MECHANISM(S) OF VASCULAR CALCIFICATION

INVESTIGATING THE ROLE OF LEPTIN AND GSK-3 IN THE OSTEOGENIC DIFFERENTIATION OF VASCULAR SMOOTH MUSCLE CELLS

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy

McMaster University

DOCTOR OF PHILOSOPHY (2014) McMaster University

(Medical Sciences) Hamilton, Ontario, Canada

TITLE: Investigating the role of leptin and GSK-3 in the osteogenic differentiation of vascular smooth muscle cells

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NUMBER OF PAGES: xvii, 224

ABSTRACT

Obesity is a major risk factor for insulin resistance, type 2 diabetes, cardiovascular disease (CVD), and vascular calcification. Vascular calcification is correlated with advanced CVD and a significant predictor of cardiovascular events. Obese individuals tend to have increased levels of circulating leptin, an adipocytokine that is a significant independent predictor of cardiovascular disease.

We have shown that daily intraperitoneal injections of exogenous leptin (125 μg/mouse/d) can promote vascular calcification in an ApoE$^{-/-}$ mouse model of atherosclerosis. This increase in calcification is associated with an increase in the expression of several osteoblast-specific markers and is independent of any affect on atherosclerotic lesion size. Our studies suggest that leptin mediates the osteogenic differentiation of vascular smooth muscle cells (VSMCs) to promote vascular calcification via a pathway involving the inhibition of glycogen synthase kinase (GSK)-3 activity.

Other studies have suggested that endoplasmic reticulum (ER) stress-induced GSK-3 activity promotes the development of atherosclerosis. Therefore, we hypothesized that during the progression of vascular disease, GSK-3 functions as a checkpoint for VSMCs at which cells can commit to: i) de-differentiation, thereby contributing to atherosclerosis, or ii) osteogenic differentiation, thereby contributing to vascular calcification. We investigated the effects of modulating GSK-3 activity on the differentiation of VSMCs *in vitro*. We

found that many of the molecular tools that are typically used to modulate ER stress can promote the expression of osteoblast-specific markers and the osteogenic differentiation of MOVAS cells. However, because many of these interventions affect multiple pathways in MOVAS cells, the specific role of the ER stress – GSK-3 pathway is difficult to discern. Future studies are required to determine the effects of direct modulation of GSK-3 on vascular calcification and to delineate the mechanisms/effects of various ER stressors in the osteogenic differentiation of VSMCs.

ACKNOWLEDGEMENTS

This thesis is dedicated to one very special lady, my grandma: Ani!

Someone once told me that a PhD is as much about intelligence as it is about perseverance. I could not have persevered without the support and encouragement of certain individuals to whom I will forever be indebted.

First and foremost, I would like to extend a heartfelt thank you to my supervisor, Dr. Geoff Werstuck, for welcoming me into his lab and believing in me when sometimes I didn't believe in myself. You are the epitome of what a graduate supervisor should be. Words can never adequately express how much I have appreciated your expertise and mentorship over the past several years.

To my committee members, Dr. Dino Trigatti and Dr. Carl Richards, thank you for always challenging me and helping to guide my project. To every past and present member of the Werstuck lab, thank you for all of the fond memories, the much needed pep talks, and for making graduate school just a little sweeter. It has been a sincere pleasure to learn from all of you.

To my dearest friends who are the sisters I never had: thank you for always willing to listen and offer words of comfort, for the endless laughter and support. You make my life richer. A special thank you to Dr. Terry McCurdy for her continued mentorship, friendship and delicious lattes.

I am eternally grateful to my two biggest fans: my Mom and my Dad without whom I would be lost. Your unwavering love, support and confidence is the reason I am where I am today! Thank you for always making me feel like I can do anything. (Dad, we can finally open that bottle of Cognac) Last but not least, to my husband, Alex: you always believed in me, encouraged me and supported me. I know this has been a bumpy road but I am so lucky to have been on it with you by my side. You three are my pillars of strength!

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LIST OF ABBREVIATIONS

Ad	adenovirus
AgRP	agouti-related protein
Akt/PKB	protein kinase B
ALP	alkaline phosphatase
Ang II	angiotensin II
ANOVA	analysis of variance
AP-1	activator protein - 1
APC	denomatous polyposis coli
ApoE$^{-/-}$	apolipoprotein E-deficient
ASK1	apoptosis signal-regulating kinase
ATF6	activating transcription factor 6
BASMC(s)	bovine aortic smooth muscle cell(s)
BMI	body mass index
BMP	bone morphgenetic protein
BSA	bovine serum albumin
CAC	coronary artery calcification
CAD	coronary artery disease
Cbfa1	core-binding factor subunit alpha-1
CCHS	Canadian Community Health Survey
C/EBPα	CCAAT/enhancer binding protein-α
cGMP	cyclic guanosine monophosphate
CHOP	C/EBP homologous protein
CK1	casein kinase 1
Col1a1	type I collagen α1
Col1a2	type I collagen α2
CPM	counts per minute
CRP	C-reactive protein
CVC	calcifying vascular cells
CVD	cardiovascular disease
db/db	leptin-receptor deficiency
Dlx5	distal-less homeobox 5
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
Dvl	disheveled
EBCT	electron beam computer tomography

EDTA	ethylenediaminetetraacetic acid
eIF2α	eukaryotic translation initation factor 2 alpha
ENPPI	ectonucleotide pyrophosphate/phosphodiesterase I
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ERK 1/2	extracellular regulated kinase 1/2
FBS	fetal bovine serum
FC	free cholesterol
FGF	fibroblast growth factor
FOXO1	forkhead box O1
FRAT-1	frequently rearranged in advanced T-cell lymphomas 1
Fz	frizzled
GADD153	growth arrest and DNA damage 153
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAT	glutamine:fructose-6 phosphate amidotransferase
Grb2	growth factor receptor-bound protein 2
βGP	β-glycerophosphate
Grp78/BiP	glucose-regulated protein 78/94
GSK-3	glycogen synthase kinase – 3
HBP	hexosamine biosynthetic pathway
HepG2	human hepatocarcinoma
H_2O_2	hydrogen peroxide
ICAM-1	intracellular adhesion molecule - 1
IL-6	interleukin-6
IRAK4	interleukin-1 receptor-associated kinase 4
IRE1	inositol-requiring transmembrane kinase/endonuclease 1
IRS	insulin receptor substrate
IVUS	intravascular ultrasound
JAK2	janus kinase 2
KC	ketocholesterol
KDEL	lysine-aspartic acid-glutamic acid-leucine
LDL	low density lipoproteins
$LDLR^{-/-}$	low density lipoprotein receptor-deficient
LEF/TCF	lymphoid enhancer factor/T-cell factor
LPL	lipoprotein lipase
LRP 5/6	LDLR - related protein 5/6
MAPK	mitogen activated protein kinase
MCP-1	monocyte chemoattractant protein -1

MEF	mouse embryonic fibroblast
MEK	mitogen activated protein kinase kinase
MGP	matrix gla protein
MGP$^{-/-}$	MGP-deficient
MOPS	morpholino propanesulfonic acid
MOVAS	murine vascular smooth muscle cell line
MSC	mesenchymal stem cell
α-MSH	α-melanocyte-stimulating hormone
Msx2	msh homeobox 2
MYD88	myeloid differentiation primary response gene 88
NF-κB	nuclear factor kappa beta
NK	natural killer
NLS	nuclear localization sequence
NO	nitric oxide
NPY	neuropeptide Y
ob/ob	leptin-protein deficiency
Ob-R	leptin receptor
Ob-Rb	leptin receptor (long isoform)
OCT	optimal cutting temperature
OPN	osteopontin
OPPG	osteoporosis pseudo-glioma
OSE	osteoblast-specific element
Osx	osterix
PBA	4-phenylbutyric acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDI	protein disulphide isomerase
PERK	RNA-dependent-protein kinase-like ER kinase
PKA	protein kinase A
PKC	protein kinase C
PI3K	phosphoinositide 3- kinase
PMSF	phenylmethanesulfonylfluoride
pNPP	*p*-nitrophenyl phosphate
POMC	proopriomelanocortin
PPARγ2	peroxisome proliferator-activated receptor γ2
PPi	inorganic pyrophosphate
PTH	parathyroid hormone
PTP1B	protein tyrosine phosphatse 1B

