model of diabetes

Chapter 2 : Materials and Methods

2 Materials and Methods

2.1 *In Vitro* Studies

I derived BM-MPCs from cultures of BM mononuclear cells (MNCs; Lonza Inc., Walkersville, MD) on plastic tissue culture dishes coated with 1 μg/cm^2 fibronectin (FN) (Millipore, Temecula, CA) in complete Endothelial Basal Media-2 (EBM-2; Lonza Inc.) supplemented with 20% fetal bovine serum (FBS; Life Technologies, Burlington, ON), 1% antibiotic-antimycotic solution of penicillin, streptomycin, and fungizone (amphotericin) (PSF; Mediatech Inc., Manassas, VA), and SingleQuots (Lonza Inc.) containing VEGF, IGF-1, human epidermal growth factor, human basic fibroblast growth factor, ascorbic acid, heparin, hydrocortisone, and gentamicin/amphotericin B. The media was changed three times per week. Cultures were maintained in an incubator with 5% CO_2 at 37^{o}C. In order to generate human adipocytes and osteoblasts, I prepared BM-MPCs as described above, with the exception of FN coating of culture dishes. I then induced differentiation by exposing cells seeded at a density of 50,000 cells/cm^2 to specific differentiation media. Differentiation into adipocytes was induced through a seven-day culture of BM-MPCs in StemPro Adipogenesis Differentiation Media (Life Technologies), supplemented with 1% PSF. BM-MPC differentiation into osteoblasts was induced through a 14-day culture of confluent BM-MPCs in StemPro Osteogenesis Differentiation Media (Life Technologies) with 1% PSF. Differentiation media were changed three times per week.

I utilized Poietics™ human umbilical cord blood CD133+ cells (Lonza Inc.) to serve as VSCs, as CD133 is, at present, the single best marker of precursor cells with endothelial potential[145, 147]. Our laboratory and others have previously shown derivation of outgrowth EPCs from CD133+ umbilical cord and bone marrow cells[143, 332, 333]. As the expression of CD133 tends to be lost very rapidly in these cells upon culture, I utilized the cells without prior culture in all experiments in order to minimize variability[146]. For some experiments, I maintained CD133+ cells in short-term culture in complete EBM-2, with 20% FBS, 1% PSF, and SingleQuots. For co-culture experiments, 10,000 CD133+ cells/cm^2 were seeded onto confluent cultures of BM-MPCs, or MPC-derived adipocytes or osteoblasts and maintained for one to 21 days. Twenty-one days was selected as a suitable endpoint in order to allow for the potential differentiation of CD133+ cells into ECs, which occurs after approximately three weeks of culture[143, 334].

2.2 *In Vivo* Model

All experiments were conducted in accordance with Western University and Animal Care and Veterinary Services Guidelines. Six-week old male Sprague-Dawley rats (175 g) were obtained from Charles River (Wilmington, MA). A single intraperitoneal injection of 65 mg/kg streptozotocin (STZ) in a pH 5.6 citrate buffer was administered. Control animals were subjected to an intraperitoneal injection of an equivalent volume of citrate buffer alone. Changes in body weight and blood glucose in rats were monitored for four weeks following STZ injections and confirmation of hyperglycemia. After four weeks, the rats were then euthanized and their femurs were extracted. Four weeks has previously been shown to be a sufficient length of time to

elicit significant changes in skeletal composition and bone deposition in models of STZ-induced diabetes in both mice and rats[335-338].

2.2.1 RNA Isolation from Marrow Samples for qPCR

The BM of the medullary cavity of femurs of each rat was then extracted in order to conduct gene expression analyses. To remove the BM, the epiphyses of the bones were removed, followed by centrifugation of the diaphyses at 200 xg for 12 minutes. The resulting fluid was then suspended in RLT lysis buffer (from RNeasy Mini Plus kit; Qiagen, Mississauga, ON) before being passed through an 18-gauge syringe.

2.3 Measurement & Assessment

2.3.1 RNA Isolation and qRT-PCR

Total RNA was extracted from cells and isolated rat marrow using RNeasy Mini Plus kits and according to established protocol[339]. cDNA was synthesized using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Inc., Mississauga, ON) and the PTC-100 Thermal Cycler (MJ Research, St. Bruno, QC). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was conducted using custom PrimePCR arrays (Bio-Rad Laboratories, Inc.) (Tables 2.1 and 2.2) or individual primers as listed in Tables 2.3 and 2.4 (Qiagen). PCR reactions for *in vitro* experiments consisted of 10 µL of SsoFast Evagreen Supermix (Bio-Rad Laboratories, Inc.), 1 µL of cDNA, 1 µL of forward and reverse primers at 10 µM (except when using PrimePCR arrays), and 8-9 µL nuclease-free H_2O (total reaction volume of 20 µL). PCR reactions for *in vivo* experiments utilized 10 µL of RT^2 SYBR Green Mastermix (Qiagen), 2 µL of

Table 2.1. Primer sequence information for niche gene qRT-PCR custom plate array.

Gene	Species	Length (bp)	Source (Catalogue Number/Assay ID)
SC Markers			
CD90	Human	120 bp	Bio-Rad (qHsaCED0036661)
CD133	Human	146 bp	Bio-Rad (qHsaCID0017657)
cKit	Human	63 bp	Bio-Rad (qHsaCID0008692)
Nanog	Human	116 bp	Bio-Rad (qHsaCED0023824)
Octamer-binding transcription factor 4 (Oct4)	Human	100 bp	Bio-Rad (qHsaCED0038334)
SCF	Human	63 bp	Bio-Rad (qHsaCID0008692)
	Human	115 bp	Bio-Rad (qHsaCID0008103)
Sox2	Human	98 bp	Bio-Rad (qHsaCED0036871)
Endothelial Markers			
VE-cadherin	Human	112 bp	Bio-Rad (qHsaCID0016288)
vWF	Human	113 bp	Bio-Rad (qHsaCED0033955)
Hematopoietic Markers & Signalling			
CD34	Human	99 bp	Bio-Rad (qHsaCID0007456)
CD45	Human	69 bp	Bio-Rad (qHsaCED0038908)
Hemoglobin-α1 (HBA1)	Human	90 bp	Bio-Rad (qHsaCED0020775)
Myeloproliferative leukemia protein (MPL)	Human	119 bp	Bio-Rad (qHsaCID0015934)
Thrombopoietin (THPO)	Human	92 bp	Bio-Rad (qHsaCED0002654)

Adipogenic Markers			
C/EBP-α	Human	69 bp	Bio-Rad (qHsaCED0019045)
C/EBP-β	Human	117 bp	Bio-Rad (qHsaCED0019041)
PPARγ	Human	117 bp	Bio-Rad (qHsaCID0011718)
Osteogenic Markers			
BGLAP	Human	69 bp	Bio-Rad (qHsaCED0038437)
Runx2	Human	80 bp	Bio-Rad (qHsaCID0006726)
Sp7	Human	139 bp	Bio-Rad (qHsaCED0003759)
BMP Signalling			
BMP4	Human	127 bp	Bio-Rad (qHsaCED0003208)
BMP4R1A	Human	164 bp	Bio-Rad (qHsaCED0003308)
BMP4R1B	Human	120 bp	Bio-Rad (qHsaCID0021330)
BMP4R2	Human	119 bp	Bio-Rad (qHsaCID0008240)
Notch Receptors			
NOTCH1	Human	141 bp	Bio-Rad (qHsaCID0011825)
NOTCH2	Human	72 bp	Bio-Rad (qHsaCED0005739)
NOTCH3	Human	115 bp	Bio-Rad (qHsaCID0006529)
NOTCH4	Human	163 bp	Bio-Rad (qHsaCID0037298)
Notch Ligands			
Delta-like ligand 1 (DLL1)	Human	100 bp	Bio-Rad (qHsaCID0011257)
	Human	110 bp	Bio-Rad (qHsaCED0048350)
DLL3	Human	100 bp	Bio-Rad (qHsaCED0003364)
DLL4	Human	84 bp	Bio-Rad (qHsaCID0008450)
Jagged-1 (JAG1)	Human	99 bp	Bio-Rad (qHsaCID0006831)

	Human	96 bp	Bio-Rad (qHsaCED0042862)
JAG2	Human	127 bp	Bio-Rad (qHsaCED0003193)
	Human	72 bp	Bio-Rad (qHsaCED0047702)
Angiopoietin (Ang) Signalling			
Ang1	Human	140 bp	Bio-Rad (qHsaCID0008671)
Ang2	Human	148 bp	Bio-Rad (qHsaCID0017615)
Tie-1	Human	75 bp	Bio-Rad (qHsaCID0006540)
	Human	115 bp	Bio-Rad (qHsaCED0042231)
Tie-2	Human	101 bp	Bio-Rad (qHsaCID0015119)
Cell Adhesion Molecules			
CD38	Human	110 bp	Bio-Rad (qHsaCID0006586)
Integrin-α4	Human	73 bp	Bio-Rad (qHsaCID0007441)
Integrin-β1	Human	104 bp	Bio-Rad (qHsaCED0005248)
Neuronal cadherin (N-cadherin)	Human	151 bp	Bio-Rad (qHsaCID0015189)
Vascular cell adhesion molecule (VCAM)	Human	137 bp	Bio-Rad (qHsaCID0016779)
SDF-1 Signalling			
SDF-1	Human	94 bp	Bio-Rad (qHsaCID0012398)
C-X-C chemokine receptor 4 (CXCR4)	Human	142 bp	Bio-Rad (qHsaCED0002020)
Immunoglobulin Superfamily Genes			
CD33	Human	148 bp	Bio-Rad (qHsaCID0006439)
Telomerase			
Telomerase reverse transcriptase (TERT)	Human	150 bp	Bio-Rad (qHsaCID0009247)

Housekeeping Genes

β-actin	Human	62 bp	Bio-Rad (qHsaCED0036269)
β2-microglobulin	Human	123 bp	Bio-Rad (qHsaCID0015347)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Human	117 bp	Bio-Rad (qHsaCED0038674)

Table 2.2. Primer sequence information for ECM gene qRT-PCR custom plate array.

Gene	Species	Length (bp)	Source (Catalogue Number/Assay ID)
A Distintegrin and Metalloproteinase (ADAM) Peptidases			
ADAM 9	Human	144 bp	Bio-Rad (qHsaCID0018553)
ADAM 10	Human	95 bp	Bio-Rad (qHsaCED0001377)
ADAM 12	Human	99 bp	Bio-Rad (qHsaCID0011870)
ADAM 17	Human	103 bp	Bio-Rad (qHsaCID0016420)
ADAM 19	Human	67 bp	Bio-Rad (qHsaCID0008717)
ADAM 20	Human	98 bp	Bio-Rad (qHsaCED0003133)
ADAM 21	Human	148 bp	Bio-Rad (qHsaCED0019636)
ADAM 28	Human	112 bp	Bio-Rad (qHsaCED0004497)
ADAM 30	Human	114 bp	Bio-Rad (qHsaCED0006925)
ADAM 33	Human	113 bp	Bio-Rad (qHsaCED0001073)
MMPs			
MMP 1	Human	69 bp	Bio-Rad (qHsaCID0017039)
MMP 2	Human	144 bp	Bio-Rad (qHsaCID0015623)
MMP 3	Human	148 bp	Bio-Rad (qHsaCID0006170)
MMP 7	Human	138 bp	Bio-Rad (qHsaCID0011537)

MMP 8	Human	135 bp	Bio-Rad (qHsaCID0023232)
MMP 9	Human	82 bp	Bio-Rad (qHsaCID0011597)
MMP 10	Human	69 bp	Bio-Rad (qHsaCID0008481)
MMP 11	Human	139 bp	Bio-Rad (qHsaCID0022136)
MMP 13	Human	138 bp	Bio-Rad (qHsaCID0008487)
MMP 14	Human	60 bp	Bio-Rad (qHsaCED0001628)
MMP 15	Human	84 bp	Bio-Rad (qHsaCED0002668)
MMP 16	Human	66 bp	Bio-Rad (qHsaCID0016162)
MMP 17	Human	164 bp	Bio-Rad (qHsaCED0005565)
MMP 19	Human	93 bp	Bio-Rad (qHsaCID0010428)
MMP 24	Human	118 bp	Bio-Rad (qHsaCID0017196)
MMP 25	Human	91 bp	Bio-Rad (qHsaCED0004540)
Tissue Inhibitors of Metalloproteinases (TIMPs)			
TIMP 1	Human	82 bp	Bio-Rad (qHsaCID0007434)
TIMP 2	Human	145 bp	Bio-Rad (qHsaCID0022953)
TIMP 3	Human	119 bp	Bio-Rad (qHsaCID0015238)
TIMP 4	Human	77 bp	Bio-Rad (qHsaCID0016129)

Aminopeptidases			
Glutamyl Aminopeptidase (Aminopeptidase A)	Human	79 bp	Bio-Rad (qHsaCID0014953)
Fibronectin & Inhibitors			
Fibronectin	Human	138 bp	Bio-Rad (qHsaCID0012349)
Tenascin C	Human	125 bp	Bio-Rad (qHsaCID0020888)
Collagens			
Collagen 1A1	Human	114 bp	Bio-Rad (qHsaCED0002181)
Collagen 2A1	Human	102 bp	Bio-Rad (qHsaCED0001057)
Collagen 3A1	Human	90 bp	Bio-Rad (qHsaCID0014986)
Collagen 4A4	Human	114 bp	Bio-Rad (qHsaCID0016411)
Collagen 5A1	Human	193 bp	Bio-Rad (qHsaCID0014514)
Collagen 6A1	Human	90 bp	Bio-Rad (qHsaCID0007091)
Basement Membrane Component Proteins			
Laminin C1	Human	104 bp	Bio-Rad (qHsaCID0006254)
Laminin C2	Human	150 bp	Bio-Rad (qHsaCID0021924)
Laminin C3	Human	119 bp	Bio-Rad (qHsaCID0018418)
Nidogen 1	Human	106 bp	Bio-Rad (qHsaCED0036445)

Nidogen 2	Human	127 bp	Bio-Rad (qHsaCID0009675)
Heparan Sulfate			
Heparan sulfate proteoglycan 2 (HSPG2)	Human	69 bp	Bio-Rad (qHsaCED0036599)
Housekeeping Genes			
β-actin	Human	62 bp	Bio-Rad (qHsaCED0036269)
β2-microglobulin	Human	123 bp	Bio-Rad (qHsaCID0015347)

Table 2.3. Primer sequence information for qRT-PCR individual primers used for *in vitro* experimental analyses.

Gene	Species	Length (bp)	Source (Catalogue Numbers/Assay ID)
SC Markers			
CD133	Human	105 bp	Qiagen (QT00075586)
Nanog	Human	90 bp	Qiagen (QT01025850)
Oct4	Human	77 bp	Qiagen (QT00210840)
Sox2	Human	64 bp	Qiagen (QT00237601)
Endothelial Markers			
CD31	Human	144 bp	Qiagen (QT00081172)
Adipogenic Markers			
C/EBP-α	Human	88 bp	Qiagen (QT00203357)
C/EBP-β	Human	121 bp	Qiagen (QT00237580)
C/EBP-δ	Human	90 bp	Qiagen (QT00219373)
PPARγ	Human	113 bp	Qiagen (QT00029841)
FABP4	Human	100 bp	Qiagen (QT01667694)
Angiopoietin Signalling			
Ang1	Human	111 bp	Qiagen (QT00046865)
Ang2	Human	79 bp	Qiagen (QT00100947)
Tie-1	Human	63 bp	Qiagen (QT00013797)
Tie-2	Human	134 bp	Qiagen (QT01666322)
Cell Cycling Molecules			
Cyclin D1	Human	96 bp	Qiagen (QT00495285)
Housekeeping Genes			
β-actin	Human	104 bp	Qiagen (QT01680476)

Table 2.4. Primer sequence information for qRT-PCR primers used for *in vivo* experiment analyses.

Gene	Species	Length (bp)	Source (Catalogue Numbers/Assay ID)
SC Markers			
Nanog	Rat	112 bp	Qiagen (QT01300579)
Oct4	Rat	134 bp	Qiagen (QT00455028)
Sox2	Rat	128 bp	Qiagen (QT00544649)
Endothelial Markers			
CD31	Rat	96 bp	Qiagen (QT01289939)
VEGFR2	Rat	118 bp	Qiagen (QT00408352)
vWF	Rat	107 bp	Qiagen (QT01588713)
MPC Markers			
CD73	Rat	93 bp	Qiagen (QT00190876)
CD90	Rat	82 bp	Qiagen (QT00195825)
CD105	Rat	65 bp	Qiagen (QT00492870)
Adipogenic Markers			
C/EBP-α	Rat	63 bp	Qiagen (QT00395010)
C/EBP-β	Rat	113 bp	Qiagen (QT00366478)
C/EBP-δ	Rat	94 bp	Qiagen (QT00368599)
PPARγ	Rat	146 bp	Qiagen (QT00186172)
FABP4	Rat	127 bp	Qiagen (QT01290072)
Osteogenic Markers			
BGLAP	Rat	92 bp	Qiagen (QT00371231)
Runx2	Rat	172 bp	Qiagen (QT01300208)

Sp7	Rat	111 bp	Qiagen (QT00423206)
Angiopoietin Signalling			
Ang1	Rat	92 bp	Qiagen (QT00199346)
Ang2	Rat	89 bp	Qiagen (QT01592045)
Tie-1	Rat	106 bp	Qiagen (QT01592031)
Tie-2	Rat	133 bp	Qiagen (QT01592038)
ECM Genes			
Collagen 1A1	Rat	92 bp	Qiagen (QT00370622)
Collagen 4A4	Rat	108 bp	Qiagen (QT02346085)
Collagen 6A1	Rat	73 bp	Qiagen (QT00440839)
Fibronectin	Rat	92 bp	Qiagen (QT00179333)
Chemokines			
SDF-1	Rat	120 bp	Qiagen (QT00194152)
Housekeeping Genes			
β-actin	Rat	145 bp	Qiagen (QT00193473)

cDNA, and 8 µL of nuclease-free H_2O, for a total reaction volume of 20 µL. PCR reactions were performed at the temperature profiles outlined in Table 2.5 and Table 2.6 for SsoFast EvaGreen Supermix and qBiomarker SYBR Green Fluor Mastermix, respectively, using the CFX Connect Real Time PCR Detection System (Bio-Rad Laboratories, Inc.). Data were analyzed according to the ΔΔCT method using CFX Manager Software (Bio-Rad Laboratories, Inc.) and normalized to β-actin expression.

2.3.2 Immunofluorescence Cell Staining

BM-MPCs were cultured and induced to differentiate as described above on collagen 1-coated 4- or 8-chambered slides. CD133+ cells were overlaid for 48 hours and culture slides were then stained for SC markers through one hour incubations of cells at room temperature using primary antibodies (Table 2.7). Subsequent to the primary antibody incubation, cells were incubated with the appropriate Alexa488-conjugated secondary antibody (Life Technologies) for one hour at room temperature. Slides were then counterstained using ProLong® Diamond Antifade Mountant with DAPI (Life Technologies). Imaging was performed using the Olympus BX-51 fluorescent microscope (Olympus Canada Inc., Richmond Hill, ON) and SPOT Basic Image Capture & SPOT Advanced Microscope Imaging Software (SPOT Imaging Solutions, Sterling Heights, MA).

Table 2.5. Temperature profiles for qRT-PCR with SsoFast EvaGreen Supermix.

Cycling Step	Temperature	Time (min:sec)	No. of Cycles
Enzyme Activation	95°C	2:00	1
Denaturation	95°C	0:02	45
Annealing/Extension	55°C	0:12	
Melt Curve	65°C + 0.2°C to 95°C	0:10 / step	1

Table 2.6. Temperature profiles for qRT-PCR with qBiomarker SYBR Green Fluor Mastermix.

Cycling Step	Temperature	Time (min:sec)	No. of Cycles
Enzyme Activation	95°C	10:00	1
Denaturation	95°C	0:15	40
Annealing/Extension	60°C	1:00	
Melt Curve	65°C + 0.5°C to 95°C	0:10 / step	1

Table 2.7. Primary antibodies used for immunofluorescence staining.

Antigen	Host	Source (Catalogue Number)	Dilution
CD133	Rabbit polyclonal	Abcam (ab19898)	1:200
Sox2	Goat polyclonal	R&D Systems (AF2018)	1:200
Nanog	Rabbit polyclonal	Abcam (ab21624)	1:200
Oct4	Rabbit polyclonal	Abcam (ab19857)	1:200
Ki67	Rabbit monoclonal	Abcam (ab16667)	1:200

2.3.3 Statistical Analyses

Data are expressed as mean expression ± standard error of the mean (SEM). Significance was determined using two-tailed student's unpaired t-tests. P-values < 0.10 were considered to be statistically significant.

Chapter 3 : Results

3 Results

3.1 Effect of ECM Substrates on Adipogenesis

My first objective was to establish adipogenic differentiation of BM-MPCs to be used in creating an *in vitro* SC niche. In order to enhance MPC adhesion to glass chamber plates, surfaces are typically coated in a FN substrate to improve cell adherence. It has previously been demonstrated that FN may significantly alter MPC differentiation, including inhibition of adipogenesis[340-344]. To evaluate the impact FN has on adipogenesis and to identify a novel coating substrate that does not interfere with adipogenic differentiation, BM-MPCs were cultured on uncoated plastic culture dishes, or plastic dishes coated with 1 μg/cm^2 FN or 10 μg/cm^2 type 1 collagen. MPCs were induced to develop into adipocytes through culture in the appropriate differentiation medium for seven days. I observed a delay in adipogenic differentiation on FN-coated plates, with increased expression of early differentiation factor C/EBP-β and repression of late adipogenic marker PPARγ (Figure 3.1). Collagen 1-coated plates appeared to have no significant effect on adipogenesis, identifying it as a suitable substrate for cultures involving MPC differentiation.

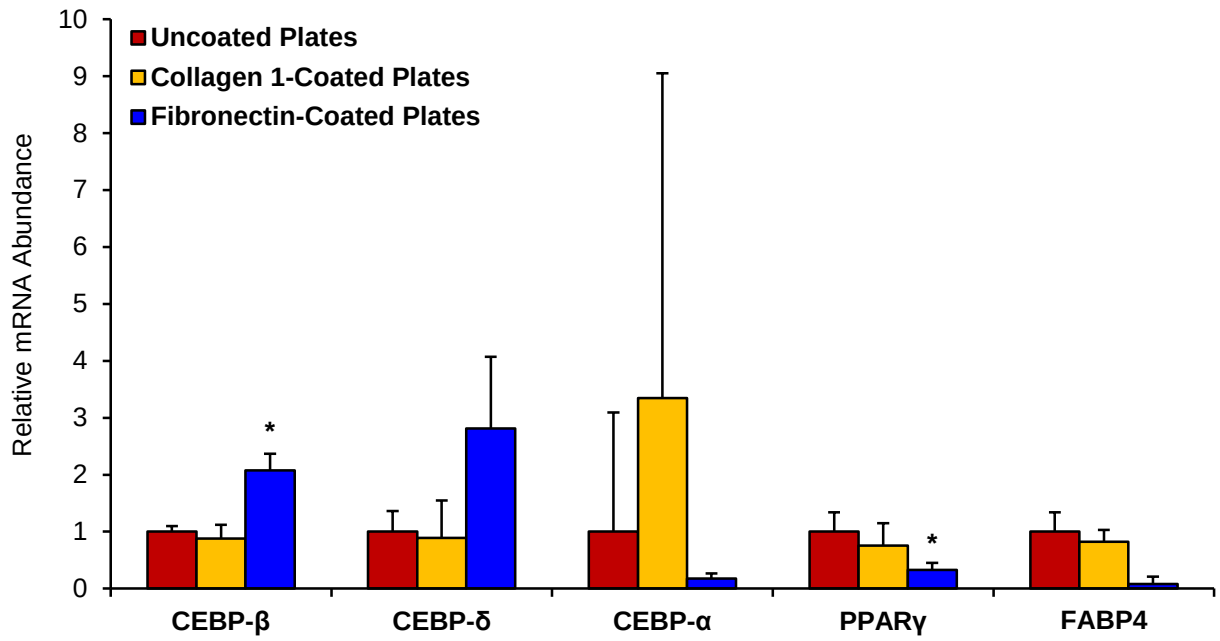


Figure 3.1. Effect of ECM substrates on adipogenesis.

BM-MPCs were induced to differentiate into adipocytes over seven days on uncoated plastic culture dishes, or plates coated with 10 μg/cm^2 collagen 1 or 1 μg/cm^2 FN. FN appeared to delay adipogenesis, with up-regulation of early adipogenic marker C/EBP-β and repression of late marker PPARγ. Collagen 1 coating did not have any significant effects on the expression of markers of adipogenic differentiation.

[C/EBP = CCAAT-enhancer-binding protein; ECM = extracellular matrix; FABP4 = fatty acid binding protein 4; PPARγ = peroxisome proliferator-activated receptor gamma]

[* $p < 0.05$ compared to uncoated plate control; data expressed as mean ± SEM, normalized to β-actin expression and expressed as fold difference of uncoated plate control]

3.2 Establishing Co-culture of CD133-Positive Cells with Adipocytes and Osteoblasts

Based on the results garnered from the previous experiment, collagen 1 was selected as an ECM substrate for coating of glass chamber slides with minimal interference on differentiation. Chamber slides were incubated for 3 hours with 10 μg/cm^2 collagen 1 in acetic acid collagen coating buffer. BM-MPCs were seeded at a high density and induced to differentiate into adipocytes or osteoblasts. CD133+ cells were then added to each chamber slide system at a cell density of 10,000 cells/cm^2. After 48 hours of co-culture, slides were subjected to immunofluorescence microscopy for CD133, Nanog, and Oct4. Figures 3.2-3.4 serve as representative images demonstrating CD133+ cells in co-culture with MPCs, adipocytes, and osteoblasts after 48 hours of culture. Although I detected CD133+ cells in these cultures, the numbers were too low for proper quantification (Figures 3.2-3.4). Therefore, I explored the possibility of using qPCR to quantify CD133 and other SC markers as a measure of the quantity of SCs in each culture. qRT-PCR was performed on CD133+ cells, MPCs, adipocytes, and osteoblasts in order to determine the expression of SC markers CD133, Nanog, and Oct4. CD133+ cells were positive for the expression of CD133, with no detection of CD133 mRNA in MPCs, adipocytes, or osteoblasts (Figure 3.5). Further, CD133+ cells were also positive for Oct4 and Nanog mRNA. The undetectable level of CD133, Nanog, and Oct4 in MPCs, adipocytes, and osteoblasts supported the use of these three factors as identifiers of the SC phenotype within this co-culture system.

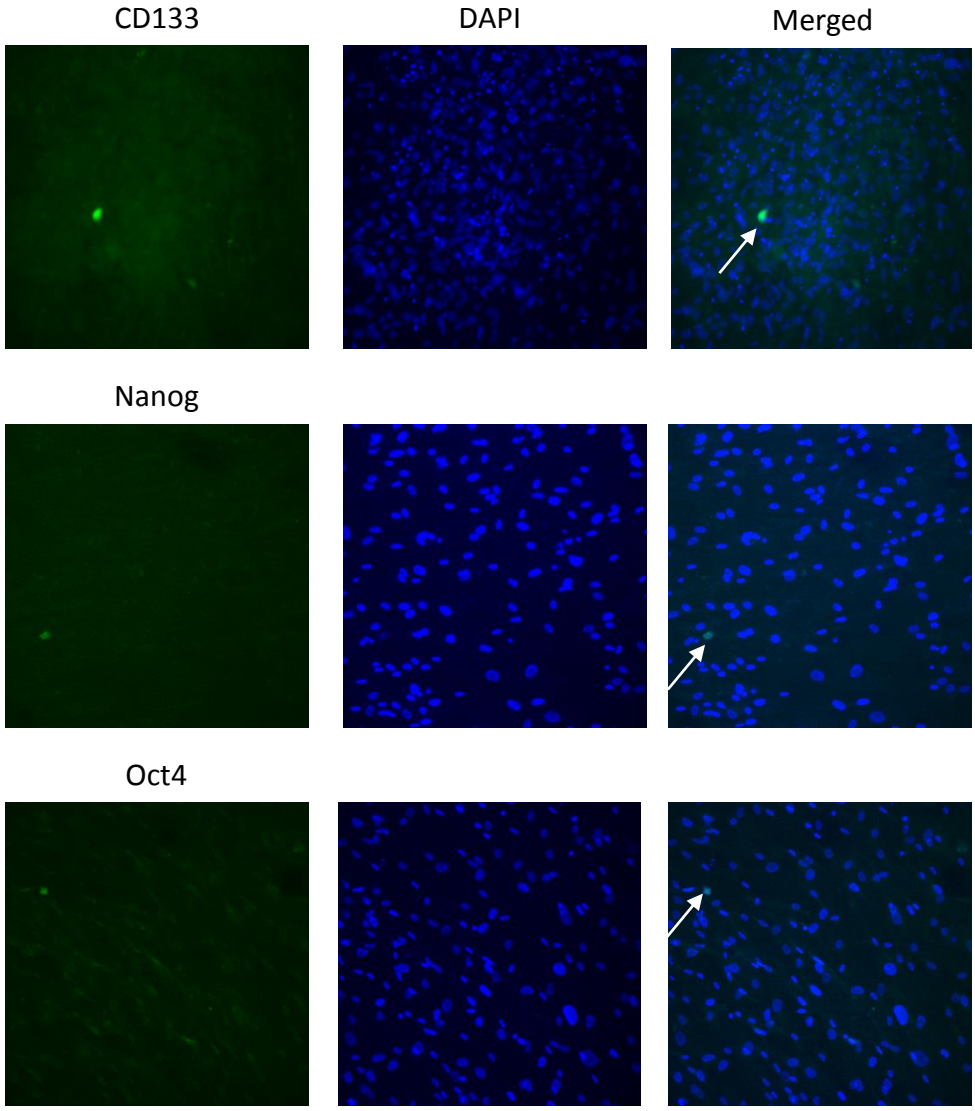


Figure 3.2. Immunofluorescence staining of CD133+ cells in BM-MPC co-culture.

CD133-positive SCs were cultured for 48 hours in co-culture with BM-MPCs before being stained for the expression of SC markers, CD133, Nanog, and Oct4.

[20x magnification]

[Oct4 = octamer-binding transcription factor 4]

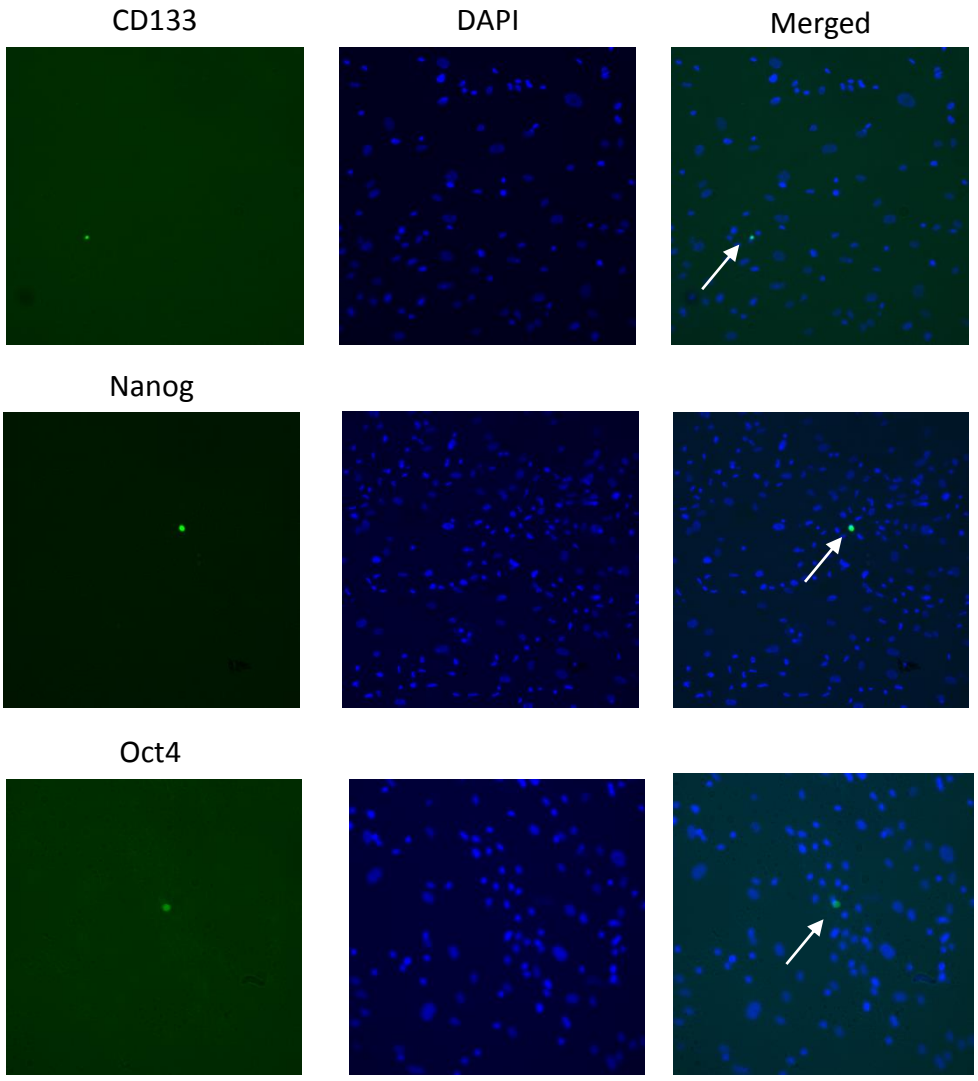


Figure 3.3. Immunofluorescence staining of CD133+ cells in adipocytic co-culture.

CD133-positive SCs were cultured for 48 hours in co-culture with adipocytes before being stained for the expression of SC markers, CD133, Nanog, and Oct4.

[Taken at 20x magnification]

[Oct4 = octamer-binding transcription factor 4]

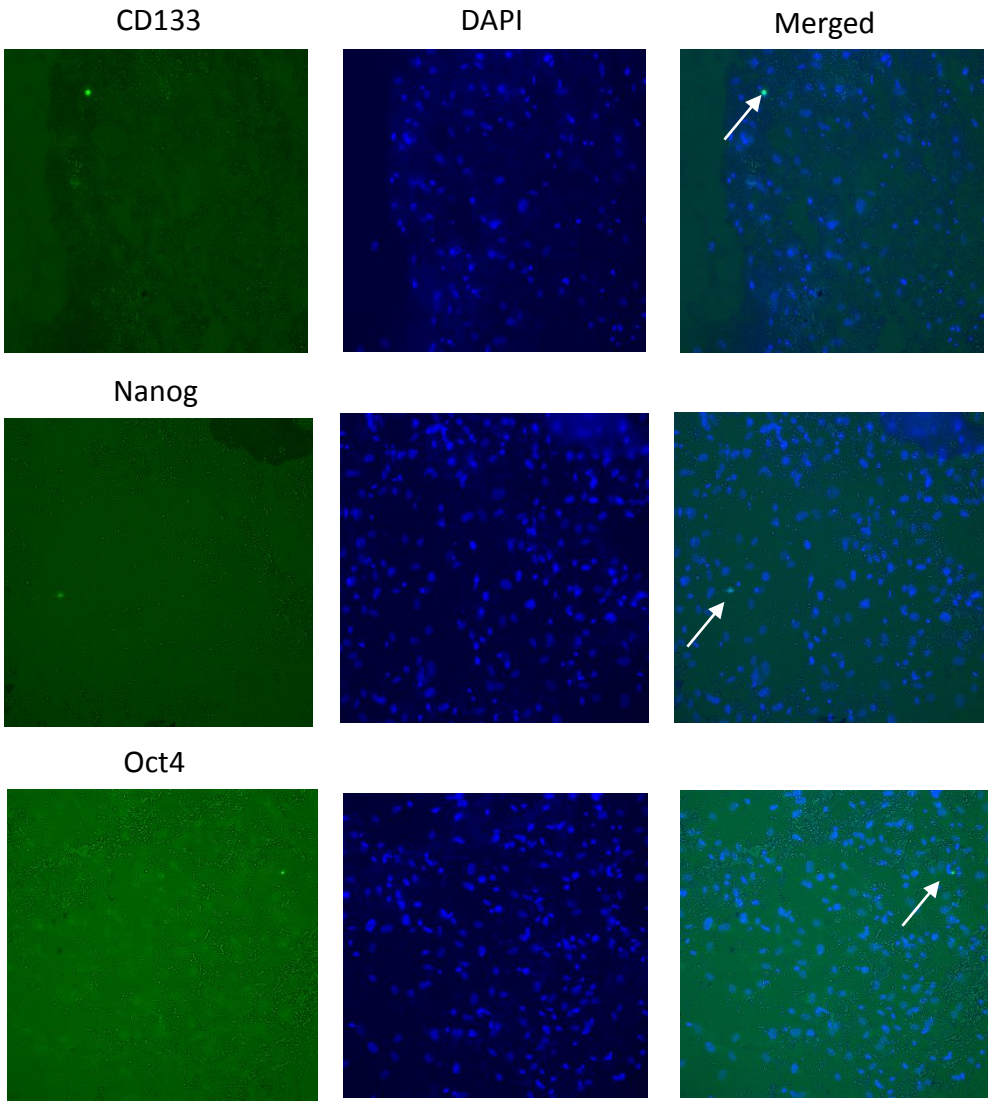


Figure 3.4. Immunofluorescence staining of CD133+ cells in osteoblastic co-culture.

CD133-positive SCs were cultured for 48 hours in co-culture with osteoblasts before being stained for the expression of SC markers, CD133, Nanog, and Oct4.

[Taken at 20x magnification]

[Oct4 = octamer-binding transcription factor 4]

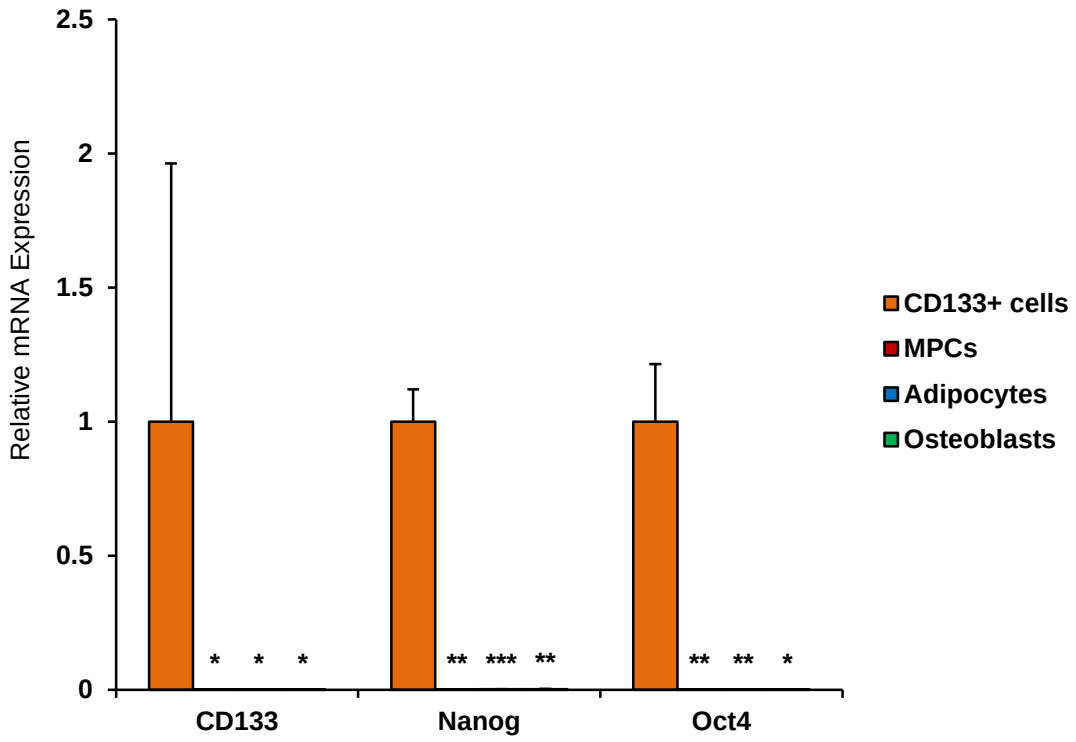


Figure 3.5. SC gene expression in CD133+ cells, MPCs, adipocytes, and osteoblasts.

There was no detectable expression of CD133, Oct4, or Nanog in MPCs or MPC-derived adipocytes and osteoblasts.

[* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to CD133-positive SC controls; data expressed as mean ± SEM, normalized to β-actin and expressed as fold difference of CD133+ cells]

[MPCs = mesenchymal progenitor cells; Oct4 = octamer-binding transcription factor 4]

3.3 Effect of Co-culturing CD133-Positive Cells with BM-MPCs, Adipocytes, or Osteoblasts

BM-MPCs and BM-MPC-derived adipocytes and osteoblasts were cultured with CD133-positive SCs for one or 21 days with regular media changes three times per week, aspirating existing media containing any non-adherent cells. After 21 days of co-culture, qRT-PCR was performed to quantify the expression of three SC markers, CD133, Nanog, and Oct4, to serve as proxies for "stemness" of the cells remaining in culture. There was a significant reduction in the mRNA levels of CD133 in co-culture with MPCs after 21 days, with non-significant decreases in the mRNA abundance of the two other stem cell markers Nanog and Oct4 (Figure 3.6). A similar pattern occurred following co-culture with adipocytes (Figure 3.7). In osteoblasts however, relatively greater expression of CD133, Nanog, and Oct4 were noted after 21 days as compared to day 1 (Figure 3.8). These results may indicate an increased number of SCs in osteoblast co-culture or induction of SC genes.