PTHrP PTH-related protein
qRT-PCR quantitative real-time PCR
R26R Rosa26 Cre reporter
redox reduction and oxidation
ROS reactive oxygen species
Runx2 runt-related transcription factor 2
S1P site 1 protease
SH2 Src homology 2
SHP2 SH2- containing protein tyrosine phosphatase
SM22α smooth muscle 22 alpha
αSM-actin smooth muscle α-actin
SMC(s) smooth muscle cell(s)
SM-MHC smooth muscle – myosin heavy chain
SOCS3 suppressor of cytokine signaling - 3
Sost sclerostin
SREBP sterol regulatory element-binding protein
STAT signal transducer and activator of transcription
TGFβ transforming growth factor β
ThT Thioflavin T
TNFR tumor necrosis factor receptor
TLR toll-like receptor -2
TRAF2 tumor necrosis factor receptor-associated factor 2
TRAIL TNF-related apoptosis-inducing ligand
TRB3 tribbles homolog 3
β-TrCP β-transducin repeat-containing protein
TRH thyrotropin-releasing hormone
TSA trichostatin A
TUDCA tauroursodeoxycholic acid
TUNEL terminal deoxynucleotidyl transferase dUTP nick end labeling
UDP-GlcNAc UDP-*N*-acetylglucosamine
UPR unfolded protein response
VCAM-1 vascular cell adhesion molecule – 1
VLDL very low density lipoprotein
VSMC(s) vascular smooth muscle cell(s)
WOSCOPS West of Scotland Coronary Prevention Study
WSF1 Wolfram syndrome 1

CHAPTER 1: General Introduction

1.1 Atherosclerosis

Risk Factors

Cardiovascular diseases (CVD) are defined as “diseases and injuries of the heart, the vessels of the heart and the system of vessels throughout the body.” CVD continues to be the principal cause of mortality among Canadians accounting for 29% of all deaths and representing a significant expenditure of the national health budget. As of 2009, CVD costs to the Canadian health care system were estimated to be over $22 billion (2009). CVD is now understood to be a multifactorial disease that has both genetic and environmental components (Glass and Witztum 2001;Yusuf *et al.* 2004). Cardiovascular risk factors including dyslipidemia, smoking, hypertension, diabetes and obesity accelerate the development and progression of atherosclerosis (Yusuf *et al.* 2004).

Pathophysiology of Atherosclerosis

The arterial wall is made up of three distinct layers: adventitia, media and intima. The adventitia is the outermost layer made up largely of connective tissue and its role is to provide structure and support to the blood vessel. The media, the thickest layer found in the vessel wall, is composed of elastic tissue and vascular smooth muscle cells (VSMCs) which can synthesize collagen, elastin, and proteoglycans. This allows the tissue to provide strength and elasticity by

modulating vasoconstriction and vasodilation of the vessel wall. The intima, the innermost layer, is composed of a single, continuous layer of endothelial cells. Despite its simplicity, the endothelium has several important regulatory functions: (1) it acts a semi-permeable membrane to blood borne factors; (2) it maintains the hemostatic balance between coagulation and fibrinolysis; and (3) regulates blood vessel reactivity through the synthesis and appropriate release of vascular mediators including nitrix oxide (NO) and endothelin (Ross 1999).

Atherosclerosis is contemporarily viewed as a chronic inflammatory response to injury of the endothelial layer within the vessel wall mediated predominantly by interactions between modified lipoprotein particles, monocyte-derived macrophages, T-lymphocytes, and smooth muscle cells (SMCs). Neutrophils, B-cells, natural killer (NK) cells, mast cells and dendritic cells have also been localized to the atherosclerotic lesion and may participate in the local immune response (Weber and Noels 2011). Atherosclerosis tends to occur at arterial sites with disturbed laminar blood flow such as branches, bifurcations and curvatures where shear stress is low and blood flow exhibits directional variations (Warboys *et al.* 2011). Chronic injury alters the characteristics of the endothelium by increasing its permeability, inducing procoagulant activity and stimulating the production of vasoactive molecules, cytokines and growth factors (Ross 1999). For example, the damaged endothelium up-regulates the expression of selective leukocyte adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (Libby *et al.*

2002;Ross 1999). These changes promote the adhesion, migration and accumulation of monocytes and T-cells within the intimal space. During the inflammatory response, low density lipoproteins (LDLs) also become modified by oxidation. Oxidized-LDLs are internalized by monocyte-derived macrophages via their scavenger receptors resulting in the formation of lipid-laden macrophage foam cells. Further aggravating lesion development is the migration and proliferation of SMCs from the medial layer into the intimal space, which together with macrophages engulf modified lipoprotein particles (i.e. lipids). In addition, SMCs secrete extracellular matrix including collagen and proteoglycans to produce a fibrous cap that functions to stabilize the growing lesion. The apoptosis of lipid engorged macrophage foam cells contributes to the formation of a necrotic core. Ischemic symptoms may arise due to narrowing of the vessel lumen whereas cardiovascular events such as myocardial infarction and stroke usually result from plaque rupture and thrombosis. This latter event is exacerbated by activated macrophages that produce metalloproteinases and other proteolytic enzymes which degrade the matrix of the fibrous cap compromising plaque stability (Glass and Witztum 2001;Ross 1999).

Obesity and Cardiovascular Disease

The prevalence of obesity, defined as a BMI of 30 kg/m^2 or higher, has increased significantly from 6.1% in 1985 to 18% in 2010, according to the Canadian Community Health Survey (CCHS). Alarmingly, this number is

predicted to reach 21% by 2019 (Twells *et al.* 2014). This is of great concern, as obesity is a major risk factor for CVD. It has been recognized for some time now that obese individuals are predisposed to CVD, suggesting that there must exist a link between the two conditions, but we are just beginning to understand the mechanisms linking obesity to these pathologies (Grundy 2004;Kenchaiah *et al.* 2002;Kopelman 2000;Lakka *et al.* 2001;Van Gaal *et al.* 2006;Yusuf *et al.* 2004). For example, the seminal Framingham Heart Study followed the 26-year incidence of CVD in Framingham men and women. This study reported that men and women in the heaviest group experienced more than twice the risk of coronary disease compared with the leanest group (Hubert *et al.* 1983).

These observations have led many researchers to propose that adipose tissue may be more than just a passive organ for “fat” storage, and that the various factors it secretes, functionally called adipocytokines (i.e. leptin, resistin, visfatin, apelin), may in fact be playing a role in promoting CVD. Adipocytokines are known to circulate at elevated levels in obese individuals and to correlate positively with the risk of CVD (Azuma *et al.* 2003;Burnett *et al.* 2005;Clarke *et al.* 2009;Degawa-Yamauchi *et al.* 2003;Heinonen *et al.* 2005;Lee *et al.* 2009;Maugeri *et al.* 2002;Norata *et al.* 2007;Pagano *et al.* 2006;Reilly *et al.* 2005;Romero-Corral *et al.* 2008;Wallace *et al.* 2001). The possible pro-atherogenic effects of these adipocytokines include the induction of endothelial dysfunction (Jung *et al.* 2006;Konstantinides *et al.* 2001;Verma *et al.* 2003), stimulation of inflammatory processes (Bokarewa *et al.* 2005;Loffreda *et al.*

1998), increased levels of oxidative stress (Yamagishi *et al.* 2001) and increased migration and proliferation of VSMCs (Calabro *et al.* 2004;Jung *et al.* 2006;Oda *et al.* 2001). Much of the CVD research to date has focused on the role of the adipocytokine, leptin.