I next set out to analyze the effect BM-MPCs, BM-MPC-derived adipocytes, and BM-MPC-derived osteoblasts have on CD133-positive SCs in a modified survival assay. CD133+ cells were again co-cultured with MPCs, adipocytes, and osteoblasts for one or 21 days. Media changes were conducted three times per week, in which existing media was aspirated and any non-adherent cells were centrifuged and seeded back with fresh media. qRT-PCR was performed after one and 21 days in order to evaluate the expression of SC markers CD133, Nanog, and Oct4.

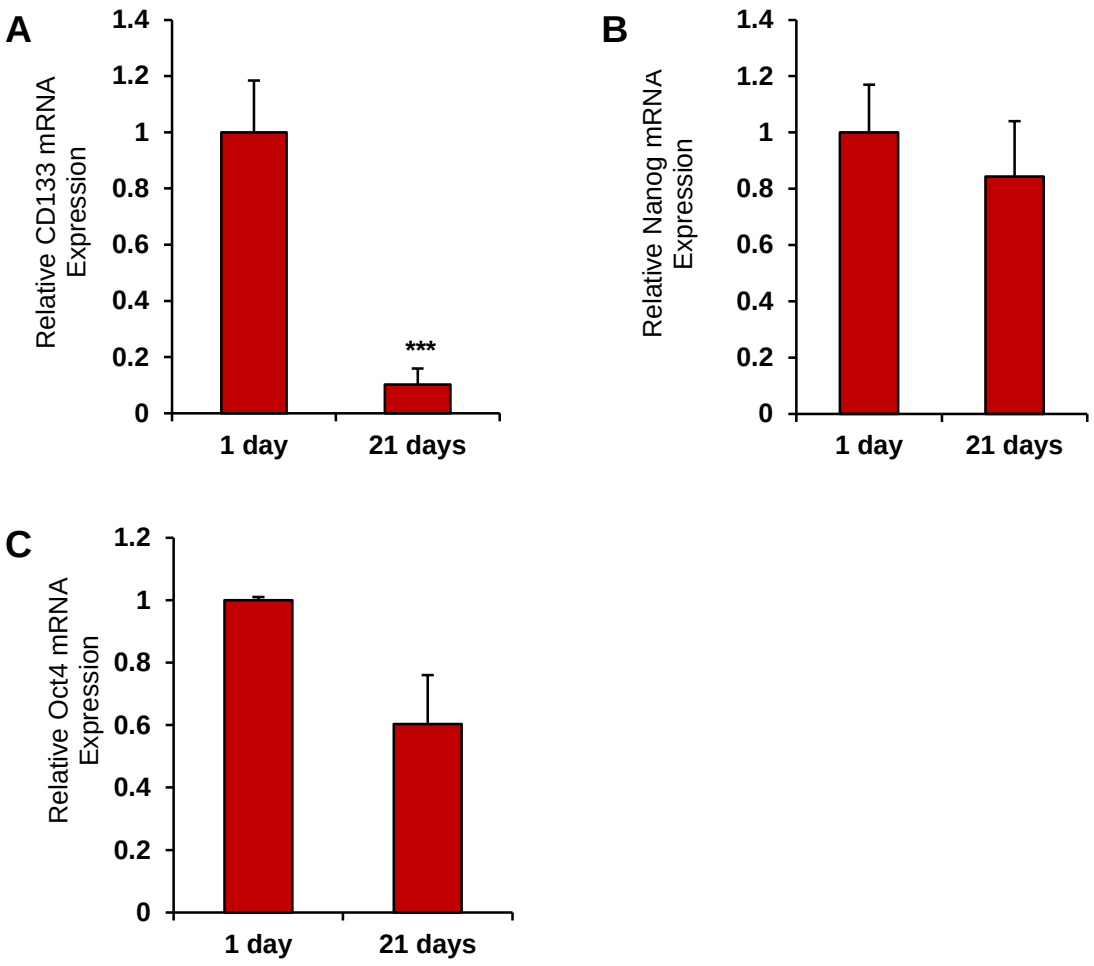


Figure 3.6. SC gene expression in adherence co-culture of CD133+ cells with MPCs.

After one and 21 days of co-culture of CD133+ cells with BM-MPCs, the expression levels of three SC markers (CD133, Nanog, and Oct4) were quantified as a measure of the stemness of cells remaining in culture. Relative to one day of co-culture, there was a significant reduction in the mRNA abundance of CD133, with non-significant reductions in Nanog and Oct4.

[*** $p < 0.001$ compared to one-day MPC co-culture controls; data expressed as mean ± SEM, normalized to β-actin and expressed as fold change of one-day MPC co-culture controls]

[Oct4 = octamer-binding transcription factor 4]

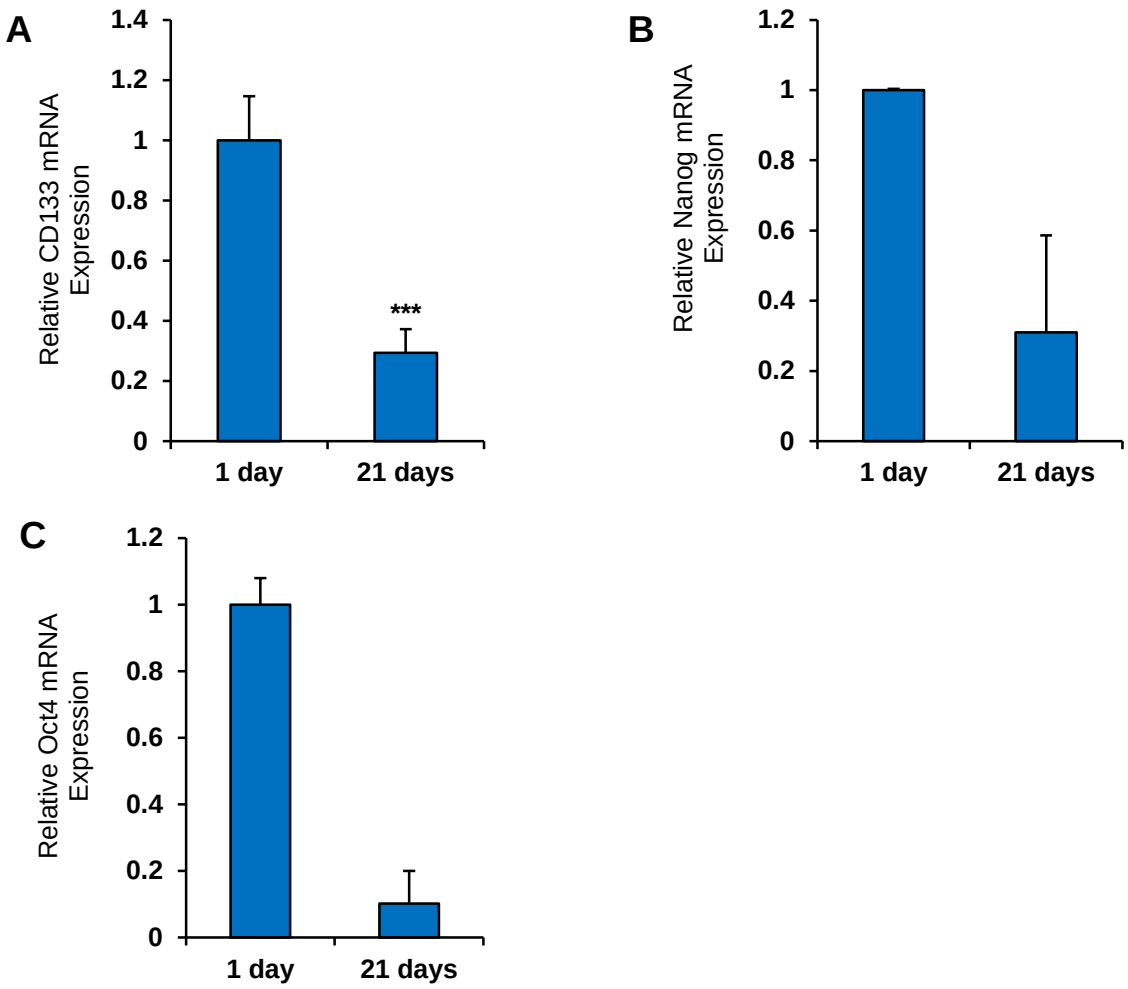


Figure 3.7. SC gene expression in adherence co-culture of CD133+ cells with adipocytes.

After one and 21 days of co-culture of CD133+ cells with BM-MPC-derived adipocytes, the expression levels of three SC markers (CD133, Nanog, and Oct4) were quantified as a measure of the stemness of cells remaining in culture. Relative to one day of co-culture with adipocytes, there was a significant reduction in the mRNA abundance of CD133, with non-significant reductions in Nanog and Oct4.

[*** $p < 0.001$ compared to one-day adipocyte co-culture controls; data expressed as mean ± SEM, normalized to β-actin and expressed as fold change of one-day adipocyte co-culture controls]

[Oct4 = octamer-binding transcription factor 4]

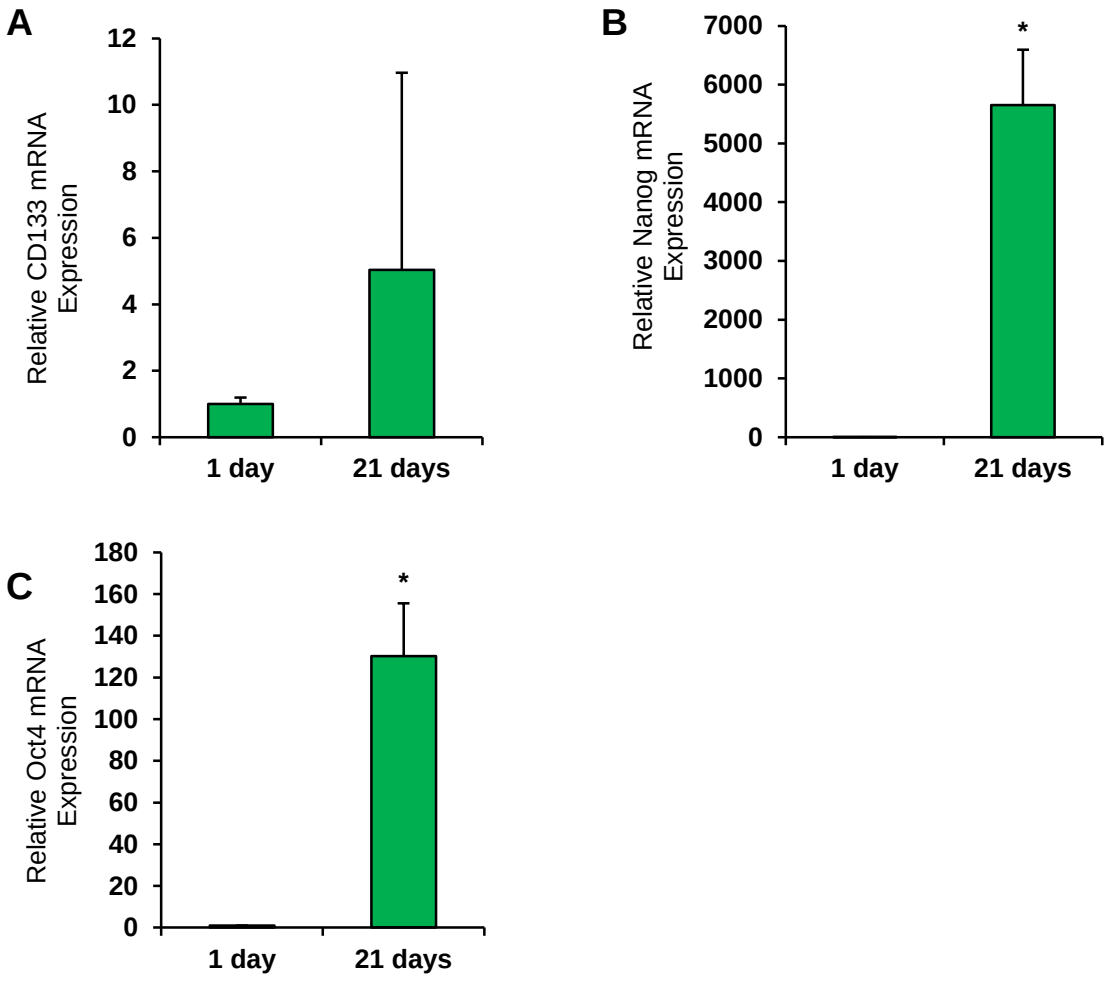


Figure 3.8. SC gene expression in adherence co-culture of CD133+ cells with osteoblast co-culture.

After one and 21 days of co-culture of CD133+ cells with BM-MPC-derived osteoblasts, the expression levels of three SC markers (CD133, Nanog, and Oct4) were quantified as a measure of the stemness of cells remaining in culture. Relative to one day of co-culture with osteoblasts, there were substantial increases in the mRNA abundance of Nanog and Oct4.

[* $p < 0.001$ compared to one-day osteoblast co-culture controls; data expressed as mean ± SEM, normalized to β-actin and expressed as fold change of one-day osteoblast co-culture controls]

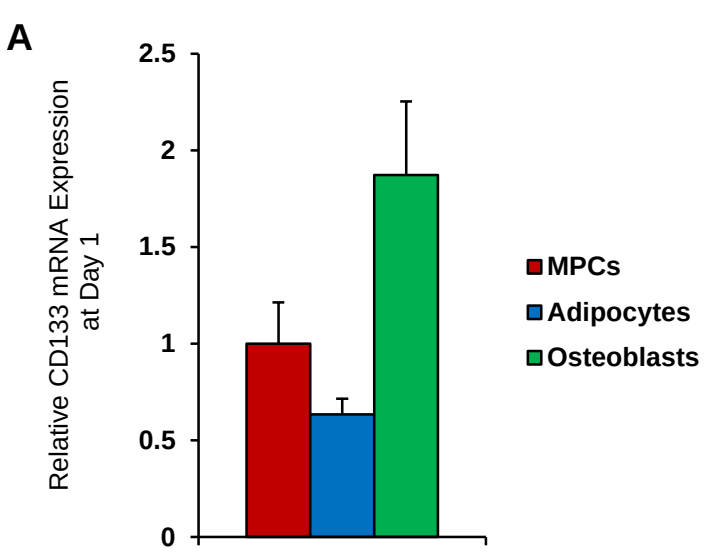
[Oct4 = octamer-binding transcription factor 4]

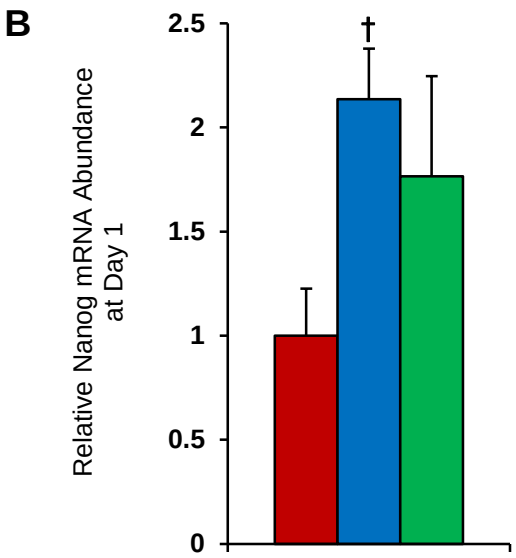
After one day of co-culture (Figure 3.9), there were only minor, non-significant differences in the mRNA levels of the three SC markers investigated. However after 21 days (Figure 3.10), there was a significant reduction in the mRNA levels of each of the SC markers in co-culture with adipocytes, suggesting that adipocytes may impair the survival of CD133-positive SCs, either by reducing cell numbers or down-regulation of SC genes.

One potential explanation for the differences observed in my model systems may be differential rates of proliferation of the background cell populations, modifying the mRNA abundance of the chosen housekeeping gene, β-actin, to dilute the expression of our genes of interest. To eliminate this as a possibility, BM-MPCs were induced to differentiate into adipocytes and osteoblasts on chamber slides before being stained with the Ki67 to identify proliferating cells. Under these experimental settings, Ki67-positive MPCs, adipocytes, or osteoblasts were rarely found (Figure 3.11 A, C, & E). Few dividing cells may be seen in culture with each of the three cell types under high magnificantion (Figure 3.11 B, D, & F).

3.4 BM-MPC, Adipocyte, and Osteoblast Contribution to SC Niche

After establishing alterations in CD133+ cell phenotype (due to either survival, adherence, or gene expression changes), I wanted to investigate the potential mechanisms underlying these changes. Therefore, I utilized qRT-PCR to quantify differences in the gene expression of various genes involved in ECM composition and


A
Relative CD133 mRNA Expression at Day 1
2.5
2
1.5
1
0.5
0
MPCs
Adipocytes
Osteoblasts


B
Relative Nanog mRNA Abundance at Day 1
2.5
2
1.5
1
0.5
0
†

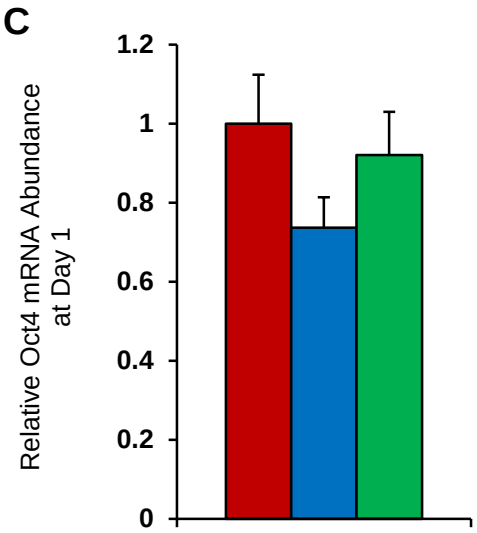
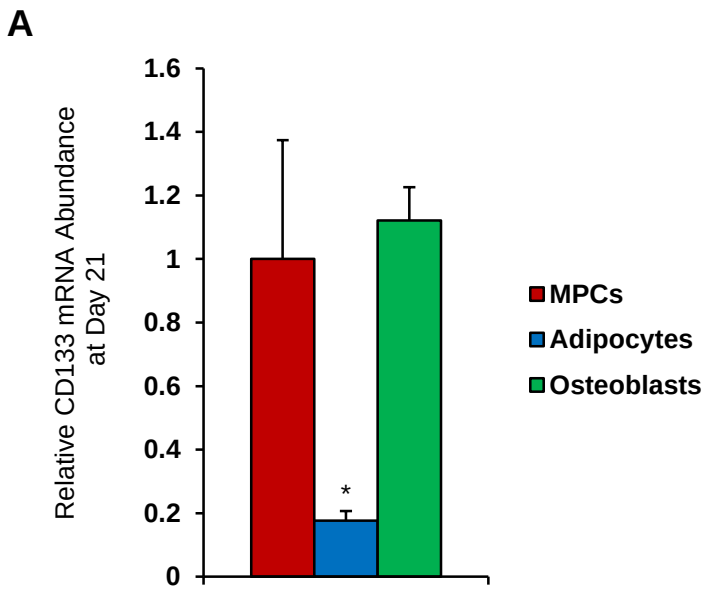

C
Relative Oct4 mRNA Abundance at Day 1
1.2
1
0.8
0.6
0.4
0.2
0

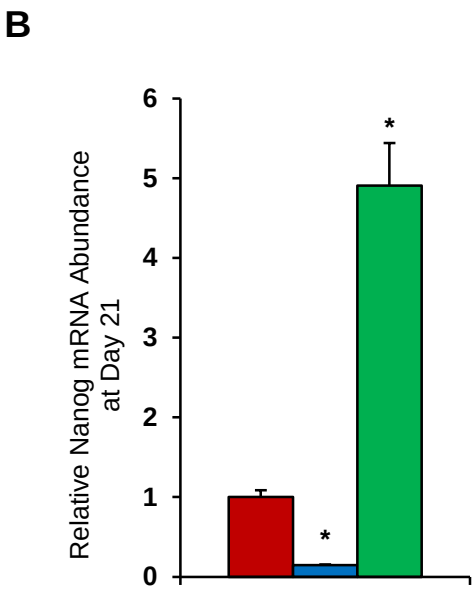
Figure 3.9. SC gene expression in CD133+ cell co-cultures for survival after one day.

CD133+ cells were cultured in co-culture systems with BM-MPCs or MPC-derived adipocytes or osteoblasts with modified media changes to assess SC survival. After one day of co-culture, SC markers CD133, Nanog, and Oct4 were quantified. The expression levels of each of the three SC markers were consistent across culture with each of the cell types.

[† $p < 0.1$ compared to one-day BM-MPC co-culture controls; data expressed as mean ± SEM, normalized to β-actin and expressed as fold change of one-day BM-MPC co-culture controls]

[MPCs = mesenchymal progenitor cells; Oct4 = octamer-binding transcription factor 4]


A
Relative CD133 mRNA Abundance at Day 21
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
MPCs
Adipocytes
Osteoblasts


B
Relative Nanog mRNA Abundance at Day 21
6
5
4
3
2
1
0
*
*

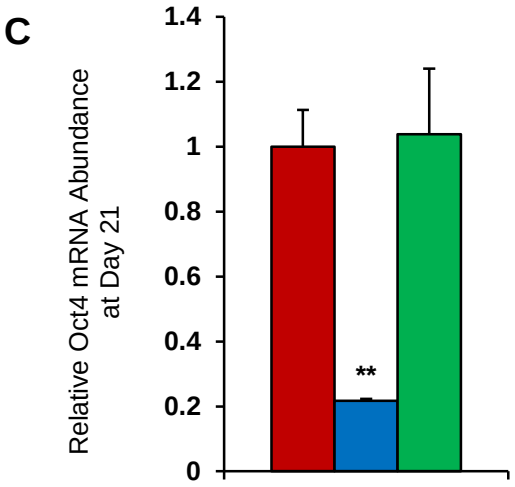
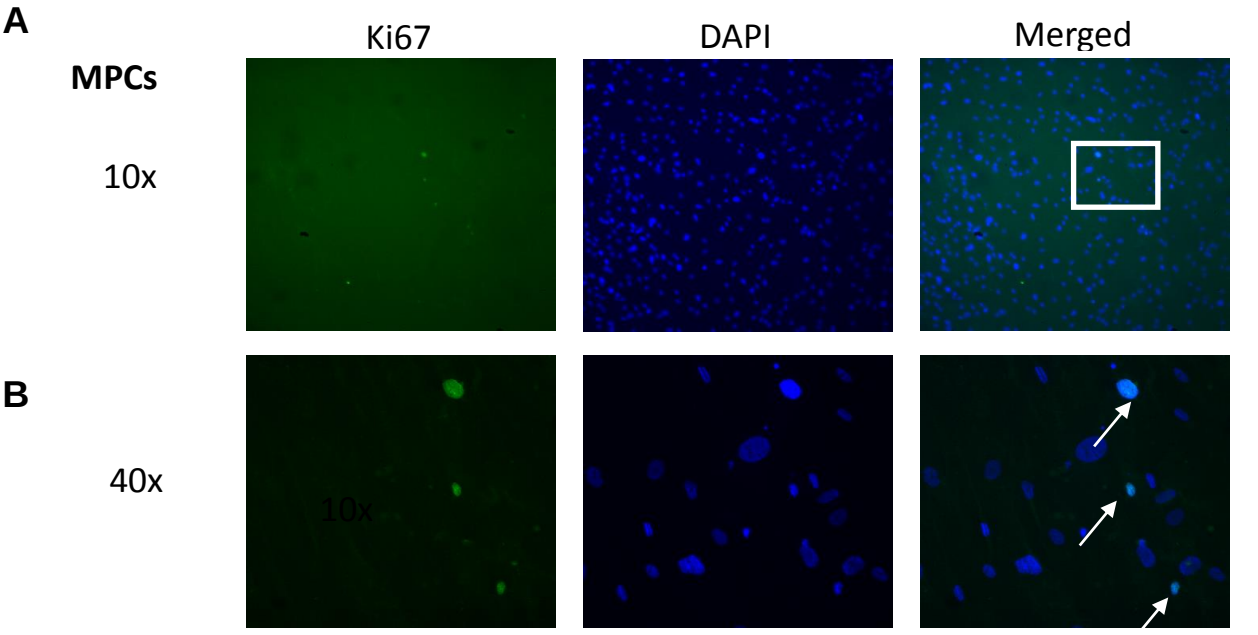

C
Relative Oct4 mRNA Abundance at Day 21
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**

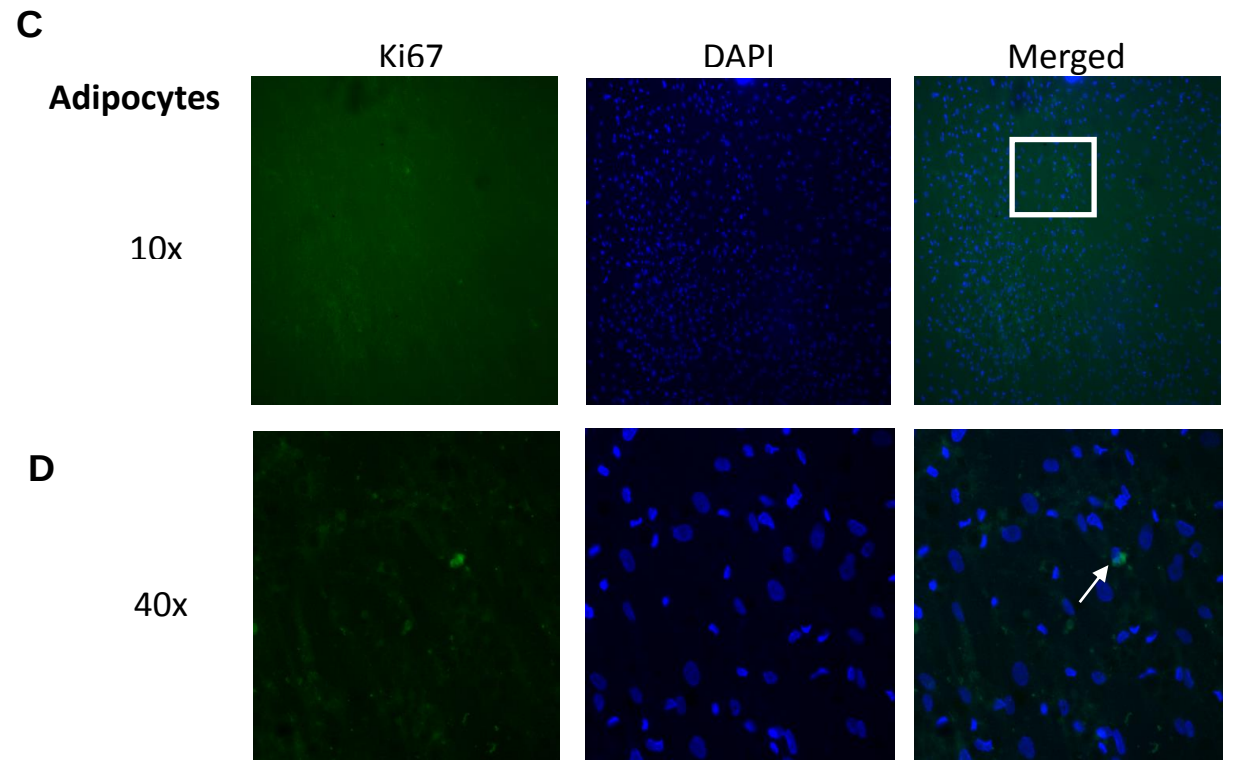
Figure 3.10. SC gene expression in CD133+ cell co-culture for survival after 21 days.

CD133+ cells were cultured in co-culture systems with BM-MPCs or MPC-derived adipocytes or osteoblasts with modified media changes to assess SC survival. After 21 days of co-culture, SC markers CD133, Nanog, and Oct4 were quantified. While BM-MPCs and osteoblasts appear able to support the maintenance of the SC population, co-culture with adipocytes led to a significant decrease in the mRNA abundance of CD133, Nanog, and Oct4, potentially indicative of a reduction in the stem cell phenotype of the cells remaining in culture.

[* $p < 0.05$, ** $p < 0.01$ compared to 21-day BM-MPC co-culture controls; data expressed as mean ± SEM, normalized to β-actin and expressed as fold change of 21-day BM-MPC co-culture controls]

[MPCs = mesenchymal progenitor cells; Oct4 = octamer-binding transcription factor 4]


A
Ki67
DAPI
Merged
MPCs
10x
B
40x
10x


C
Ki67
DAPI
Merged
Adipocytes
10x
D
40x

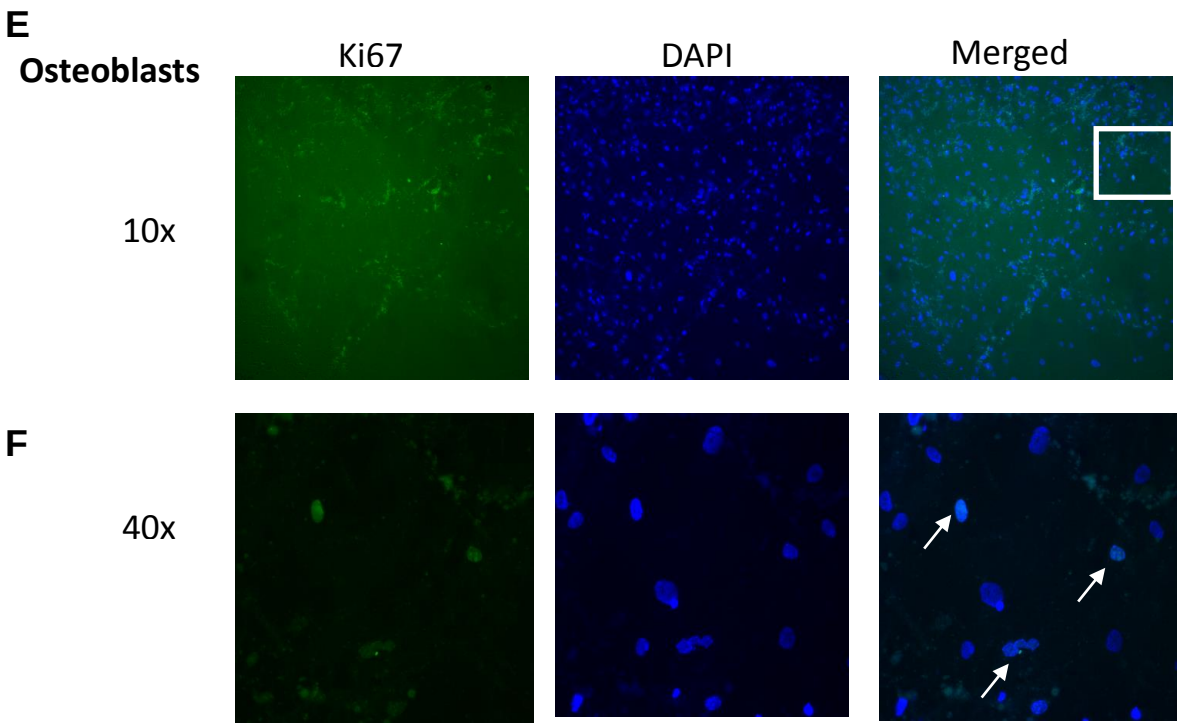


Figure 3.11. Ki67 stain for proliferation of BM-MPCs, adipocytes, and osteoblasts.

(A, C, & E) The number of Ki67-positive cells was low among MPCs, as well as cells maintained under adipogenic and osteoblastogenic differentiation conditions, indicative of low rates of proliferation. (B, D, & F) Few proliferating cells under each culture condition can be seen under 40-fold magnification.

[A, C, & E taken at 10x magnification; B, D, & F taken at 40x magnification]

[MPCs = mesenchymal progenitor cells]

remodeling between BM-MPCs, adipocytes, and osteoblasts that may contribute to the cell types' unique effects on CD133+ cells.

Major proteins involved in the composition of the ECM and basement membrane were uniquely expressed between the three cell types (Figure 3.12). In adipocytes, I observed higher levels of collagen 3A1, 4A1, and 6A1, while osteoblasts displayed reductions in the expression of collagen 1A1, 4A1, 5A1, and 6A1 (Figure 3.12 A). FN was repressed in both adipocytes and osteoblasts relative to MPCs, while the FN inhibitor tenascin C was increasingly expressed among both adipocytes and osteoblasts relative to MPC controls (Figure 3.12 B). HSPG2 (also known as perlecan) was found to be significantly repressed in osteoblasts relative to control MPCs (Figure 3.12 C). Other basement membrane component proteins were also modulated (Figure 3.12 D). Laminin C1, nidogen 1, and nidogen 2 were up-regulated, while laminin C2 was significantly down-regulated in adipocytes. Osteoblasts displayed repression of laminin C1 and C2, though the expression of nidogen 1 was substantially increased.

Genes involved in remodeling the ECM were also uniquely regulated between the cell types. The majority of ADAM peptidases were down-regulated in osteoblasts, with significant repression of ADAMs 9, 10, 12, 15, 19, 21, and 28 relative to MPCs (Figure 3.13 A). ADAMs were selectively regulated in adipocytes, with repression of ADAMs 9 and 19 and increased expression of ADAMs 10, 15, and 17. Adipocytes and osteoblasts also displayed unique expression of MMPs (Figure 3.13 B). In adipocytes, there were significant decreases in the mRNA abundance of MMPs 1, 2, 11, 14, and 16, and up-regulated expression of MMPs 13, 15, 17, 19, and 24. Alternatively, osteoblasts displayed repression of MMPs 1, 2, 10, 11, 14, 15, 16, 17, and 24 relative to MPCs,

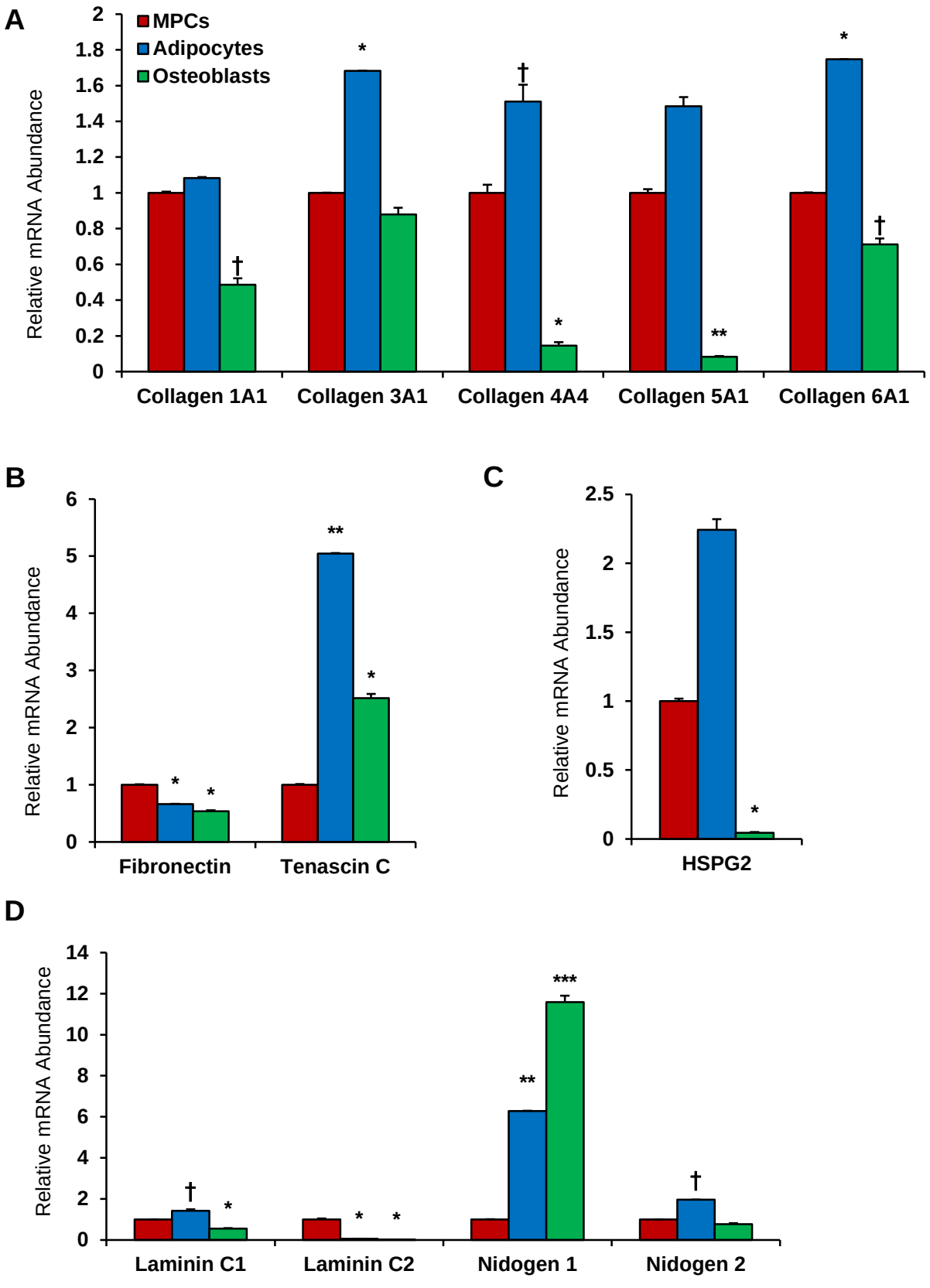

A
MPCs
Adipocytes
Osteoblasts
Relative mRNA Abundance
Collagen 1A1
Collagen 3A1
Collagen 4A4
Collagen 5A1
Collagen 6A1
B
Fibronectin
Tenascin C
C
HSPG2
D
Laminin C1
Laminin C2
Nidogen 1
Nidogen 2

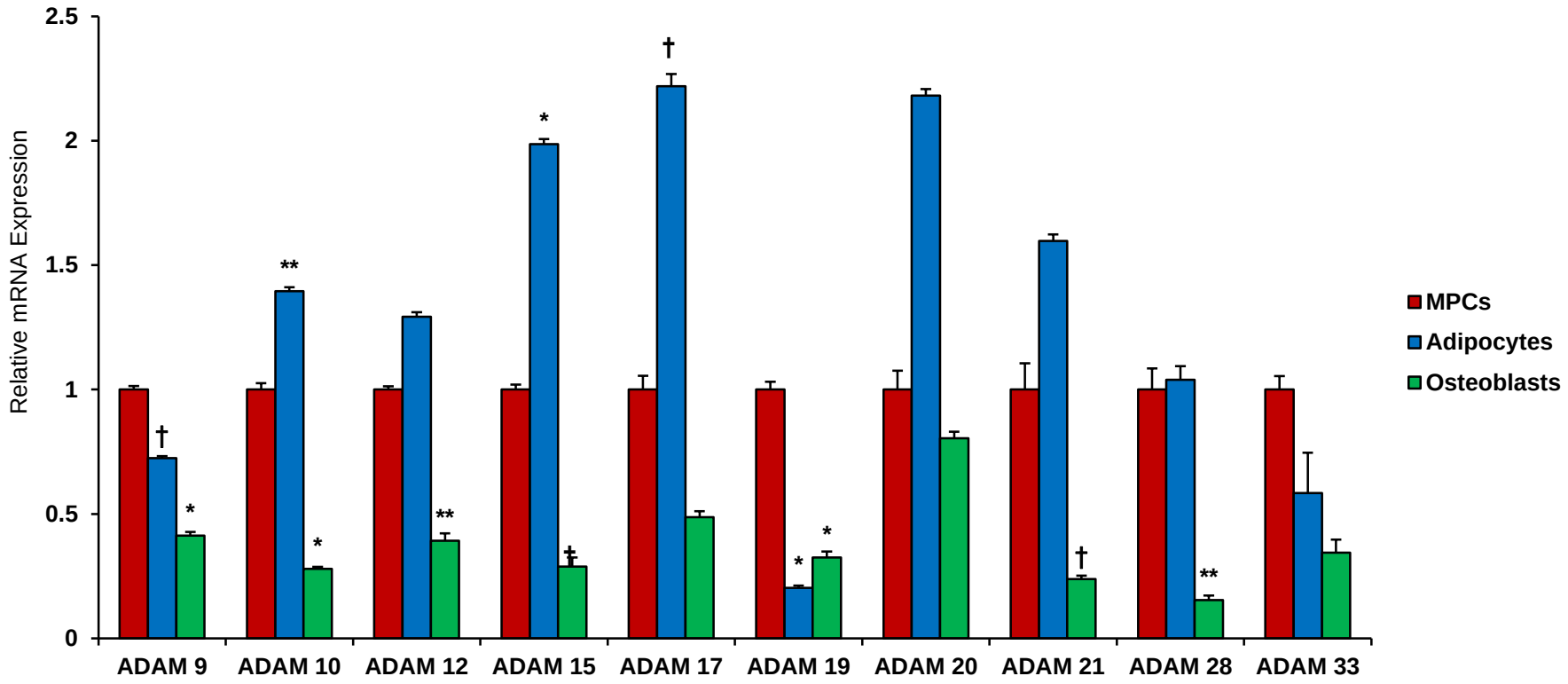
Figure 3.12. ECM gene expression by MPCs, adipocytes, and osteoblasts.