1.2 Leptin

Biology of Leptin

Leptin is a 16 kDa protein (Tartaglia 1997). Initially, circulating levels were considered to be a mere reflection of the body's energy reserves. During times of inadequate nutrient intake, leptin levels decrease resulting in increased appetite and decreased energy metabolism. However, during times of adequate nutrient intake, leptin levels increase to attenuate appetite and increase metabolic function through its effects on the neurons of the basomedial hypothalamus (Myers, Jr. 2004). In support of this, Halaas *et al* (1995) demonstrated that leptin-deficient mice given daily intraperitoneal injections of recombinant leptin experienced a 30% reduction in body weight, a reduction in food intake and an increase in metabolic rate (Halaas *et al.* 1995). Notably, in human subjects, very few cases of severe obesity have been associated with leptin deficiency (Gibson *et al.* 2004;Licinio *et al.* 2004;Montague *et al.* 1997;Strobel *et al.* 1998). On the contrary, the majority of obese individuals display significantly elevated levels of leptin and appear to be selectively resistant to leptin's centrally-mediated effects

whereas its peripheral effects remain largely unaffected. Leptin receptors are expressed ubiquitously throughout the body and can affect systematic processes such as reproduction, bone hemostasis, immunity and cardiovascular function (Margetic *et al.* 2002) .

Multiple forms of the leptin receptor have been identified (OB-Ra -to- OB-Rd) and are considered to be alternative splice variants of a single *lepr* gene. OB-Ra-d are membrane bound forms of the leptin receptor that share identical extracellular and transmembrane domains as well as the first 29 amino acids within the intracellular domain (Tartaglia 1997). OB-Re is referred to as the soluble leptin receptor because it has only the extracellular leptin-binding domain. It is still unclear whether Ob-Re is a splice variant of the leptin receptor or whether it is derived from ectodomain shedding of membrane-bound leptin receptors (Ge *et al.* 2002;Li *et al.* 1998). Its function is to bind leptin and thus regulate its availability and bioactivity. The function(s) of the short receptor isoforms (Ob-Ra, c, d) is still unclear although OB-Ra may be involved in leptin transport across the blood-brain barrier (Golden *et al.* 1997). The long-form receptor, OB-Rb appears to be responsible for most, if not all, of leptin's physiological effects. Ob-Rb does not have intrinsic enzymatic activity but signals via a tyrosine kinase, Janus Kinase (JAK), which is noncovalently bound to the cytoplasmic proline-rich Box 1 motif of the long-form leptin receptor. In addition, the Ob-Rb intracellular domain contains a less-conserved sequence COOH-

terminal to Box 1 referred to as Box 2 that dictates JAK2 selectivity. This motif is absent from all of the other short leptin receptor isoforms (Myers *et al.* 2008).

Leptin Signaling

Serum leptin levels in lean subjects are typically between 5 - 15 ng/mL but levels have been reported to be ≥ 25 - 30 ng/mL in obese individuals (Sinha *et al.* 1996). Importantly, serum levels above this threshold do not translate into a proportionate increase in cerebrospinal or brain leptin levels which may contribute to central leptin resistance (Caro *et al.* 1996). Leptin expression is increased by overfeeding, insulin, endotoxins and cytokines and decreased by fasting, testosterone, thyroid hormone and cold temperatures. The binding of leptin to its receptor can activate multiple signal transduction pathways (**Figure 1.1**). Ob-Rb constitutively binds JAK2. When leptin interacts with Ob-Rb it promotes Ob-Rb dimerization resulting in JAK2 activation and autophosphorylation. Activated JAK2 also phosphorylates the leptin receptor on the following cytoplasmic residues: Tyr985, Tyr1077 and Tyr1138 (Morris and Rui 2009).

JAK2/STAT3/STAT5 Pathway

Cytokine-stimulated tyrosine phosphorylation of signal transducer and activator of transcription *(*STATs) induces their homodimerization, phosphorylation/activation and nuclear translocation to promote transcriptional regulation. Leptin is known to activate STAT3 and STAT5 in the hypothalamus.

STAT3 binds to the phosphorylated Tyr1138 residue on Ob-Rb which allows JAK2 in turn to phosphorylate and activate STAT3. The JAK2/STAT3 signaling pathway is present in various subpopulations of Ob-Rb neurons and must act coordinately to mediate leptin's anorexigenic actions (Morris and Rui 2009;Yang and Barouch 2007). In addition, STAT5 has been shown to bind to the phosphorylated Tyr1077 residue on Ob-Rb. Deletion of STAT3, and to a lesser degree STAT5, causes leptin resistance, hyperphagia and obesity (Morris and Rui 2009). This indicates an important role for both the JAK2/STAT3 and JAK2/STAT5 cascades in leptin's ability to regulate energy balance and body weight.

Insulin Receptor Substrate/Phosphoinositide 3- Kinase (IRS/PI3K)

Leptin has also been shown to stimulate insulin receptor substrate-2 (IRS-2) and phosphoinositide 3-knase (PI3K) in cultured cells, including myotubes, insulinoma cells and heptocytes cells, as well as hypothalamic neurons (Morris and Rui 2009). The IRS-2/PI3K pathway plays a role in mediating leptin's anorexigenic action by inactivating transcriptional factor, forkhead box O1 (FOXO1) via protein kinase B (PKB/Akt). When active, FOXO1 stimulates the expression of orexigenic proteins, neuropeptide Y (NPY) and agouti-related protein (AgRP) but inhibits the expression of anorexigenic protein proopriomelanocortin (POMC) - a precursor for α-melanocyte-stimulating hormone (α-MSH) (Kim *et al.* 2006). α-MSH stimulates thyrotropin-releasing

hormone (TRH) biosynthesis and secretion whereas NPY and AgRP suppress TRH secretion. TRH activates the pituitary-thyroid axis thus increasing metabolic rate and energy expenditure.

Mitogen Activated Protein Kinase (MAPK)

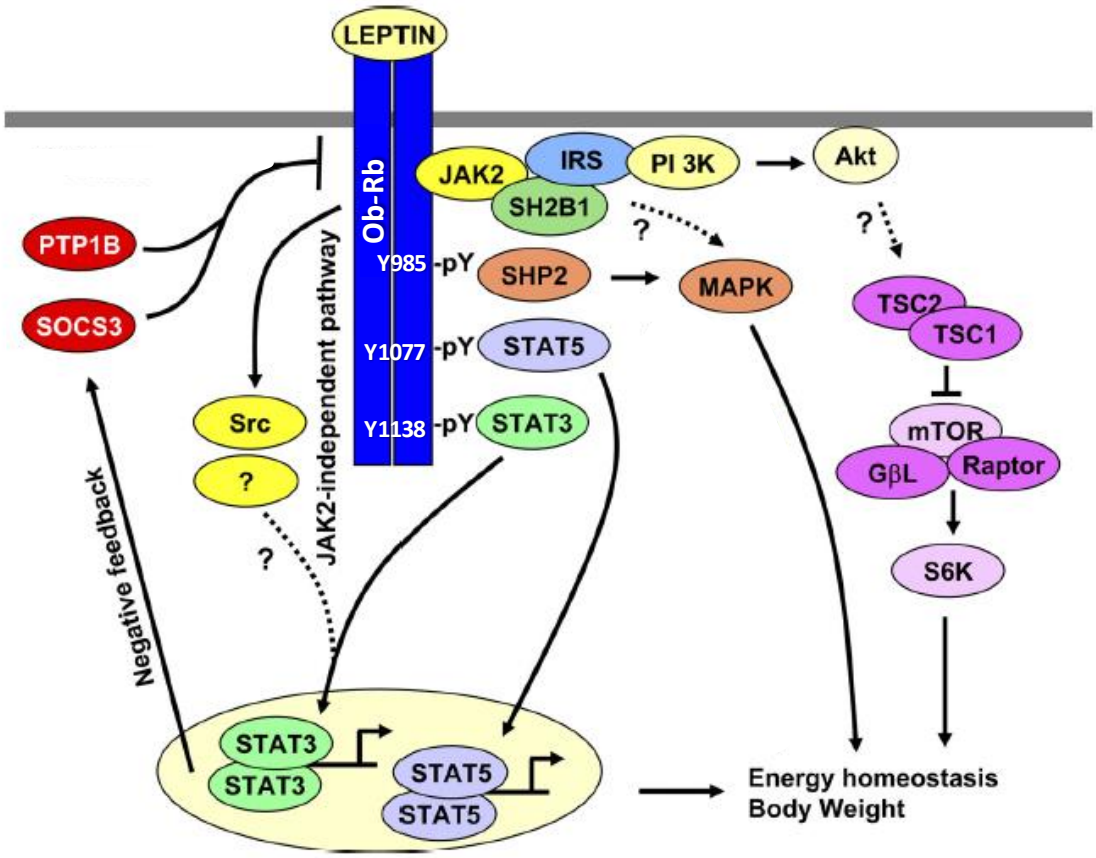
Leptin can also stimulate the activation of extracellular-regulated kinase (ERK) 1/2 via Src homology 2 (SH2) - containing protein tyrosine phosphatase (SHP2). SHP2 binds phosphorylated Tyr985 on Ob-Rb via its SH2 domain (Morris and Rui 2009;Yang and Barouch 2007). Hypothalamic inhibition of ERK1/2 results in leptin resistance and obesity (Rahmouni *et al.* 2009).

Negative Feedback Regulation

Leptin can also induce its own negative feedback through STAT3-mediated expression of suppressor of cytokine signaling-3 (SOCS3) expression. SOCS3 binds to and inhibits JAK2 activity during prolonged leptin stimulation. Protein tyrosine phosphatase 1B (PTP1B) is also able to inhibit leptin signaling by dephosphorylating JAK2 (Morris and Rui 2009;Yang and Barouch 2007).

Figure 1.1 - Leptin signaling pathways.

Leptin binds to the long-form of the leptin receptor (Ob-Rb) and activates JAK2 which is noncovalently bound to Ob-Rb. JAK2 autophosphorylates on Tyr813, which can stimulate activation of the IRS/PI3K pathway via SH2B1. JAK2 also phosphorylates Ob-Rb on Tyr 985/1077/1138 which results in the activation of other signaling pathways. STAT5 and STAT3 bind to phospho-Tyr1077 and phospho-Tyr1138, respectively, and are subsequently phosphorylated and activated by JAK2. Once activated, STATs dimerize and translocate to the nucleus to induce transcription. SHP2 binds to phospho-Tyr985 and mediates the activation of MAPK pathway via growth factor receptor-bound protein 2 (Grb2). Ob-Rb signaling is negatively regulated by SOCS3 and PTP1B. This figure has been adapted from Morris, D.L. & Rui, L. (2009). Recent advances in understanding leptin signaling and resistance. Am J Physiol Endocrinol Metab 297, E1247-E1259. Permission is not required for use of this figure in a thesis/dissertation.


LEPTIN
Ob-Rb
JAK2
IRS
PI 3K
Akt
SH2B1
PTP1B
SOCS3
Y985
-pY
SHP2
MAPK
TSC2
TSC1
Y1077
-pY
STAT5
Src
?
Y1138
-pY
STAT3
mTOR
GβL
Raptor
S6K
JAK2-independent pathway
Negative feedback
STAT3
STAT3
STAT5
STAT5
Energy homeostasis
Body Weight

Leptin and Atherosclerosis in Animal Studies

The results from several *in vivo* studies have suggested that plasma leptin levels can affect atherosclerosis. This section provides a brief review of these studies before discussing how leptin effects the various cell types found within the vessel wall. Shäfer *et al* (2004) examined the outcome of leptin-receptor deficiency (*db/db*) and leptin-protein deficiency (*ob/ob*) on atherosclerotic lesion progression in mice and demonstrated that wild-type mice placed on an atherogenic, high-fat diet had significantly elevated leptin levels (9-fold) compared to normal chow fed mice. Wild-type mice maintained on the high-fat diet also developed greater neointimal thickening after carotid artery injury with ferric chloride. However, *ob/ob* and *db/db* maintained on the same atherogenic, high-fat diet showed no signs of neointimal lesion formation after injury despite severe obesity, higher glucose and plasma lipid levels. This "athero-protection" was reversed once *ob/ob* mice were supplemented with recombinant murine leptin (dosage: 0.6 µg/g body weight in 100 µL normal saline administered by intraperitoneal injection) daily for three weeks despite significant reductions in their body weight and plasma cholesterol levels (Schafer *et al.* 2004).

In a separate animal study, Bodary *et al* (2005) examined the effect of leptin treatment on atherosclerosis in apolipoprotein E - deficient ($ApoE^{-/-}$) mice. Leptin was administered at a concentration of 125 µg/mouse/day for four weeks. Their analysis of total lesion area revealed greater levels of atherosclerosis at the thoracic aorta and brachiocephalic artery in leptin-treated animals when

compared to non-treated vehicle control animals but no differences in atherosclerosis were apparent at the ascending aorta. These results were independent of any effects on triglyceride or cholesterol levels (Bodary *et al.* 2005).

Interestingly, ApoE$^{-/-}$*db/db* double knockout mice exhibit an obese, hyperglycemic, hyperinsulinemic and dyslipidemic phenotype which correlates with significantly accelerated atherosclerosis in the aorta when compared to their age-matched (20 weeks) ApoE$^{-/-}$ littermate controls (Wu *et al.* 2005). Similarly, LDLR$^{-/-}$*ob/ob* (LDLR$^{-/-}$ = low density lipoprotein receptor - deficient) double knockout mice also exhibit an increase in atherosclerotic lesions throughout the aorta, but this was also associated with severe hyperlipidemia and impaired carbohydrate metabolism (Hasty *et al.* 2001). At first glance, these findings seem to suggest that leptin may protect from atherosclerosis and appear to contradict the aforementioned studies by Shäfer *et al.* and Bodary *et al.* However, the pro-atherogenic effects in LDLR$^{-/-}$*ob/ob* mice were reversed following weight loss suggesting that the metabolic profile could be confounding the interpretation of any role that leptin may be playing in the atherosclerotic process (Verreth *et al.* 2004). Furthermore, in contrast to the phenotypes of mice with a mutation in the leptin receptor (ApoE$^{-/-}$*db/db*), exogenous leptin administered to ApoE$^{-/-}$ mice, as in the Bodary *et al.* study, does not alter the lipid profile of these animals. Therefore, it is possible that leptin's direct atherogenic effects are unmasked when leptin is administered exogenously (Beltowski 2006). In support of this,

LDLR$^{-/-}$*ob/ob* mice display a significant decrease in atherosclerotic lesion development when compared to LDLR$^{-/-}$ littermates with similar total cholesterol levels (Taleb *et al.* 2007).

Mechanisms for Leptin's Proatherogenic Effects

How is leptin exerting its proatherogenic effects and which cells are being affected? Leptin receptors are expressed ubiquitously and have also been found on cell types involved in atherosclerotic lesion development including endothelial cells, macrophages, and VSMCs.

Endothelial Cells

Several *in vitro* studies demonstrated that leptin elicits NO – mediated vasorelaxation of the endothelium (Kimura *et al.* 2000;Lembo *et al.* 2000). However, the over production of NO may result in oxidative stress and actually impair endothelial function. In support of this, leptin has been shown to induce pro-inflammatory changes to endothelial cells. For example, leptin signaling in bovine endothelial cells upregulates fatty acid oxidation and this promotes reactive oxygen species (ROS) production via the mitochondrial electron transport chain (Yamagishi *et al.* 2001). ROS production induced by leptin activation has also been shown to stimulate the expression of other pro-inflammatory markers including toll-like receptor (TLR) – 2, TLR-4, myeloid differentiation primary response gene 88 (MYD88), interleukin-1 receptor-associated kinase 4 (IRAK4) and angiotensin II (Ang II) (Chen and Stinnett 2008).