(A) There was selective regulation of several collagen subtypes compared to MPCs in adipocytes and osteoblasts, respectively. (B) Both adipocytes and osteoblasts were characterized by significant repression of FN expression and up-regulation of tenascin C. (C) HSPG2, also known as perlecan, was found to be significantly repressed in osteoblasts relative to MPC controls. (D) Adipocytes displayed increased mRNA abundance of basement membrane component proteins laminin C1, nidogen 1, and nidogen 2, with reduced expression of laminin C2. In osteoblasts, laminin C1 and C2 were both down-regulated, while nidogen 1 expression was substantially increased.

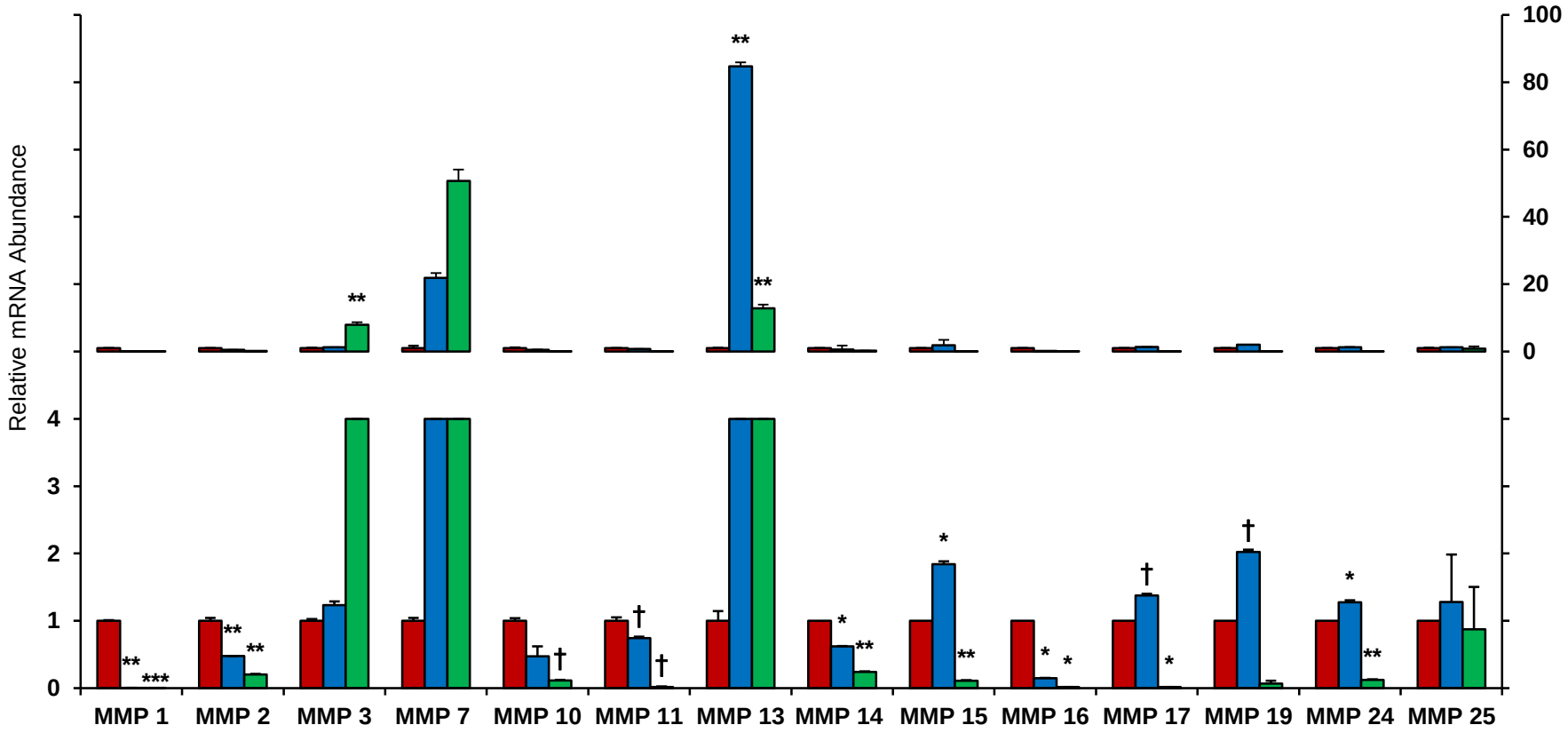
[† $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to BM-MPC controls; data expressed as mean ± SEM, normalized to β-actin and expressed as fold change of BM-MPC controls]

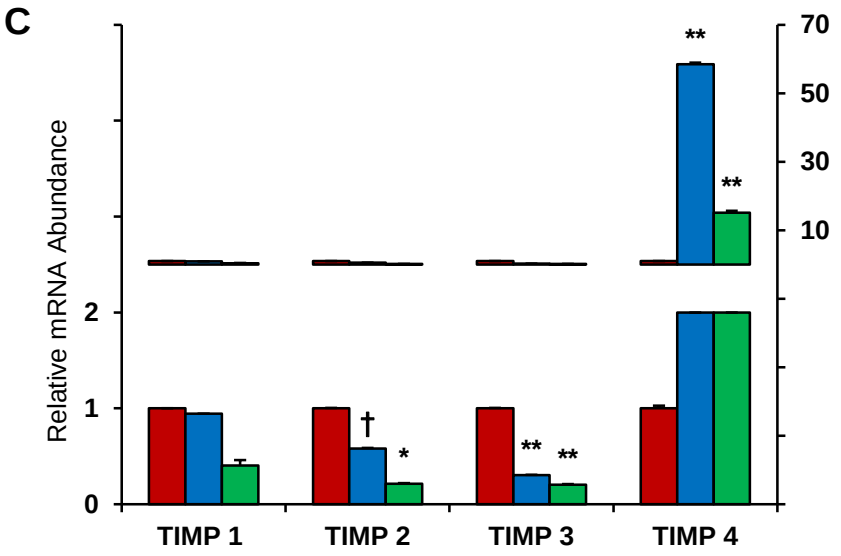
[HSPG2 = heparin sulfate proteoglycan 2; MPCs = mesenchymal progenitor cells]

A


Relative mRNA Expression
2.5
2
1.5
1
0.5
0
ADAM 9
ADAM 10
ADAM 12
ADAM 15
ADAM 17
ADAM 19
ADAM 20
ADAM 21
ADAM 28
ADAM 33
MPCs
Adipocytes
Osteoblasts

B


Relative mRNA Abundance
100
80
60
40
20
0
4
3
2
1
0
MMP 1
MMP 2
MMP 3
MMP 7
MMP 10
MMP 11
MMP 13
MMP 14
MMP 15
MMP 16
MMP 17
MMP 19
MMP 24
MMP 25


C
Relative mRNA Abundance
70
50
30
10
2
1
0
**
**
†
*
**
**
TIMP 1
TIMP 2
TIMP 3
TIMP 4

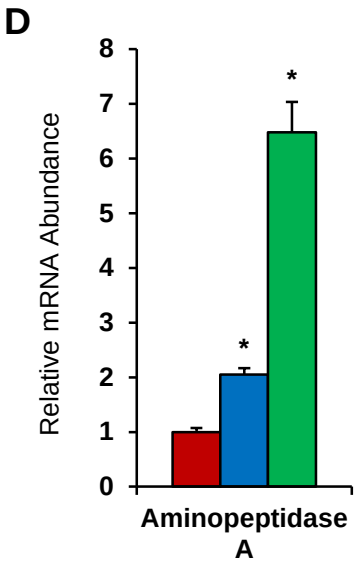

D
Relative mRNA Abundance
8
7
6
5
4
3
2
1
0
*
*
Aminopeptidase A

Figure 3.13. Expression of ECM remodeling protein genes by MPCs, adipocytes, and osteoblasts.

(A) Adipocytes and osteoblasts displayed unique patterns of expression of members of the ADAMs family of peptidases. Adipocytes were characterized by up-regulation of most ADAM subtypes, while osteoblasts demonstrated repression of most ADAM genes. (B) The expression of a number of MMPs were selectively regulated by adipocytes and osteoblasts relative to control MPCs. (C) TIMPs were also differentially expressed by the three cell types, with repression of TIMP 2 and 3 and substantial up-regulation of TIMP 4 by both adipocytes and osteoblasts. (D) Membrane-bound aminopeptidase A was also found to be up-regulated in adipocytes and osteoblasts relative to MPCs.

[† $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to BM-MPC controls; data expressed as mean ± SEM, normalized to β-actin and expressed as fold change of BM-MPC controls]

[ADAM = a disintegrin and metalloproteinase; Aminopeptidase A = glutamyl aminopeptidase; MMP = matrix metalloproteinase; MPCs = mesenchymal progenitor cells; TIMP = tissue inhibitor of metalloproteinase]

while MMPs 3 and 13 were substantially up-regulated. Tissue inhibitors of metalloproteinases (TIMPs) demonstrated similar patterns of expression in adipocytes and osteoblasts, with both cell types repressing the expression of TIMPs 2 and 3 and significantly up-regulating TIMP 4 in comparison with expression by MPCs (Figure 3.13 C). The membrane-anchored protease aminopeptidase A was also observed to be increasingly expressed by both adipocytes and osteoblasts (Figure 3.13 D).

qRT-PCR was also employed to investigate changes in the expression of 45 unique niche genes involved in cell-to-cell communication following adipogenic and osteogenic differentiation of BM-MPCs. I observed significant differences in the expression of genes involved in the Notch signalling system, with up-regulation of Notch receptors 1 and 2, as well as Notch ligand JAG1 in osteoblasts and increased expression of DLL4 in adipocytes (Figure 3.14 A and B). Integrin-α4 was the only cell adhesion molecule to be differentially regulated, with substantial up-regulation in osteoblasts (Figure 3.15). The pro-angiogenic chemokine SDF-1 was found to be repressed by osteoblasts relative to MPC controls (Figure 3.16). Adipocytes displayed significant increases in the expression of vascular growth factors Ang1 and Ang2, while the changes in the expression of either of these factors failed to reach significance in osteoblasts (Figure 3.17 A). As Ang1 and Ang2 play antagonistic roles, I elected to compare

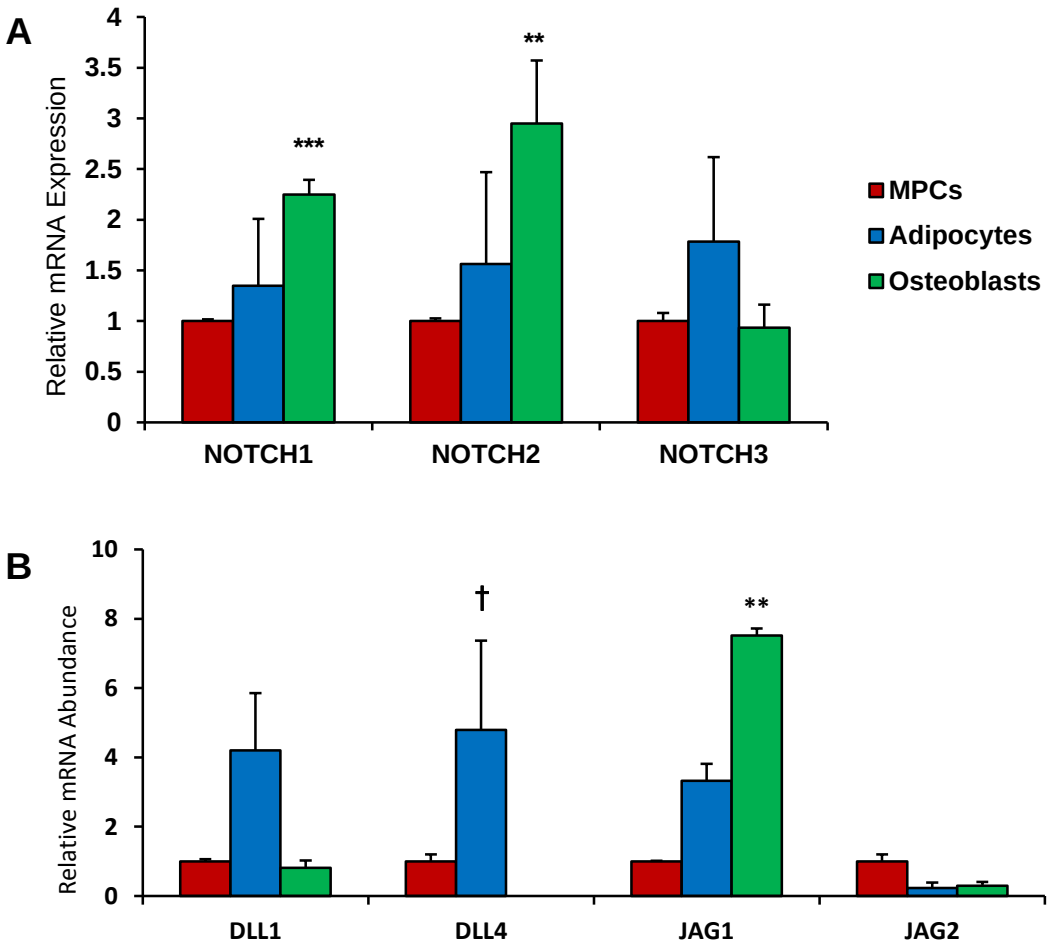


Figure 3.14. Expression of Notch signalling genes in MPCs, adipocytes, and osteoblasts.

(A) Notch receptors 1 and 2 were significantly up-regulated in osteoblasts. (B) Notch ligands DLL4 and JAG1 were up-regulated in adipocytes and osteoblasts, respectively, in comparison to control MPCs.

[† $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$ compared to BM-MPC controls; data expressed as mean ± SEM, normalized to β-actin and expressed as fold change of BM-MPC controls]

[DLL = delta-like ligand; JAG = Jagged; MPCs = mesenchymal progenitor cells]

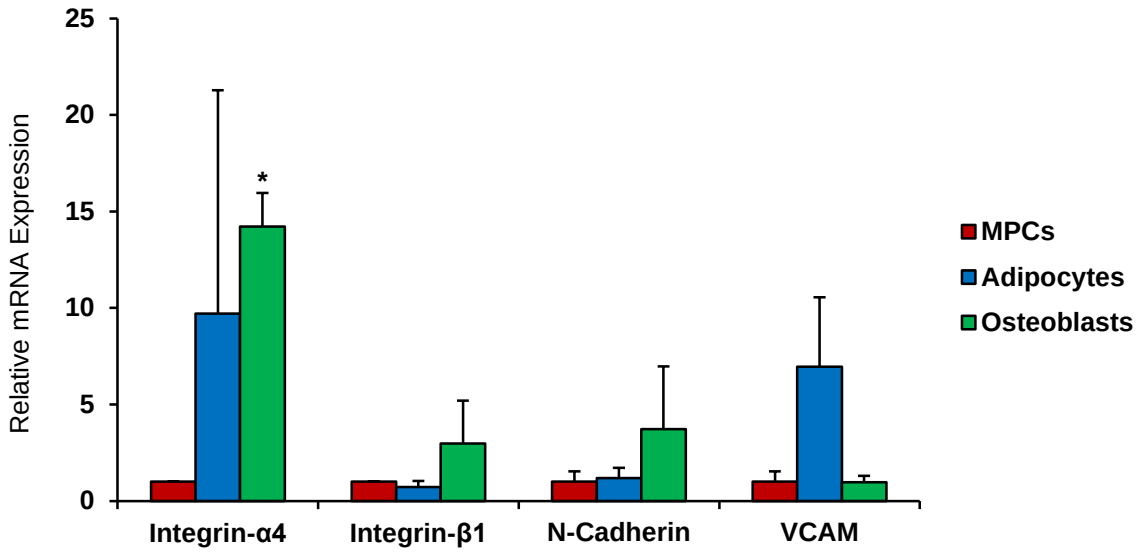


Figure 3.15. Cell adhesion molecule gene expression in MPCs, adipocytes, and osteoblasts.

Integrin-α4 was the only gene involved of cell adherence to be differentially expressed between the cell types with a significant increase in expression in osteoblasts.

[* $p < 0.05$ compared to BM-MPC controls; data expressed as mean ± SEM, normalized to β-actin and expressed as fold change of BM-MPC controls]

[MPCs = mesenchymal progenitor cells; N-cadherin = neural cadherin; VCAM = vascular cell adhesion molecule]

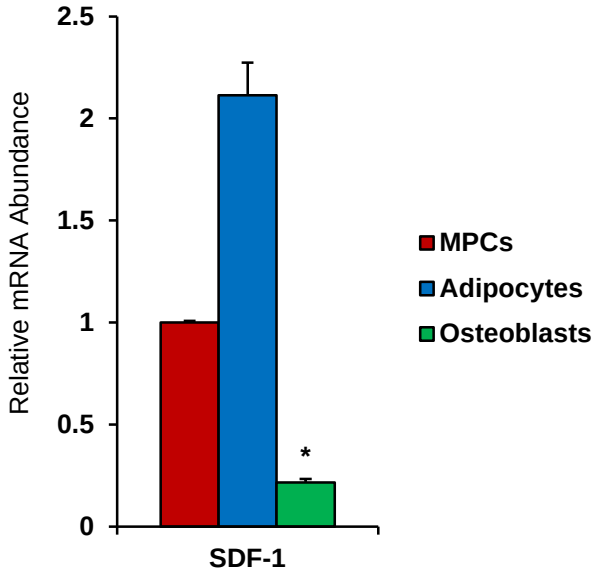


Figure 3.16. Expression of SDF-1 in MPCs, adipocytes, and osteoblasts.

Expression of the pro-angiogenic factor SDF-1 was found to be significantly decreased in osteoblasts.

[* $p < 0.05$ compared to BM-MPC controls; data expressed as mean ± SEM, normalized to β-actin and expressed as fold change of BM-MPC controls]

[MPCs = mesenchymal progenitor cells; SDF-1 = stromal cell-derived factor-1]

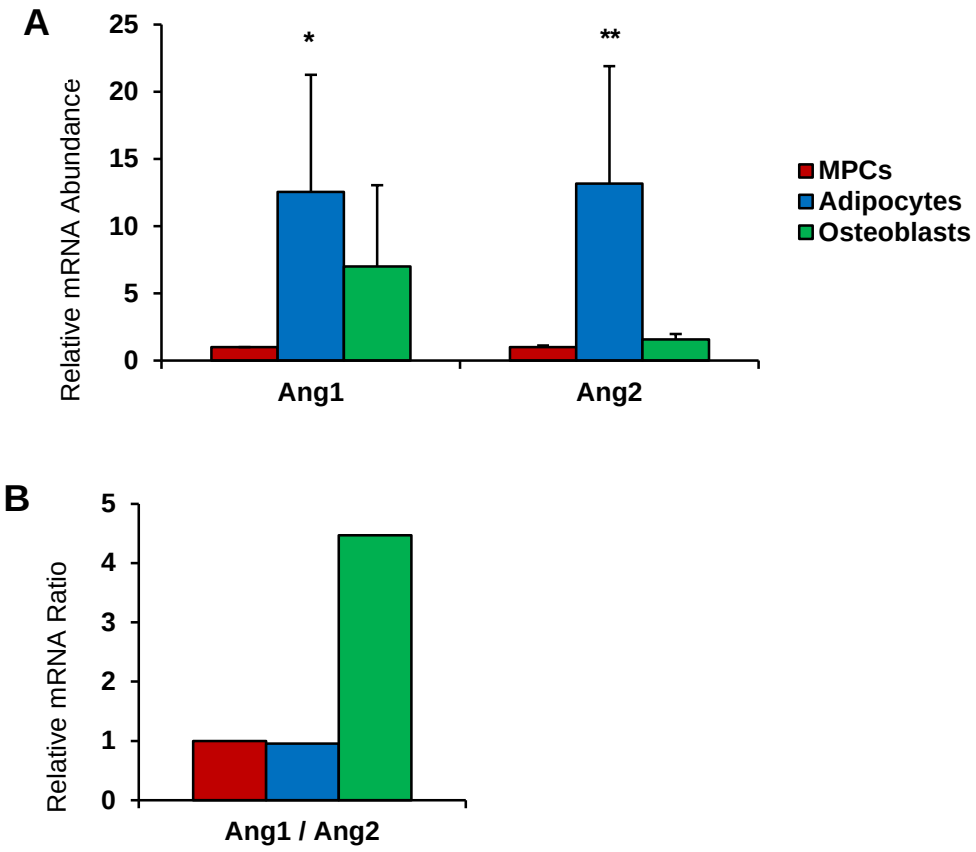


Figure 3.17. Expression of angiopoietin genes in MPCs, adipocytes, and osteoblasts.