In human umbilical vein endothelial cells, the generation of hydrogen peroxide (H_2O_2) increased the DNA-binding of activator protein-1 (AP-1) and nuclear factor kappa beta (NF-κB) and this was associated with enhanced expression of monocyte chemoattractant protein - 1 (MCP-1) (Bouloumie *et al.* 1999). Some clinical studies also suggest that leptin may contribute to endothelial damage. For example, leptin was positively correlated with the expression of soluble thrombodulin and VCAM-1, two markers of endothelial damage in obese women independent of body mass index (BMI), waist to hip ratio, C-reactive protein (CRP) levels and insulin sensitivity (Porreca *et al.* 2004).The effect of leptin on endothelial function remains controversial with somewhat conflicting results being published. Leptin exhibits biphasic effects such that acute exposure promotes positive effects on the endothelium and prolonged exposure can induce the more detrimental effects that have been discussed (Northcott *et al.* 2012).

Macrophages

There is little research available on the effect of leptin on macrophages as it relates to atherosclerosis. Leptin has been shown to promote the proliferation and activation of circulating monocytes (Santos-Alvarez *et al.* 1999) as well as stimulate the secretion of macrophage-derived lipoprotein lipase (LPL) (Maingrette and Renier 2003). Macrophages are the primary source of LPL within the vessel wall and macrophage – secreted LPL activity has been shown to be pro-atherogenic in nature. LPL found on the vessel wall mediates the lipolysis of

circulating very low-density lipoprotein (VLDL) and chylomicron particles. These remnants become smaller and richer in cholesterol ester content allowing them to be taken up more readily by macrophages (Mead *et al.* 1999).

Vascular Smooth Muscle Cells

Leptin's effects on VSMCs have been well documented. Leptin has been shown to promote the proliferation and migration of VSMCs isolated from human, rat and mouse vessels (Li *et al.* 2005a;Oda *et al.* 2001;Schafer *et al.* 2004) and to mediate VSMCs remodeling by increasing VSMC hypertrophy (Shin *et al.* 2005;Zeidan *et al.* 2005). Finally, leptin stimulates osteoblast differentiation and calcification of VSMC thus affecting plaque and vessel integrity (Parhami *et al.* 2001;Zeadin *et al.* 2009).

Leptin and Atherosclerosis in Clinical Studies

Clinical trials have demonstrated that an increase in leptin levels is a risk factor for CVD. Some of the most convincing data emerged from the five year prospective West of Scotland Coronary Prevention Study (WOSCOPS) which was initially designed to examine the effectiveness of pravastatin in the prevention of ischemic heart disease. This study, however, showed that leptin is a significant risk factor for the development of acute cardiovascular events including acute myocardial infarction, need for revascularization and mortality, even after adjustment for BMI, plasma lipid, glucose and CRP levels (Wallace *et al.* 2001). Several years later, a separate study demonstrated that high baseline

plamsa leptin levels in 382 non-diabetic patients with coronary artery stenosis was associated with an increased prevalence of cardiac death, risk for myocardial infarction, and need for coronary revascularization (Wolk *et al.* 2004).

In addition, clinical trials have shown a positive correlation between circulating levels of plasma leptin and coronary artery calcification (CAC). In a cross-sectional study, a positive correlation between plasma leptin levels and CAC was found in 200 individuals with type 2 diabetes, even after standardizing for other traditional risk factors such as BMI, waist circumference, and CRP levels (Reilly *et al.* 2004). Similar findings were reported in a separate study that examined CAC in 860 asymptomatic, non-diabetic individuals (Qasim *et al.* 2008). This study evaluated the association between several plasma cytokines (i.e. leptin, interleukin-6 (IL-6), and soluble tumor necrosis factor receptor (TNFR) - 2, and adiponectin) and CAC, but found that only leptin was a significant independent predictor. Coronary calcification also occurs more frequently in overweight or obese individuals than it does in those with a healthy BMI (Hsu *et al.* 2007;Lee *et al.* 2007). Although CAC is often used as a predictor of atherosclerosis, it is becoming more evident that calcified deposits can influence the clinical outcomes of CVD, in part, by affecting atherosclerotic lesion stability (Detrano *et al.* 1997;Ehara *et al.* 2004;Fujii *et al.* 2005).

1.3 Mesenchymal Stem Cell Differentiation

Mature osteoblasts arise from a mesenchymal stem cell (MSC) population located within the bone marrow space (Bianco *et al.* 1999;Kuznetsov *et al.* 1997). The commitment of MSCs to tissue-specific cell types is regulated by the expression of appropriate transcription factors that first allow for the differentiation of MSCs into osteoprogenitor cells, then into preosteoblasts and finally into mature and functional osteoblasts which promote matrix mineralization. Various factors affect this process including fibroblast growth factors (FGFs) and their receptors, parathyroid hormone (PTH) and PTHrP (PTH-related protein), bone morphogenetic proteins (BMPs)/ transforming growth factor-β (TGF-β), as well as the Wnt/β-catenin pathway.

Regulation of Osteoblast Differentiation by Bone Morphogenetic Proteins

BMPs belong to the TGF-β superfamily of polypeptides and are potent inducers of osteoblast differentiation. BMPs 2/4/6 are secreted by osteoblasts in an autocrine manner to induce mesenchymal progenitor cells to enter the osteoblastic lineage. The BMP receptor is a heteordimer of two serine/threonine kinases type I and II. BMP type II receptor induces the phosphorylation and activation of BMP type I receptor. This receptor complex mediates the phosphorylation of the signal-transducing molecules called Smads 1/5/8. Smads 1/5/8 complex with Smad 4 and together translocate to the nucleus to induce gene expression of osteoblast-specific transcription factors (Ebara and

Nakayama 2002;Hoffmann and Gross 2001). In addition to the effects of BMPs/Smads, other signaling pathways such as Wnt/β-catenin have been established as essential for the process of osteogenic differentiation to occur (see Section 1.5).

Osteoblast-Specific Transcription Factors

A brief discussion of the role of osteoblast-specific transcription factors is necessary, as they play a central role in the process of vascular calcification. In order to identify the transcription factors that regulate osteoblast differentiation, investigators examined the regulatory regions of osteocalcin, as it is expressed exclusively by osteoblasts (and megakaryocytes) (Thiede *et al.* 1994;Yamaguchi *et al.* 2000). Three runt-domain protein recognition sites were identified (Merriman *et al.* 1995) in addition to an osteoblast-specific cis-acting element designated osteoblast-specific element 2 (OSE2) which included conserved binding sequences for runt-domain protein (Ducy and Karsenty 1995;Geoffroy *et al.* 1995). This suggested to investigators that core-binding factor subunit alpha-1 (Cbfa1), also known as runt-related transcription factor 2 (Runx2), is one of the transcription factors which can bind to and induce the expression of osteocalcin. In addition to its effects on osteocalcin, Runx2 is a positive regulator that induces the expression of other bone matrix proteins including type I collagen α1 (Col1a1), type I collagen α2 (Col1a2), osteopontin (OPN), bone sialoprotein, fibronectin and osteoprotegrin (Komori 2005;Yamaguchi *et al.* 2000). However,

for complete osteogenic differentiation, cells also require the activation of osterix (Osx). Osx is a zinc finger-containing transcription factor. Runx2 and/or Osx -deficient mice lack functional osteoblasts and do not exhibit a mineralized cartilaginous skeleton (Komori *et al.* 1997;Nakashima *et al.* 2002). Osx-deficient mice still express Runx2, but Runx2-deficient mice do not express Osx suggesting that Osx acts downstream of Runx2. Osx expression can also be regulated by the homeobox transcription factor, msh homeobox 2 (Msx2) (Cheng *et al.* 2003;Matsubara *et al.* 2008). Msx2 promotes the osteogenic differentiation of MSCs in a Runx-2-independent manner with maximal expression in the mesenchymal osteoprogenitor population (Sumoy *et al.* 1995). Several osteoblast-specific proteins are expressed temporally as cells undergo differentiation. For example, alkaline phosphatase (ALP) is typically used as a marker for osteoblast differentiation because it is highly expressed until mineralization is well underway. The mineralization stage coincides instead with osteocalcin expression. Bone sialoprotein expression coincides with the presence of differentiated osteoblasts while OPN expression is more complex. Osteopontin expression peaks twice; once during the proliferative stage and then again later during mineralization (Lian *et al.* 2003).

MSCs also have the capacity to differentiate into chondrocytes or adipocytes depending upon the transcription factor(s) that is expressed. Adipocyte differentiation requires the expression of peroxisome proliferator-activated receptor γ2 (PPARγ2) while chondrocytes require the expression of

SOX9 followed by Runx2. In addition, pluripotent progenitor cells have the capacity to transdifferentiate. For example, an increase in the expression of PPARγ2 over Runx2 will result in the transdifferentiation of osteoblasts into adipocytes (Jeon *et al.* 2003;Nuttall *et al.* 1998;Skillington *et al.* 2002).

1.4 Vascular Calcification

Types of Vascular Calcification

Under certain conditions, blood vessels become prone to calcium deposits consisting of bone mineral apatite. Vascular calcification was once thought to be a passive event in which calcium was deposited non-specifically along the blood vessels, myocardium and cardiac valves. Currently, it is considered to be a tightly-regulated, cell-mediated process that mimics bone formation. Four types of vascular calcification have been identified; **1)** ***Atherosclerotic calcification*** is localized to the intimal layer of the vessel wall. It is characterized by distinct punctuate deposits of the atherosclerotic lesions. **2)** ***Medial artery calcification*** (or Mönckeberg's sclerosis) is frequently evident in diabetics and/or patients with chronic kidney disease (CKD). It is characterized by linear deposits along the elastic lamina. Intimal calcification can occur independently of medial calcification and *vice versa.* **3)** ***Cardiac valve calcification*** is a consequence of mechanical stress and valve inflammation. Valves also calcify in a process similar to osteogenesis. **4)** ***Calciphylaxis*** is a systematic process that involves the

calcification of small - to medium - sized arteries and arterioles. This process appears to be independent of osteogenesis (Johnson *et al.* 2006).

Clinical Consequences of Vascular Calcification

The clinical consequences of vascular calcification are well documented. The calcification of coronary arteries contributes to an increased risk of plaque rupture (plaque instability) and as such the risk of myocardial infarction/stroke (Bobryshev *et al.* 2008;Burke *et al.* 2000;Detrano *et al.* 1997;Ehara *et al.* 2004;Fujii *et al.* 2005;Li *et al.* 2007;Vengrenyuk *et al.* 2006;Vliegenthart *et al.* 2002) as well as an increased risk of vessel wall dissection during balloon angioplasty/stent placement (Fischman *et al.* 1994;Fitzgerald *et al.* 1992;Haude *et al.* 1991;Virmani *et al.* 1994). Medial artery calcification results in increased vascular stiffness and pulse pressure which leads to impaired arterial distensibility and compromised diastolic coronary perfusion (Blacher *et al.* 1998;Lian *et al.* 2003;Madhavan *et al.* 1994). Finally, the calcification of cardiac valves can lead to valvular aortic stenosis resulting in the loss of valve mobility, a major mechanism for valve failure (Johnson *et al.* 2006;Lindroos *et al.* 1993;Stewart *et al.* 1997). Vascular calcification may arise by several different, non-mutually exclusive mechanisms.

Theories Regulating the Development of Vascular Calcification

Loss of Mineralization Inhibitors

Several theories have been advanced to explain the mechanism(s) contributing to the development and progression of vascular calcification. The first is that vascular calcification arises in the absence of mineralization inhibitors such as inorganic pyrophosphate, matrix gla protein (MGP) and osteopontin (OPN) (Johnson *et al.* 2006). Inorganic pyrophosphate (PPi) is a potent inhibitor of vascular calcification. Pyrophosphate is generated by ectonucleotide pyrophosphate/ phosphodiesterase I (ENPPI) and then transported across the cell membrane to the extracellular matrix by the protein ankyrin (Johnson *et al.* 2006). A deficiency in ENPPI results in the development of infantile 'idiopathic arterial calcification' characterized by widespread arterial calcification (Johnson *et al.* 2005;Rutsch *et al.* 2003). PPi inhibits vascular calcification by preventing hydroxyapatite formation and calcium deposition within tissues (Fleisch *et al.* 1966). It has also been shown to prevent VSMCs from differentiating into osteoblast-like cells (Johnson *et al.* 2006).

Gene-knockout experiments have identified MGP as an important regulator of vascular calcification. MGP-deficient ($MGP^{-/-}$) mice develop extensive vascular calcification and die soon after birth (Luo *et al.* 1997). This vitamin-K dependent protein is hypothesized to function by chelating calcium ions

in vivo (Bostrom 2001). In addition, MGP can inhibit the signaling of BMP-2 (Bostrom *et al.* 2001;Zebboudj *et al.* 2002).

Another important regulator of calcification is OPN. OPN has been localized to sites of atherosclerotic plaques and valvular lesions and is expressed by a variety of cell types including preosteoblasts, osteoblasts, osteocytes, smooth muscle cells and macrophages (Giachelli *et al.* 1993;Mody *et al.* 2003;O'Brien *et al.* 1994;O'Brien *et al.* 1995;Speer *et al.* 2005). OPN is believed to regulate mineralization by inhibiting apatite crystal growth and promoting osteoclast activity through its interaction with the $\alpha_v\beta_3$ integrin (Giachelli *et al.* 2005;Speer *et al.* 2005;Steitz *et al.* 2002). In support of these *in vitro* findings, $MGP^{-/}OPN^{-/-}$ mice showed a more rapid onset of calcification when compared with $MGP^{-/}OPN^{+/+}$ mice (Speer *et al.* 2002).

Nucleation Complexes

Alternatively, vascular calcification has been linked to the process of bone remodeling. This theory hypothesizes that sites of enhanced osteoclastic bone resorption generate crystal nuclei that are released into the circulation and eventually become lodged in soft tissue (vasculature) thereby inducing mineralization (Speer and Giachelli 2004). For example, calcium phosphate mineral has been detected in the bloodstream of rats (Price *et al.* 2002b). The release of these complexes was attenuated when inhibitors of bone resorption (calcitonin, osteoprotegrin and alendronate) were administered (Price *et al.*

2002a). This theory has been used to explain the link between vascular calcification and osteoporosis in postmenopausal women (Price *et al.* 2001a;Price *et al.* 2001b).

Vascular Smooth Muscle Cell Apoptosis

One of the key hallmark features of atherosclerosis is VSMC apoptosis. *In vitro*, inhibition of apoptosis with caspase inhibitor ZVAD.fmk was shown to inhibit calcification by ~40%. Conversely, stimulation of apoptosis in nodular cultures increased calcification 10-fold. VSMC-derived apoptotic bodies may act as platforms to concentrate calcium and phosphate thereby providing a suitable niche for nucleation (Proudfoot *et al.* 2000). Interestingly, in studies of human arteries, apoptosis was localized to areas of calcification in regions with Mönckeberg's sclerosis and atherosclerosis. Osteoprotegrin and TNF-related apoptosis-inducing ligand (TRAIL) were colocalized to these areas of apoptosis (Schoppet *et al.* 2004). Osteoprotegrin, produced by endothelial cells and VSMCs, can bind the pro-apoptotic cytokine, TRAIL. Its expression may be upregulated as a mechanism to limit apoptosis, and thus calcification, *in vivo*.