(A) Adipocytes were characterized by significantly increased mRNA abundance of both Ang1 and its antagonist, Ang2, in comparison to BM-MPC control cells. (B) When compared as the ratio between the pro-angiogenic Ang1 and anti-angiogenic Ang2, there was no difference relative to MPCs in adipocytes, though osteoblasts demonstrated a four-fold increase in this ratio.

[* $p < 0.05$, ** $p < 0.01$ compared to BM-MPC controls; data expressed as mean ± SEM, normalized to β-actin and expressed as fold change of BM-MPC controls]

[MPCs = mesenchymal progenitor cells; Ang = angiopoietin]

the expressions of these two factors as the ratio of Ang1 relative to Ang2 (Figure 3.17 B). In adipocytes, this ratio was very similar to that of MPCs, although the ratio approached 4.5 in osteoblasts, suggesting a much greater contribution to the surrounding niche by Ang1 in osteoblast-predominant culture.

3.5 Identifying Potential Role of Ang1 & Ang2 in SC Niche

I was particularly intrigued by the finding in outlined in Figure 3.17, with increased expression of Ang1 and Ang2 in adipocytes and an increased Ang1-to-Ang2 ratio in osteoblasts and set out to determine the effects that varying concentrations of Ang1 and Ang2 have on CD133-positive SCs. To do this, CD133+ cells were cultured at 5,000 cells/cm^2 for seven days in DMEM with 10% FBS containing one of six concentrations of Ang1 or Ang2 (Table 3.1). qRT-PCR was then performed to quantify the expression of SC marker CD133 and EC marker CD31.

Although meaningful significance was not attained, several important points could be raised. CD133 mRNA was reduced in each culture condition relative to 0 Ang controls, though this was not statistically significant (Figure 3.18 A). Ang1, which is responsible for promoting endothelial differentiation, seemed to increase the expression of EC marker CD31 when added to culture at 100 ng/mL or 75 ng/mL + 25 ng/mL Ang2 (Figure 3.18 B)[345].

Table 3.1. Treatment conditions for Ang1-Ang2 overabundance experiment.

CD133+ cells were cultured for seven days in DMEM containing the concentrations of Ang1 and Ang2 listed below.

Label	Ang1	Ang2
0 Ang	0 ng/mL	0 ng/mL
A1 100	100 ng/mL	0 ng/mL
A1 75 / A2 25	75 ng/mL	25 ng/mL
A1 50 / A2 50	50 ng/mL	50 ng/mL
A1 25 / A2 75	25 ng/mL	75 ng/mL
A2 100	0 ng/mL	100 ng/mL

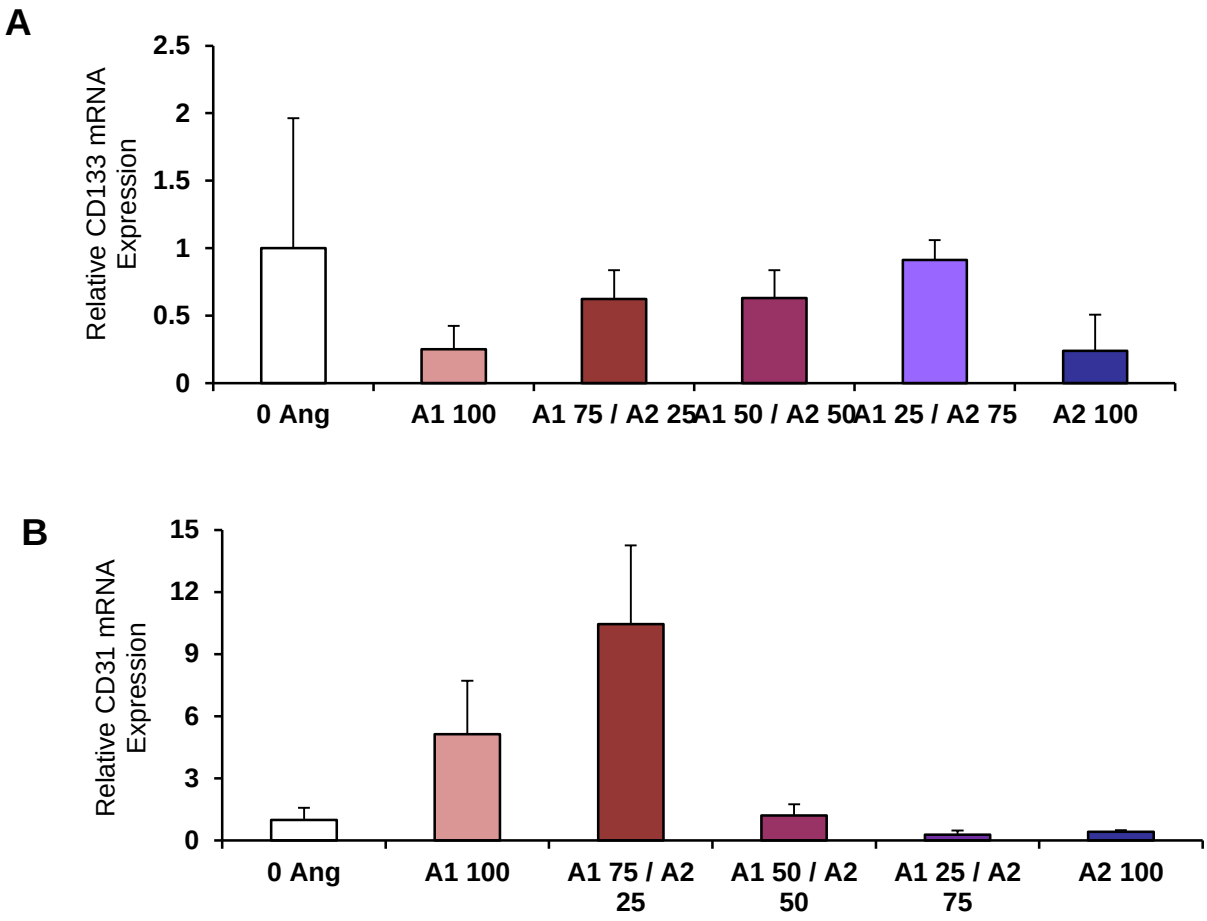


Figure 3.18. Expression of SC and EC markers in CD133+ cells upon Ang exposure.

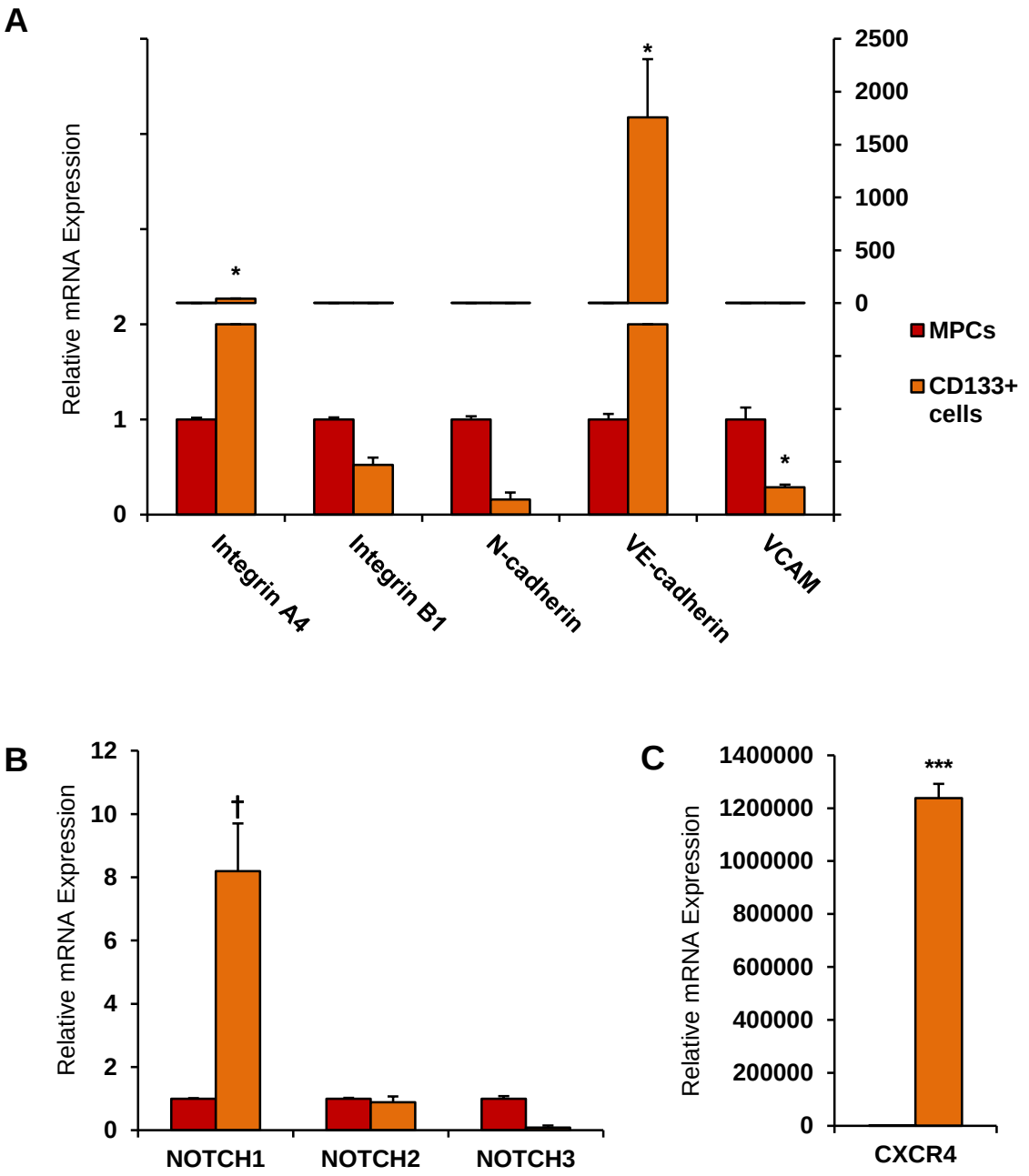
CD133+ cells were maintained for seven days in DMEM containing varying concentrations of Ang1 and Ang2 before quantification of markers of SC and EC phenotypes. (A) SC marker CD133 mRNA levels (B) EC marker CD31 mRNA levels

[Data expressed as mean ± SEM, normalized to β-actin and expressed as fold change of 0 Ang controls]

[Ang = angiopoietin]

3.6 CD133-Positive Cell Adhesion Molecule & Receptor Expression

I conducted qRT-PCR analyses of various niche receptor and cell adhesion molecule genes in CD133+ cells in order to confirm the expression of reciprocal factors that correspond to genes in which we observed differential expression patterns between mesenchymal cell types. I utilized MPCs as controls to compare to the relative mRNA levels in CD133+ cells. I observed substantive expression of integrin-α4, along with robust expression of VE-cadherin, which is a characteristic endothelial marker (Figure 3.19 A). CD133+ cells also displayed a reduced expression of VCAM relative to BM-MPCs. NOTCH1 was the predominant Notch receptor protein expressed by CD133+ cells (Figure 3.19 B). CXCR4, which serves as the receptor for SDF-1, was expressed in significantly higher levels in CD133+ cells relative to control MPCs (Figure 3.19 C). I also observed robust expression of the Tie-1 and Tie-2, which serve as receptors in the angiopoietin signalling system (Figure 3.19 D). These findings suggest the potential for the unique niche gene expression patterns of BM-MPCs, adipocytes, and osteoblasts to be parlayed into functional changes within the CD133+ cell population.


A
Relative mRNA Expression
2500
2000
1500
1000
500
0
2
1
0
*
*
*
MPCs
CD133+ cells
Integrin A4
Integrin B1
N-cadherin
VE-cadherin
VCAM
B
Relative mRNA Expression
12
10
8
6
4
2
0
†
NOTCH1
NOTCH2
NOTCH3
C
Relative mRNA Expression
1400000
1200000
1000000
800000
600000
400000
200000
0
***
CXCR4

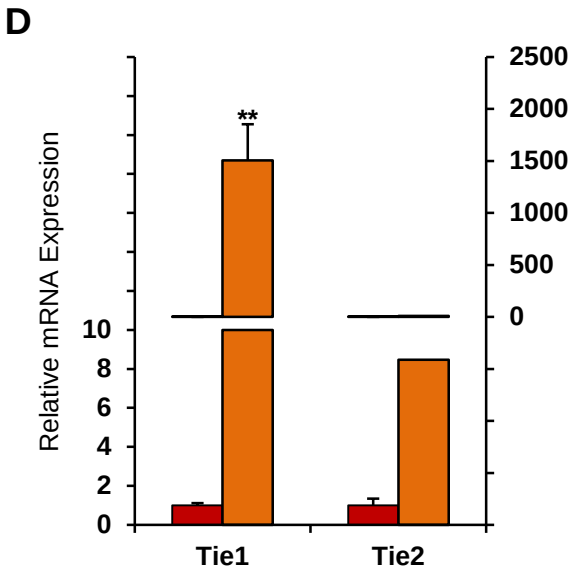


Figure 3.19. Expression of cell adhesion molecules and niche receptors in CD133+ cells.

qRT-PCR analyses of the expression of niche gene receptors in CD133+ cells were performed and compared to expression in cultured BM-MPCs. (A) mRNA levels of select integrins and cadherins (B) mRNA levels of Notch receptors (C) mRNA levels of SDF-1 receptor CXCR4 (D) mRNA levels of Tie receptors involved in Ang1 and Ang2 signalling

[† $p < 0.1$, * $p < 0.05$, ** $p < 0.01$ compared to BM-MPC controls; data expressed as mean ± SEM, normalized to β-actin and expressed as fold change of BM-MPC controls]

[CXCR4 = C-X-C chemokine receptor type 4; MPCs = mesenchymal progenitor cells; N-cadherin = neural cadherin; Tie = tyrosine kinase with immunoglobulin-like and EGF-like domains; VCAM = vascular cell adhesion molecule; VE-cadherin = vascular endothelial cadherin]

3.7 *In Vivo* Diabetic Model

Clinical variables of diabetic and control rats were recorded and reported in Table 3.2. All rats receiving injections of STZ were confirmed to be diabetic with blood glucose measurements in excess of 25 mmol/L. There were significant differences between diabetic and control animals in body weight ($p < 0.01$), blood glucose levels ($p < 0.01$), heart weight ($p < 0.1$), and tibia length ($p < 0.01$).

My data confirmed an increase in marrow adipogenesis in diabetic rats, as demonstrated by the increased expression of adipogenic transcription factors C/EBP-α and C/EBP-β (Figure 3.20 A). Although non-significant, expression of pro-osteoblastic factors BGLAP, Runx2, and Sp7 seemed lower in diabetic animals (Figure 3.20 B). Potentially increasing the sample size may show significantly lower levels. Expression of MPC marker CD105 was not altered in the diabetic animals. While the putative marker of EPCs in humans, CD133 is not exclusive to SCs in rats. Therefore, I elected to utilize other SC-specific markers: Nanog, Oct4, and Sox2. Expression of Nanog, Oct4, and Sox2 all displayed slight decreases in mRNA abundance in diabetic animals, though this difference was not significant (Figure 3.20 D).

Table 3.2. Clinical variables of control and diabetic rats used in *in vivo* model of diabetes.

	Control Mean	Diabetic Mean	P-Value
Body Weight	551.67 g	302.67 g	$p < 0.01$
Blood Glucose	6.87 mmol	29.2 mmol	$p < 0.01$
Heart Weight	1.78 g	1.03 g	$p < 0.1$
Tibia Length	5.57 cm	4.57 cm	$p < 0.01$

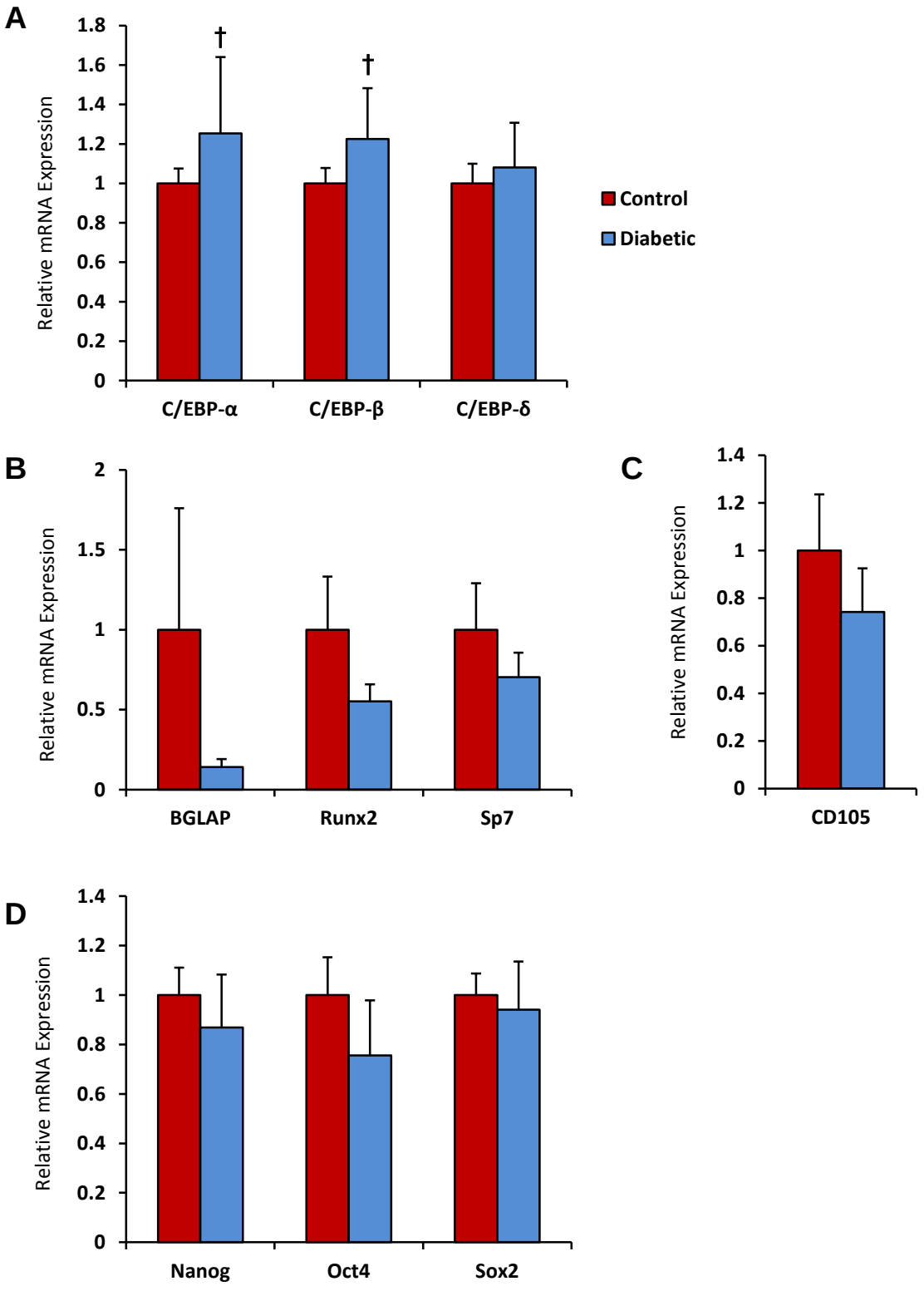

A
Relative mRNA Expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
†
†
C/EBP-α
C/EBP-β
C/EBP-δ
Control
Diabetic
B
Relative mRNA Expression
2
1.5
1
0.5
0
BGLAP
Runx2
Sp7
C
Relative mRNA Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CD105
D
Relative mRNA Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Nanog
Oct4
Sox2

Figure 3.20. *In vivo* expression of adipogenic, osteogenic, MPC, and SC markers in marrow of diabetic rats.

(A) Increased expression of adipogenic transcription factors were found in the BM of diabetic rats. (B) The decrease in the mRNA expression of osteogenic markers failed to reach significance. (C) The expression of MPC marker CD105 was not significantly altered in the BM of diabetic rats. (D) A non-significant reduction in the expression of SC markers was observed in diabetic marrow.