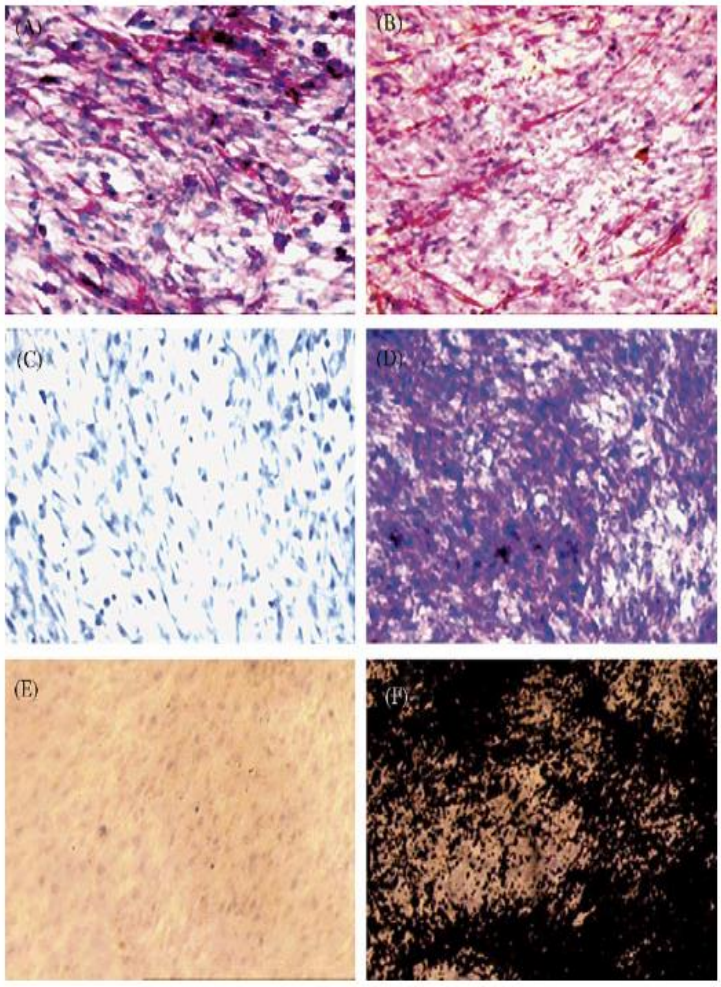
Differentiation of Vascular Smooth Muscle Cells

The predominant school of thought is that a subpopulation of VSMCs can undergo a phenotypic change and behave much like the bone-forming cells known as osteoblasts. The expression of smooth muscle α-actin (αSM-actin), smooth muscle 22 alpha (SM22α), and smooth muscle – myosin heavy chain

(SM-MHC) allow VSMCs to maintain vascular tone in the medial layer of the arterial wall. Under pathological conditions, however, VSMCs can be induced to undergo osteogenic differentiation. For example, we and others have shown that primary cultures of bovine aortic smooth muscle cells (BASMCs) can differentiate into osteoblast-like cells when cultured *in vitro* (**Figure 1.2**) (Bear *et al.* 2008;Steitz *et al.* 2001;Yang *et al.* 2005). These “osteoblast-like” cells are characterized by an increase in the expression of osteogenic-specific markers, such as runt-related transcription factor 2 (Cbfa1/Runx2) (Iyemere *et al.* 2006).

Figure 1.2 - The effects of β-glycerophosphate (βGP) on primary cultures of BASMCs.

BASMCs cultured in the absence **(Panel A, C & E)** or presence **(Panel B, D &F)** of βGP were examined for osteoblast differentiation and mineralization. **Panels A and B:** the cells were stained for smooth muscle cell α-actin. **Panels C and D:** the cells were stained for the marker of osteoblast differentiation: alkaline phosphatase. **Panels E and F:** the cells were stained for evidence of calcium deposition (von Kossa). (Yang *et al.* 2005).



Reprinted with permission from Yang, L., Butcher, M., Simon, R. R., Osip, S. L., and Shaughnessy, S. G. (2005). The effect of heparin on osteoblast differentiation and activity in primary cultures of bovine aortic smooth muscle cells. *Atherosclerosis* **179**, 79-86. (License # 3438761001671).

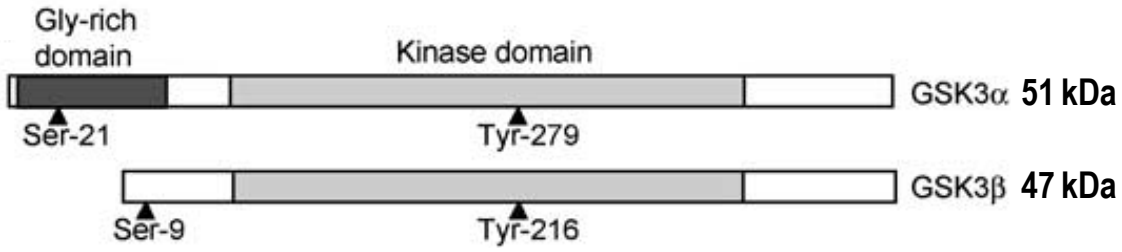
1.5 GSK-3

Composition and Structure

Glycogen synthase kinase - 3 (GSK-3) is a multifunctional serine/threonine kinase that was initially identified as the enzyme that phosphorylates and inactivates glycogen synthase in glucose metabolism (Embi *et al.* 1980;Rylatt *et al.* 1980). GSK-3 is now known to be involved in a variety of cellular processes including signal transduction, protein synthesis, cell migration, mitosis, apoptosis and cell fate determination. Dysregulated GSK-3 activity is implicated in a number of human diseases such as diabetes, Alzheimer's and cancer. GSK-3 is encoded by two genes, *gsk-3α* and *gsk-3β. gsk-3α is* located on mouse chromosome 7 and human chromosome 9; *gsk-3β* is located on mouse chromosome 16 and human chromosome 3 (Kaidanovich-Beilin and Woodgett 2011). GSK-3α (51 kDa) and GSK-3β (47 kDa) share 98% homology within the kinase domain, but GSK-3α has an additional glycine-rich N-terminal domain and the two gene products share only 36% homology within the last 76 C-terminal residues (Woodgett 1990) (**Figure 1.3**). GSK-3 homologues are expressed in most eukaryotes and retain a high degree of homology between different species (reviewed by (Ali *et al.* 2001)). Both GSK-3α and GSK-3β appear to be expressed ubiquitously, and more recent studies suggest that GSK-3α and GSK-3β may function redundantly in the Wnt/β-catenin signaling pathway while performing more distinct functions in other signaling pathways (Doble *et al.* 2007).

Figure 1.3 - Composition of GSK-3α and GSK-3β.

GSK-3α and GSK-3β share a high degree of homology in their kinase domains but differ at their N- and C- terminal regions. GSK-3α has an additional glycine-rich N-terminal domain and the two gene products share only 36% homology within the last 76 C-terminal residues. Phosphorylation on a Tyr residue within their kinase domain is required for GSK-3 activity, whereas, phosphorylation of a conserved Ser residue inhibits GSK-3 activity. (Sugden *et al.* 2008)



Reprinted with permission from Sugden, P.H., Fuller, S.J., Weiss, S.C. and Clerk, A. (2008). Glycogen synthase kinase 3 (GSK3) in the heart: a point of integration in hypertrophic signaling and a therapeutic target? A critical analysis. *British Journal of Pharmacology* **153**, S137-S153. (License # 3438390343191).