[† $p < 0.1$; data expressed as mean ± SEM, normalized to β-actin and expressed as fold change relative to control rat femurs; n = 3]

[BGLAP = bone gamma-carboxyglutamic acid-containing protein; C/EBP = CCAAT-enhancer-binding protein; Oct4 = octamer-binding transcription factor 4; Runx2 = runt-related transcription factor 2; Sox2 = sex determining region Y-box 2]

Chapter 4 : Conclusions

4 Conclusions

4.1 Discussion

I have demonstrated a reduction in the mRNA expression of SC markers CD133, Nanog, and Oct4 following co-culture of CD133+ cells with adipocytes after 21 days. I first set out to identify a suitable substrate on which to culture and differentiate BM-MPCs which was essential for creating the *in vitro* SC niche model and downstream experimentation. Adipogenic differentiation has been shown to be hindered by FN and unaffected by collagen. FN is believed to exert its anti-adipogenic effect by preventing the necessary changes in morphology required for development of a fibroblast-like MPC into a large, rounded adipocyte[343]. Through the induction of differentiation of BM-MPCs on collagen 1-coated and FN-coated plates, I was able to confirm a role for FN in delaying adipogenesis. Collagen 1 did not have any significant effect on the expression of differentiation factors, confirming its potential as an ECM substrate in differentiation culture systems[346].

I also analyzed the expression of three factors, CD133, Nanog, and Oct4, believed to be specific to SCs in BM-MPCs, adipocytes, and osteoblasts. These

three cell types are thought to be negative for the expression of the aforementioned SC markers, though some reports have identified their expression in more differentiated cell types and MSCs[347-351]. I observed no detectable expression of CD133 by BM-MPCs, adipocytes or osteoblasts, supporting its use as the ideal marker to identify SCs in mixed cultures. The relative expression levels of Nanog and Oct4 were also undetectable in each of the three mesenchymal cell types in comparison to fresh CD133+ cells, supporting the use of these genes as secondary markers for the SC phenotype in our co-culture experiments.

I next investigated how altering the cellular composition of the microenvironment affected the adherence of CD133+ cells within an *in vitro* co-culture system. EPCs have previously been shown to have poor adherence in culture, as well as an overall loss of CD133 expression over time, while improved EPC adhesion may correlate to enhanced endothelial function[352-355]. My findings confirmed this characteristic of CD133+ cells, as co-culture with BM-MPCs or adipocytes led to significant reductions in the expression of CD133. Interestingly, co-culture with osteoblasts led to an increase in the mRNA of SC markers Nanog and Oct4, with a non-significant increase in the expression of CD133 after 21 days. Osteoblasts are characterized by a strong production of collagen 1, which has been shown to enhance cell adhesion in a number of cell types[356-358]. Additionally, collagen 1 has been shown to specifically facilitate the survival and

proliferation of EPCs *in vitro*, which may contribute to the increased mRNA abundance of SC markers in this co-culture system after 21 days[359].

I also explored the effects of co-culture of CD133+ cells with BM-MPCs, adipocytes, or osteoblasts on the survival or maintenance of these cells in an SC-like state. Via a modified survival assay, I profiled how the expression of SC markers changed over time in co-cultures of CD133+ cells with MPCs or MPC-derived adipocytes or osteoblasts. Interestingly, I noted a significant reduction in the mRNA abundance of CD133, Nanog, and Oct4 in co-culture with adipocytes relative to co-culture with BM-MPCs after 21 days. This suggests that adipocytes are responsible for the production of signals or cell-cell interactions that are detrimental to the survival and maintenance of the CD133+ cell population. The reduced expression of SC markers may be the consequence of depletion of the SC population, a loss of an SC-like phenotype, reduced cell proliferation, or a combination of the above. Unfortunately, this model is unable to differentiate between a reduction in overall SC marker-positive cell number and a down-regulation of SC factors with no change in the size of the population. Thus, my results, although important, are limited to describing changes to the “stemness” of these cells – the maintenance of these cells in an SC-like state – rather than CD133+ cell quantity or cellularity.

A potential confounding factor to my quantification of SC gene expression is proliferation of background cell populations. Division of the background cells

would increase the expression of housekeeping genes and dilute the expression of genes of interest. I anticipated minimal effect of cell proliferation in differentiated cell types as adipocytes and osteoblasts are typically considered to be post-mitotic cell types[81, 99, 360, 361]. Alternatively, MPCs are a highly expandable and proliferative cell type[362]. However, this property is believed to decrease in high density cultures[363]. To minimize the potential for proliferation of background cell types to artificially decrease SC marker expression, co-culture cell populations were maintained at high density. I also performed immunofluorescence staining of MPCs, adipocytes, and osteoblasts for the proliferation marker Ki67 to confirm a low rate of division in these cells. I observed very few Ki67-positive cells in each of the three culture conditions. High seeding density and exposure to differentiation media appear to be sufficient to inhibit the division of MPCs. From this stain, I can conclude that the changes in SC gene expression under co-culture systems were not the artefact of differential proliferation of background cells.

Particularly within the HSC niche, adipocytes are considered to play a pivotal role in guiding SC development[364]. While some research advocates for a positive role for adipocytes, general consensus supports an inhibitory function for adipocytes on the maintenance of the HSC population[365-367]. At birth, HSCs are found throughout the skeleton[368]. During postnatal development, conversion of areas of red BM to adipocytic yellow marrow results in the confinement of

hematopoiesis to specific regions within the skeletal system, such as flat bones and the epiphyseal ends of long bones[364]. Large, round adipocytes may compress walls of sinusoids to restrict blood flow, consequently transforming regions previously of red marrow into fatty yellow BM, which are characterized by a limited blood supply[364, 369]. *In vivo*, an overabundance of adipocytes within diabetic BM may similarly occlude capillaries to limit the accessibility of VSCs and EPCs to the vasculature. This may restrict the exposure of these cells to pro-vasculogenic chemotactic signals, as well as the ability of these cells to mobilize from the BM and enter into circulation. As reductions in blood supply are not a factor in my co-culture systems, I have identified the novel potential for adipocyte-specific cell-cell interactions or alterations to the extracellular microenvironment to negatively regulate the stemness of CD133+ cells.

My second objective sought to quantify differential expression of niche and ECM-related genes between adipocytes and osteoblasts relative to control BM-MPCs that may account for the differences in SC adherence and survival I observed in the first aim. A secondary goal was also to provide the groundwork for future studies investigating individual changes and the contribution to SC dysfunction. My results demonstrate that adipocytes are responsible for the creation of a distinct microenvironment, with modulated expression of factors known to exert influence over SC development and vasculogenesis.

The ECM has recently been shown to play a critical role in governing the fate of SCs, through both cell interactions and mechanical forces. Differential ECM production by the stromal cells comprising the SC niche regulates SCs through direct physical interactions with cells, ECM-receptor signalling, and modified mechanical properties that allow or disallow for SC migration and morphological changes necessary for differentiation[370, 371]. I found that adipogenic matrix is characterized by increased expression of most collagen subtypes, with significant up-regulation of collagens 3A1, 4A4, and 6A1. This finding corroborates previous findings of enhanced synthesis of type 4 collagen following adipogenesis, though the reported expression of other collagen subtypes varies between studies[372-380]. Notably not up-regulated however, was collagen 1A1, which has been shown to facilitate the *in vitro* survival and proliferation of EPCs[359]. Culture of EPCs on type 1 collagen-coated growth surfaces has been shown to improve their proliferative and migratory capabilities[381]. The observed up-regulation of various collagen subtypes is likely to contribute to an increase in the overall concentration of collagen within the ECM. A 2010 study by Critser et al. determined that increasing the concentration of collagen in a three-dimensional matrix influenced the vasculogenic capacity of EPCs, resulting in a significant decrease in the number of functional EPC-derived vessels in a given area[382]. Other investigations have confirmed that more malleable matrix substrates favour blood vessel formation over high density ECM[383-385]. Thus, the increased collagen production by adipocytes may result in

reduced profusion of the marrow microenvironment in which VSCs and EPCs reside.

I also observed a significant decrease in the mRNA expression of most collagen subtypes in osteoblasts, with significant repression of collagens 1A1, 4A4, 5A1, and 6A1. Osteoblastic matrix has typically been characterized by significant expression of type 1 collagen, which comprises 90-95% of organic matrix within mature bone[386]. This discrepancy in collagen mRNA production may be the result of the time point at which gene expression was assessed. I induced osteoblastic differentiation over 14 days, though significant matrix deposition could be observed visually within one week of induction[387]. After 14 days of differentiation, the culture growth surface was fully covered with osteogenic matrix, which may have resulted in down-regulation of ECM-related genes through a negative feedback mechanism. Pursuing a time-course analysis of ECM gene expression throughout the osteoblastic differentiation period may reveal the characteristic up-regulation of type 1 collagen typically seen in osteoblasts.

Various collagen subtypes have been reported to influence the differentiation of MPCs. We and others have reported either positive or no effects of collagen on adipogenesis, though this finding is not unanimous[388-391]. Types 1, 2, and 3 collagens have been shown to play important roles in promoting the differentiation and function of osteoblasts[390, 392-396]. Collagen-

coated culture dishes also increased the adhesion, proliferation, and function of a differentiated osteoblastic cell line[397]. The changes I observed in collagen gene expression in adipocytes may facilitate negative feedback to limit further adipogenic differentiation of MPCs by promoting osteoblastic lineage commitment.

I also observed a significant decrease in the expression of FN, coupled with up-regulation of its inhibitor, tenascin C, in both adipocytes and osteoblasts, which corroborates existing literature. FN has previously been shown to be down-regulated throughout adipogenic differentiation, as the existing FN network becomes degraded to allow for morphological changes to cell shape[377-380, 389, 398, 399]. FN is one of the earliest matrix proteins up-regulated during bone development, though its expression falls significantly throughout the bone maturation process[400, 401]. Additionally, tenascin C, the glycoprotein inhibitor of FN, has been shown to be up-regulated during osteogenic differentiation[402, 403].

FN has been shown to exert significant influence over SCs and EPCs. Culture of EPCs on a FN substrate was found to accelerate the appearance of endothelial colonies relative to coating of cell growth surfaces with type 1 collagen[359]. EPCs cultured on FN also demonstrate superior adhesion in comparison to culture on collagen or laminin substrata, and improved migration over culture on laminin-coated surfaces[381]. FN also accelerates the differentiation process of EPCs into ECs[146, 404]. Thus, my observed down-

regulation in the expression of FN in adipocytes and osteoblasts may exert a negative effect on EPC adhesion and function. Interestingly, advanced glycation of FN, as often seen diabetes, has been shown to impair EPC attachment and migration, as well as chemotactic targeting to sites in need of vascular repair[405].

One study by Ballard et al. has identified a potential role for tenascin C in EPC homing, with EPCs preferentially incorporating into regions of tenascin C expression in the heart[406]. This effect is further manifested as a failure for tenascin C-knockout mice to induce angiogenesis into cardiac allografts[406]. Tenascin C is also well-known to possess SC-modulatory effects in a number of other SC niches[407, 408]. Tenascin C is partially responsible for the generation of a neural SC niche within the subventricular zone and is involved in facilitating neural SC development[409]. Tenascin C-knockout studies reveal a role for tenascin C in regulating the differentiation of neural SCs and maintaining the cell population in an SC-like state[409]. Within the BM, tenascin C is also a key factor in regulating hematopoiesis[410]. Tenascin C-knockout mice also displayed reduced hematopoietic activity and HSC long-term survival, though these effects could be reversed with the addition of soluble tenascin C to culture[410]. Although a definitive function for tenascin C within the VSC niche has yet to be elucidated, the involvement of this protein in other SC niches highlights its importance and pervasiveness in developmental processes.

As described above, FN has previously been shown to drastically reduce the adipogenic capability of MPCs[341, 343, 411]. FN has also been reported to influence osteoblastic development, accelerating the expression of genes involved in osteogenic adhesion, proliferation, differentiation and function[412-414]. The effects of FN on osteoblast differentiation have been shown to be mediated via interactions with integrins[340]. Following differentiation, FN may continue to have an anabolic effect on bone by preventing the apoptosis of mature osteoblasts[415]. Interestingly, although it typically serves as an inhibitor of FN, tenascin may also have a stimulatory effect on osteoblastic progenitor cell proliferation, differentiation, and osteogenic function[416].

I observed selective regulation of other genes involved in the formation of the basement membrane between BM-MPCs, adipocytes, and osteoblasts. I found HSPG2 to be expressed by adipocytes, which confirms existing literature, while I report a novel finding in that HSPG2 is significantly down-regulated in osteoblasts[378, 417]. HSPG2 has been shown to demonstrate strong anti-adhesive properties, which may contribute to our observed differences in CD133+ cell adherence in co-culture with adipocytes and osteoblasts[418]. Although there is no known function for HSPG2 in regulating VSCs or EPCs within the BM, HSPG2 may bind to VEGFR of ECs to stimulate angiogenesis[419, 420]. Within the marrow, HSPG2 has dual effects on MPC differentiation, with exogenous HSPG2 promoting osteoblastic differentiation while inhibiting adipogenesis[421]. Taking

into account my results, this suggests the possibility of a negative feedback mechanism between HSPG2 expression and MPC differentiation.

I have also identified laminin as being uniquely regulated between the three mesenchymal cell types. In adipocytes, I observed up-regulation of laminin C1, with repression of laminin C2, as has been previously described[399, 422]. Adipocytes have been characterized as having a distinct expression of laminin proteins, with predominant expression of the laminin-8 (α4β1γ1) isoform, which is in agreement with my finding of increased expression of the laminin C1 gene[373, 422]. Osteoblasts, however, were characterized by significant down-regulation of both laminin C1 and laminin C2. Laminin is abundant in the early osteoid matrix produced by developing osteoblasts, though this expression is later supplanted by collagen, osteocalcin, bone sialoprotein, and others[386]. Laminin is known to exert a significant influence over the differentiation of MPCs by accelerating the rate of osteoblastogenesis[412, 423]. Specifically, laminin-5 (α3Aβ3γ2) increases the expression of osteogenic genes via a focal adhesion kinase (FAK) and ERK-dependent mechanism[424-427]. The reduced expression of laminin C2 by adipocytes and osteoblasts observed in my study may contribute to an inhibitory mechanism to suppress further osteoblastogenesis.

In my investigation, up-regulation of nidogen-1, also known as entactin, was observed in both osteoblastic and adipocytic cultures, although nidogen-2 was increasingly expressed only by adipocytes. Previous studies have also

identified nidogen-1 and nidogen-2 as components of the adipocytic secretome, which become up-regulated during or following adipogenesis[373, 379, 428, 429]. One study has shown decreased expression of nidogen-2 in osteoblasts, though they also observed repression of the nidogen-1 gene[430]. A critical role for nidogen-1 has been identified in promoting adhesion of a variety of cell types to the matrix[431-433]. Additionally, nidogen-laminin complexes have been found to possess both stimulatory and inhibitory actions on angiogenesis[434]. While concentrations between 30 and 300 µg of laminin-nidogen complexes per mL promoted vessel development and elongation, a concentration of 3,000 µg/mL proved inhibitory to vasculogenesis, providing credence to the notion that the basement membrane is able to dynamically regulate vessel development[434].

My profiling studies also revealed significant cell type-dependent differences in the expression of matrix remodeling proteins. I identified repression of ADAM-9 and -19 and up-regulation of ADAM-10, -15, and -17 in adipocytes. Early adipogenesis has been characterized by a transient increase in the expression of ADAM-12, which promotes matrix reorganization to accommodate changing cell morphology, though my study reports a novel change in ADAM expression by fully developed *in vitro* adipocytes[435, 436]. In osteoblasts, I noted a repression of most ADAMs, with significant down-regulation of ADAM-9, -10, -12, -15, -19, -21, and -28. In mice, knockout of the ADAM-15 gene resulted in increased activity of osteoblasts, ultimately leading to

enhanced bone mass[437]. This role for ADAM-15 in skeletal homeostasis corresponds with my observed regulation of the gene following adipogenic and osteogenic differentiation.

Substantial differences in the expression of MMPs were also observed, with repression of MMP-1, -2, -10, -11, -14, -15, -16, -17, and -24 and up-regulation of MMP-3 and -13 in osteoblasts. Down-regulation of MMPs has previously been demonstrated following osteogenesis of BM-MPCs[438]. In adipocytes, I observed down-regulation of MMP-1, -2, -11, -14, and -16 and increased expression of MMP-13, -15, -17, -19, and -24. Inhibitors of MMPs, or TIMPs, were also selectively regulated following differentiation, with repression of TIMP-2 and -3 and induction of TIMP-4 in both adipocytes and osteoblasts. A prior study observed significant induction of MMP-2 in the early stage of adipogenesis, though its expression returned to a low level after six days of differentiation[439]. Also observed was a progressive increase in the expression of MMP-19 throughout the course of differentiation, aligning with the results attained in my study[439]. As with ADAMs, the temporal activation of specific MMPs is necessary for the differentiation of MPCs into mature adipocytes and thus, the observed changes in the expression of MMPs and TIMPs by adipocytes and osteoblasts may have feedback effects on MPC differentiation[439-441]. Additionally, though little is known about the process, MMP and ADAM

metalloproteinases are believed to be involved in the mobilization of EPCs from the BM[166, 442].

Aminopeptidase A is a transmembrane peptidase involved in the cleavage of glutamic and aspartic amino acid residues from the N-terminus of various proteins. I observed up-regulation of aminopeptidase A in both adipocytes and osteoblasts in comparison to BM-MPCs. Another study has previously identified expression of aminopeptidase A in mature osteoblasts, but I believe my study is the first to characterize the induction of this peptidase following the adipogenic or osteogenic differentiation of MPCs[443]. Aminopeptidase A has been reported to possess a role in ischemia-induced angiogenesis, promoting migration and proliferation of ECs[444]. Knockout of aminopeptidase A impairs angiogenesis in a hind limb ischemia model, through a reduction in the stability of HIF-1α[445].

I also investigated the changes in expression of a variety of niche genes between BM-MPCs and BM-MPC-derived adipocytes and osteoblasts. I first investigated the expression of components that comprise the Notch signalling system that plays integral roles in directing SC fate and developmental processes. In osteoblasts, I observed significant up-regulation of Notch receptors 1 and 2, along with increased expression of the Notch ligand JAG1. Alternatively, adipocytes were characterized by induction of Notch ligand DLL4. The Notch signalling pathway is critically involved in governing the development of a number of SC types[446-448]. Within the BM, Notch has been implicated in

preventing osteoblastic differentiation to preserve the MPC population, though some studies contradict this notion[449-452]. A stimulatory role for the Notch pathway has been identified in adipogenesis, though again, this finding is not unanimous[449, 451, 453, 454].

In a profile of niche gene expression in CD133-positive SCs, I identified NOTCH1 as the predominant receptor within the Notch signalling system in these cells. The expression of Notch signalling components in EPCs suggests that the observed changes in Notch gene expression by adipocytes and osteoblasts may be parlayed into functional differences in the angiogenic capacity of EPCs. Notch signalling in EPCs has previously been shown to promote the proliferation of these cells and their mobilization and migration towards sites of vascular injury[455, 456]. Notch also regulates the *in vitro* adhesion of EPCs to the ECM by modulating integrin expression, and inhibition of this pathway impairs the ability of these cells to form tube-like structures[457]. *In vivo* activation of Notch signalling increased the angiogenic capacity of EPCs to improve wound healing in a mouse model[457].

The effects of changes in the abundance of Notch ligands on EPC survival and function have also been investigated. Conditional knockout of JAG1 in mice was associated with a reduction in the expression of EC markers within the BM, limited colony-formation by EPCs, as well as impaired EPC proliferation, migration, survival, and vasculogenic ability, while knockout of DLL1 had no

effect[458]. Overexpression of JAG1 by stromal cells in a co-culture system was able to counter the deficits in EPC differentiation and function seen in the murine model[458]. In ECs, JAG1 has been shown to antagonize the interaction between DLL4 and Notch, which has anti-angiogenic effects on vessel sprouting[459-462]. My findings of increased JAG1 in osteoblasts and repression of DLL4 in adipocytes provide a possible mechanism underlying the EPC impairment observed in diabetic BM adipogenesis.

I observed no significant changes in the expression of genes involved in mediating cell-cell and cell-ECM interactions between the three cell types, with the exception of integrin-α4, which was significantly up-regulated in osteoblasts. In contrast to my results of increased integrin-α4 expression in osteoblasts, one study that profiled gene expression throughout osteoblastic differentiation failed to observe expression of integrin-α4 at any time points[463]. This study made use of the murine MC3T3-E1 pre-osteoblast cell line, which may not be applicable to my model of human BM-MPC differentiation. My gene expression profiling of CD133+ cells revealed that one of integrin-α4's binding partners, VCAM, was expressed at a low level by these cells. This may still be sufficient to mediate binding between CD133+ cells and nearby stromal cells, but this requires further investigation. The observed increase in the expression of integrin-α4 by osteoblasts may have contributed to the enhanced SC adhesion in osteoblastic co-culture (data from first objective).

In my profiling of CD133+ cells niche gene expression, I noted that integrin-α4 was expressed in this cell type. This corresponds with existing literature, as integrin-α4β1 has been identified as a key regulator of EPC adhesion and mobilization within the BM. *In vivo* inhibition of integrin-α4 resulted in an increased propensity of EPCs to mobilize from the BM and enter into circulation, demonstrating the importance of this molecule in regulating SC adhesion[464]. Integrin-α4β1 has also been found to play a role in the homing of EPCs from the BM to areas of neovascularization, though it may be redundant in its function[464, 465]. At sites of acute lung injury, integrin-α4β1 expression by BM-derived EPCs has been implicated in promoting vessel sprouting and preventing vascular injury, demonstrating roles for integrin-α4β1 throughout the angiogenic process[466].

In osteoblastic cells, I observed a significant reduction in the expression of SDF-1, which binds to its receptor, CXCR4, to facilitate chemotaxis of lymphocytes during embryogenesis and EPCs during postnatal life. SDF-1 binds to CXCR4 to induce the concentration-dependent migration of EPCs via activation of a pathway involving PI3K, PKB, and eNOS[467]. During development, SDF-1 is expressed early in osteoblast differentiation and is believed to act as a homing agent to localize SCs to the marrow[468]. High expression of SDF-1 has been identified in immature osteoblasts and osteosarcoma cell lines, though its regulation in mature human osteoblasts is not conclusive[469]. My results indicated

that CXCR4 was expressed very highly by CD133+ cells, relative to its limited expression in BM-MPCs. I believe that the reduced expression of SDF-1 by osteoblasts may make this chemotactic gradient more detectable to EPCs in the BM, allowing for enhanced mobilization and migration towards sites of vascular injury in response to hypoxia-induced SDF-1.

SDF-1-CXCR4-induced EPC activation has previously been shown to be disrupted in diabetes. Hyperglycemia-treated EPCs display reduced expression of CXCR4 and suppression of the PI3K/PKB/eNOS axis known to mediate EPC migration[470]. This finding has been confirmed in diabetic patients, who have a reduced number of CXCR4-positive cells in circulation and whose EPCs exhibit lower CXCR4 expression than health controls[470, 471]. Diabetic mice display a reduced plasma concentration of SDF-1, as well as number of CXCR4-positive cells in circulation[472]. Overexpression of CXCR4 in EPCs has been shown to enhance the colony forming ability of these cells, prevent EPC dysfunction and apoptosis in response to hyperglycemia, and attenuate ischemic damage in a model of cerebral infarction[472].

I also investigated the potential for differential regulation of vascular growth factors Ang1 and Ang2 between BM-MPCs, adipocytes, and osteoblasts. I report the novel finding that both Ang1 and Ang2 were up-regulated over 10-fold in adipocytes relative to MPCs, though no significant change was observed in osteoblasts. Ang1 and Ang2 both serve as ligands for Tie-1 and 2 receptors,

which I have shown are expressed in CD133+ cells. Tie-2 is particularly important in angiogenesis, as well as EC proliferation and maintenance of vascular networks[473]. Alternatively, Tie-1 heterodimerizes with Tie-2 to facilitate Tie-2 signalling, and is also important in establishing vessel integrity[473, 474]. When bound to Tie-2, Ang1 is pro-angiogenic and is involved in vessel development, while binding of Ang2 acts as an antagonist to Ang1, disrupting the formation of blood vessels and inducing EC apoptosis[475-479].

Because Ang1 and Ang2 serve as antagonists to one another, I believed it was prudent to investigate the expression of these factors as the ratio between them, rather than their individual expression. Although both Ang1 and Ang2 were up-regulated in adipocytes, the ratio of Ang1 relative to Ang2 remained similar to that of MPCs. Alternatively, osteoblasts displayed a four-fold increase in the Ang1/Ang2 ratio in comparison to control MPCs, suggesting that osteoblasts may preferentially stimulate BM-EPC survival and function. My finding was in agreement with previous work citing a strong preference for Ang1 production in osteoblasts[480, 481]. Additionally, a study investigating the effects of Ang1 in the HSC niche found that Ang1 was able to heighten SC adhesion to osteoblasts, which may contribute to the increased adherence of CD133+ cells with osteoblasts in my co-culture model[330].

Murine models of diabetes have revealed significant involvement of the angiopoietin signalling system on perpetuating vascular injury. Increased

expression of Ang2 has been found in the cardiac tissue of diabetic mice relative to non-diabetic control animals[345]. This study by Tuo et al. also reported increased vascular damage and tissue apoptosis following myocardial infarction in diabetic mice relative to healthy controls[345]. This was reversed through overexpression of Ang1, and exacerbated by overexpression of Ang2[345]. In humans, the plasma concentrations of Ang2 are elevated in diabetic subjects relative to healthy controls, though there was no difference in Ang1 levels[482]. In relation to diabetic complications, Ang2 levels have been found to be significantly elevated in some forms of diabetic retinopathy the vascular changes to the retina can be reversed in a rat model through local or systemic overabundance of Ang1[483-485].

Although angiopoietins are known to be critically involved in mediating angiogenesis and dysfunction in ECs, comparatively little is known about the specific effects of Ang1 and Ang2 on endothelial progenitors. Ang1 has been shown to promote the migration of early endothelial outgrowth cells (a subclass of EPCs) and improve response to ischemia[486]. Ang1 overexpression has also been associated with enhanced VSC differentiation into EPCs within the BM, though reports of its effects on the mobilization of EPCs are divided[345, 487-491].

I investigated how modulating the balance between Ang1 and Ang2 abundance influences the stemness and endothelial properties of CD133+ cells, though the results failed to reach significance. The lack of significance attained

in this experiment may be attributed to the concentrations of angiopoietins I used in the studies. In healthy human controls, Ang1 and Ang2 concentrations are usually both below 10 ng/mL (range of mean concentrations for Ang1: 1-8 ng/mL; range of mean concentrations for Ang2: (1.5-5 ng/mL), although these values may be elevated in some disease states, including diabetes[492-496]. *In vivo* animal models using an adenovirus to overexpress Ang1 result in maximal Ang1 plasma levels of 74.1 ng/mL, though this concentration may actually reduce EPC mobilization[487, 491]. *In vitro* studies on ECs and EPCs however, have generally used Ang1 and Ang2 concentrations much greater than those seen in a physiological setting. These studies have variously used exogenous Ang1 and Ang2 concentrations ranging from 0.1-1,000 ng/mL[497-502]. While the majority of these investigations have focused on the upper ends of this spectrum, lower concentrations of Ang1 (≤ 50 ng/mL) may be capable of stimulating EC migration, vascular sprouting, and survival[497-499]. I elected to utilize moderate angiopoietin concentrations closer to physiologically relevant levels than most published literature. It is possible that extreme concentrations of angiopoietins may be required in order to induce substantive biological changes in EPCs.

I next aimed to confirm my *in vitro* findings in an *in vivo* model of diabetes. As expected, there was an up-regulation of the C/EBP family of adipogenic transcription factors in diabetic animals indicative of enhanced marrow adipogenesis. Although non-significant, the expression of osteoblastic markers

BGLAP, Runx2, and Sp7 appeared to be somewhat lower than that of controls (p = 0.124, 0.164, 0.297, respectively; n=3). The induction of adipogenesis and inhibition of osteoblastogenesis are commonly observed in diabetic models[335, 338, 503]. The expression of MPC marker CD105, also known as endoglin, in rat BM was not significantly altered by diabetes. In type 1 diabetes, increased BM adipogenesis is accompanied by a reciprocal decrease in osteogenesis, with minimal change to the size of the progenitor cell population. The effect of hyperglycemia on BM-MPC populations is unclear, with reports of contrasting effects on differentiation, proliferation, and survival[183, 257, 504, 505]. Although the finding was not statistically significant, the expression of SC markers Nanog, Oct4, and Sox2 did appear to be slightly reduced in the diabetic BM, which was to be expected given the known deleterious effects of diabetes.

Although the majority of my *in vivo* results failed to breach the desired level of significance, the finding of increased marrow adipogenesis is in concert with existing literature. A possible explanation for the lack of significance attained is the relatively short duration of diabetes used in the model. Following injections of STZ and confirmation of elevated blood glucose concentrations, rats were maintained for four weeks before being euthanized. Four weeks has proven to be sufficient time to induce cellular and morphological changes to skeletal composition in other rodent models of type 1 diabetes[335-338]. A number of other studies have observed considerably longer disease durations before

analyzing changes to the BM[506-508]. My four week time point may not have allowed sufficient time for increased BM adipogenesis to fully interfere with the proliferation, differentiation, and migration of BM-resident VSCs and EPCs. Performing a time course study of the progressive changes in diabetic BM may provide more definitive insight into the pathological modifications occurring within the marrow.

4.2 Limitations

In my exploration of the effects of MPCs, adipocytes, and osteoblasts on the survival and adherence of CD133+ SCs, I assessed the mRNA expression of three SC markers – CD133, Nanog, and Oct4 – via qPCR. I utilized the levels of these three genes as a surrogate for the "stemness" of the cells remaining in culture – a composite attribute incorporating both SC quantity and SC gene expression. In order to differentiate between these two characteristics, flow cytometry may be employed in the future to quantify the adherent and surviving cells in my *in vitro* co-culture systems.

The published literature regarding the expression of specific niche and ECM genes in adipocytes and osteoblasts is widely varied. Discordance between my results and prior findings and within existing studies may be the consequence of different starting cell populations. I made use of BM-derived MPCs and BM-MPC-derived adipocytes and osteoblasts to identify unique gene

expression patterns between the three cell types. Other studies have utilized a variety of cell types in their investigations that may not share similar gene expression profiles to my cell populations. Murine pre-adipocyte cell lines 3T3-L1 and 3T3-F442A are most commonly used in the study of adipocytes and adipogenesis, though interspecies differences may exist between these cell lines and human BM-MPCs[91, 509]. Similarly, the MC3T3 cell line is commonly used in studies of osteogenesis and osteoblast function, though it represents a mouse-derived osteoblast precursor cell line rather than multi-potential precursor cells. Other studies use the murine mesenchymal precursor cell line C3H10T1/2 that better resemble MPCs. These cells display equivalent osteoblastic development as BM-MPCs, though their capacity for adipogenesis is severely limited, with only 10% of C3H10T1/2 cells undergoing adipogenic differentiation[510]. The interspecies differences that exist between commonly used murine cell lines and human BM-MPCs used in my studies complicate the comparisons that can be made between these studies.

Additionally, the culture of isolated cell types on plastic culture dishes fails to recapitulate the complexity of the BM that exists *in vivo*. My co-culture experiment served as a simplistic example of the effects of excessive adipogenesis on CD133+ cells, but the true BM SC niche would be comprised of a mixture of MPCs, adipocytes, osteoblasts, HSCs, ECs, pericytes, and fibroblasts. Similarly, the gene expression profiles for adipocytes, osteoblasts,

and MPCs is intimately linked to paracrine and cell-cell signalling from the various cell types within the BM, which my model fails to take into account. Also, the culture of cells on collagen-coated plastic culture dishes eliminates many of the cellular interactions with the ECM, which may influence gene expression and SC phenotype.

It would be advantageous to confirm the findings of augmented gene expression in diabetic human marrow specimens, though this may not provide relevant results. Over 85% of individuals with diagnosed diabetes report controlling their disease with insulin or other anti-hyperglycemic medication[511]. While these medications are invaluable in preventing severe diabetic complications, many common diabetic therapies also induce significant changes to the composition of the BM, particularly when used chronically. Insulin and TZDs are notorious for enhancing BM adipogenesis, while metformin may increase bone density, so the use of these medications by diabetic subjects would interfere any results we may find. The difficulty involved in identifying and recruiting subjects with un-medicated disease of sufficient duration and severity to induce skeletal changes hinders our ability to confirm these findings in human subjects.

4.3 Future Directions

4.3.1 Confirming Gene and Protein Expression throughout Differentiation

The majority of my findings were derived by qPCR, which solely takes into account the mRNA abundance of target genes. PCR limits us to detecting differential rates of transcription, without acknowledging changes in expression that occur post-transcriptionally or at the protein level. Thus, an important next step would be to confirm whether the findings of augmented gene expression bear out in protein expression through quantitative western blot analyses. It would also be beneficial to perform a time course experiment reporting alterations in both gene and protein expression throughout the adipogenic and osteoblastic differentiation processes and the life cycle of these cells in order to definitively establish the contributions of each cell type to the BM SC niche.

4.3.2 Effects of Identified Target Genes on CD133-Positive Cells

Many of the genes we identified as being differentially regulated between adipocytes, osteoblasts, and MPCs are known to have effects on EPCs. Culturing CD133+ cells in conditioned media from MPCs, adipocytes, and osteoblasts could help to distinguish between the effects of soluble niche factors and cell-cell interactions, such as the Notch signalling pathway. The next step in

this research project should investigate specifically how each of these factors concentration-dependently influences proliferation, differentiation, migration, and survival in a population of CD133+ cells. I have started this process through investigation of how altering the Ang1-to-Ang2 ratio affects the expression of SC and EC markers. Through a combination of overabundance, overexpression, knockout, and inhibition experiments, we could identify how the changes in gene expression in adipocytes and osteoblasts directly affect VSCs.

4.3.3 Hindlimb Ischemia Model with Altered BM Composition

My results indicate that adipocytes may negatively impact the adhesion and survival of CD133+ cells. It would be interesting to evaluate how enhanced marrow adipogenesis affects EPCs *in vivo*. In this model, animals would be subjected to treatment with a variety of substances known to affect MPC differentiation into adipocytes and osteoblasts. These substances could include bisphenol A diglycidyl ether (BADGE; adipogenic inducer), rosiglitazone (adipogenic inducer and osteoblastogenic inhibitor), GW9662 (adipogenic inhibitor), or oncostatin M (osteoblastogenesis inducer and adipogenic inhibitor)[512-515]. Hindlimb ischemia could then be induced in these animals and successful reperfusion monitored. Angiogenic ability under distinct marrow compositions could be contrasted with that of diabetic animals, as diabetes has consistently been shown to impair vascular recovery using this model[179, 516, 517]. This experiment would be of use in conclusively ascertaining the specific

contribution of marrow adipogenesis to diabetic VSC and EPC dysfunction, rather than other pathological aspects of the diabetic state such as hyperglycemia and hyperlipidemia.

Chapter 5 : References

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Curriculum Vitae

Meghan A. Piccinin

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2013 – 2015 **M.Sc.** Pathology, University of Western Ontario

2009 – 2013 **B.M.Sc.** Medical Cell Biology & Pharmacology, University of Western Ontario

ACADEMIC AWARDS

2015 Annual Pathology and Laboratory Medicine Research Day – Best Basic Science Poster Presentation by a Graduate Student

2014 – 2015 Ontario Graduate Scholarship

2014 Canadian Diabetes Association/Canadian Society for Endocrinology & Metabolism Professional Conference Abstract Travel Award

2013 – 2015 Western Graduate Research Scholarship

2010 – 2013 Dean of Science's Honour Roll

2012 University Students' Council Honour W Award of Merit

2009 – 2012 Sun Life Financial Staff Benevolent Fund Scholarship

2010 University of Western Ontario's Excellence in Leadership Award

2009 Western Scholarship of Excellence

WORK EXPERIENCE/TEACHING ACTIVITIES

2014 – 2015 Graduate Teaching Assistant, University of Western Ontario

2012 – 2015 Research Analyst, Dr. MA Gupta Medical Professional Corporation

PUBLICATIONS

Publication Summary:

	Published/ In press	Submitted	Career Totals
Book Chapters	-	-	-
Published Manuscripts	2	1	3
Abstracts (National/International)	5	3	8
Abstracts (Regional)	10	-	10
			21

PUBLISHED MANUSCRIPTS

1. **Piccinin MA**, Khan ZA. Pathophysiological role for enhanced bone marrow adipogenesis in diabetic complications. *Adipocyte* 3:4 (2014).

2. Gupta MA, Gupta AK, Vujcic B, **Piccinin MA**. Use of Opioid Analgesics in Skin Disorders: Results from a Nationally Representative US Sample. *Journal of Dermatologic Treatment* (2014).

MANUSCRIPTS SUBMITTED

1. Gupta MA, Knapp K, **Piccinin MA**, Simpson FC. Relative contributions of insomnia, hypersomnia and fatigue in the diagnosis of depression: Results from a nationally representative US sample.

   Submitted to: Scientific Reports, February 2015

ABSTRACTS & PROFESSIONAL PRESENTATIONS

A) National/International Meetings

1. **Piccinin MA**, Khan ZA. Diabetic marrow adipogenesis alters the composition of the stem cell niche and impairs CD133-positive stem cell survival. Submitted for presentation at the World Diabetes Conference, December 2015.

2. **Piccinin MA**, Khan ZA. Diabetes-induced Alteration of the Bone Marrow Microenvironment Reduces CD133-positive Stem Cell Survival. Accepted for publication in Diabetes, abstract number 2901-PO. American Diabetes Association's 75th Scientific Sessions, June 2015.

3. Gupta MA, Knapp K, **Piccinin MA**, Simpson FC. Complaints of Insomnia, Hypersomnia and Fatigue in Depressive Disorders with Medical Comorbidities: A Case-control Study from a Nationally Representative US Sample. Accepted for publication in SLEEP, abstract number 0932. SLEEP 2015, 29th Annual Meeting of the Associated Professional Sleep Societies, June 2015.

4. **Piccinin MA**, Khan ZA. Effect of enhanced marrow adiposity on CD133-positve stem cell adherence and survival. Presented at the Till & McCulloch Meetings, October 2014.

5. **Piccinin MA**, Khan ZA. Adipogenesis of Marrow Mesenchymal Progenitor Cells Results in Modulation of Extracellular Matrix Proteins. Presented at the Canadian Diabetes Association/Canadian Society of Endocrinology and Metabolism Professional Conference and Annual Meetings, October 2014. *Canadian Journal of Diabetes* 38:5 S20, 2014.

6. **Piccinin MA**, Khan ZA. Differential contribution of niche proteins by osteoblasts and adipocytes: potential mechanism of stem cell depletion in diabetes. *Diabetes* 63:S1 A2633, 2014.

7. **Piccinin MA**, Khan ZA. Adipogenesis of bone marrow mesenchymal progenitor cells is associated with selective modulation of extracellular matrix. *Diabetes* 63:S1 A2643, 2014.

8. Gupta MA, Knapp K, **Piccinin MA**, Simpson FC. Insomnia, Hypersomnia and Fatigue are Independently Associated with Depressive Disorders (ICD9-CM Codes 296, 311): Results from a Nationally Representative US Sample of 37,171 Patient Visits for Depression. *SLEEP* 37:S1 A804, 2014.

B) Regional Meetings

1. **Piccinin MA**, Khan ZA. Diabetic Bone Marrow Adipogenesis Impairs Survival of CD133-positive Stem Cells by Altering the Composition of the Marrow Stem Cell Niche. Presented at London Health Research Day, April 2015.

2. **Piccinin MA**, Khan ZA. Diabetic Marrow Adipogenesis Alters Composition of Stem Cell Niche and Impairs CD133-positive Stem Cell Survival. Presented at the Annual Pathology Research Day, March 2015.

3. **Piccinin MA**, Khan ZA. Enhanced marrow adiposity creates a distinct extracellular microenvironment and impairs CD133-positive stem cell survival. Presented at the 5th Annual Diabetes Research Day, November 2014.

4. **Piccinin MA**, Khan ZA. Selective modulation of extracellular matrix proteins following adipogenesis of marrow mesenchymal progenitor cells. Presented at Developmental Biology Annual Research Day, May 2014.

5. **Piccinin MA**, Khan ZA. Extracellular matrix is selectively regulated following diabetes-induced adipogenesis of bone marrow mesenchymal progenitor cells. Presented at Annual Pathology Research Day, March 2014.

6. **Piccinin MA**, Khan ZA. Distinctive expression of niche factors by adipocytes and osteoblasts may mediate diabetic stem cell depletion. Presented at Annual Pathology Research Day, March 2014.

7. **Piccinin MA**, Khan ZA. Adipogenesis of bone marrow mesenchymal progenitor cells is associated with selective modulation of extracellular matrix. Presented at London Health Research Day, March 2014.

8. **Piccinin MA**, Khan ZA. Potential role of diabetes-induced angiopoietin-2 in altering stem cell niche proteins. Presented at the 4th Annual Diabetes Research Day, November 2013.

9. **Piccinin MA**, Khan ZA. Adipogenesis of bone marrow mesenchymal progenitor cells is associated with selective modulation of extracellular matrix. Presented at the 4th Annual Diabetes Research Day, November 2013.

10. **Piccinin MA**, Khan ZA. Differential contribution of niche proteins by osteoblasts and adipocytes: potential mechanism of stem cell niche depletion in diabetes. Presented at the 4th Annual Diabetes Research Day, November 2013.