Canonical Wnt/β-catenin Pathway

β-catenin can be found tightly associated with cadherins at cell-cell junctions or “free” within the cytosol. The pool of β-catenin found in the cytosol/nucleus is responsible for the regulation of gene transcription that is observed as a result of Wnt signaling. β-catenin exists in what is referred to as the “destruction complex” which includes the central scaffold protein Axin in addition to the core component, adenomatous polyposis coli (APC), casein kinase 1 (CK1) and GSK-3. CK1 phosphorylates β-catenin at Ser45 priming this substrate for subsequent phosphorylation at Thr41, Ser37 and Ser33 by GSK-3. The hyperphosphorylated form of β-catenin is subject to N-terminal ubiquitination by β-transducin repeat-containing protein (β-TrCP) E3 ligase complex followed by degradation via the 26S proteasome (Ha *et al.* 2004;Kimelman and Xu 2006;Wu and Pan 2010). It is well established that Wnt signaling inhibits GSK-3 - mediated phosphorylation of β-catenin but the exact mechanism by which canonical Wnts regulate GSK-3 activity is not well understood.

Wnt ligands are a group of highly conserved, cysteine-rich glycoproteins with at least 19 different isoforms identified in mammals. Wnt ligands (e.g. Wnt 1, Wnt 3a, Wnt 8, Wnt 10b) bind the cell-surface Frizzled (Fz) receptors that in turn complex with co-receptors LDLR - related protein 5/6 (LRP 5/6) and cytoplasmic protein, Disheveled (Dvl). The mechanism by which this receptor pair and Dvl initiate signaling is not completely understood. It has been proposed that Wnt proteins bind to the extracellular domain of both LRP 5/6 and Fz receptors that

interact with Dvl via the intracellular portion of Fz (Cong *et al.* 2004). Dvl then recruits Axin to the cell membrane inducing its conformational change and inhibiting GSK-3 – dependent phosphorylation of Axin (Yamamoto *et al.* 1999). The culminating event of Wnt protein binding, irrespective of the exact signaling cascade, is that β-catenin remains unphosphorylated and evades degradation. β-catenin accumulates instead in the cytoplasm and excess β-catenin translocates to the nucleus, and heterodimerizes with members of the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors to induce gene expression (Wu and Pan 2010;Yokoyama *et al.* 2007;Zeng *et al.* 2008).

Several additional studies have attempted to identify other key events which lead to the stabilization of β-catenin. The phosphorylation of the Ser/Thr residue in the PPP(S/T)P motif, which is reiterated five times on the cytoplasmic domain of LRP5 and LRP6, has been shown to play a critical role in mediating Wnt signaling (He *et al.* 2004;Tamai *et al.* 2004). For example, it has been demonstrated that the Wnt-receptor complex regulates β-catenin phosphorylation by promoting the sequential phosphorylation of the Ser/Thr residue in the PPP(S/T)P motif on LRP6 by GSK-3 and then CK1. The dual-kinase phosphorylation underlies at least LRP6 activation by promoting Axin recruitment. This same study demonstrated that there may in fact be different pools of GSK-3 where the membrane-bound pool of GSK-3 antagonizes β-catenin phosphorylation and degradation by cytosolic GSK-3 in the presence of Wnt-ligand binding. This was supported by the finding that there exist significant

amounts of membrane-associated GSK-3 and CK1 which do not change in either the absence or presence of Wnt signaling (Zeng *et al.* 2005). This finding is in contrast to the classic dogma that GSK-3 is solely an inhibitor of Wnt signaling by means of its role in β-catenin phosphorylation and degradation.

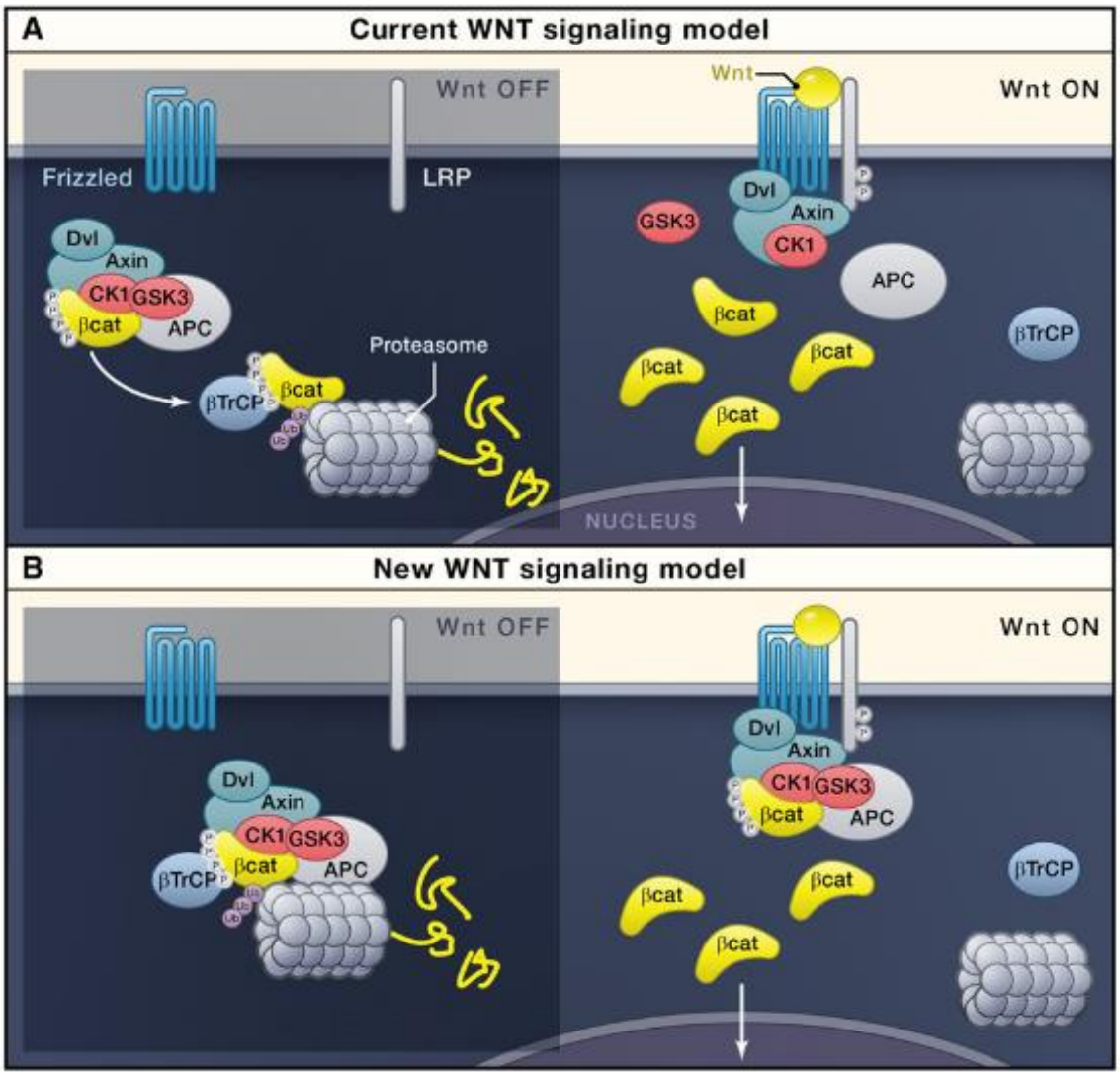
More recently, Li *et al.* (2012) demonstrated that Wnt induces the association of the destruction complex (APC/Axin/CK1/GSK-3) with phosphorylated LRP6 at the cell membrane in primary intestinal epithelium and colorectal cancer cells. In this model, the destruction complex remains compositionally intact and can therefore still bind and phosphorylate β-catenin but the association of Axin to LRP6 results in the dissociation of β-TrCP such that ubiquitination of β-catenin cannot occur (**Figure 1.4**). Ultimately, any newly synthesized β-catenin accumulates in the cytoplasm and translocates to the nucleus to induce gene expression (Li *et al.* 2012).

The canonical Wnt signaling pathway has been established as a key regulator in various aspects of cell biology including bone formation. The most striking evidence that supports a role for Wnt signaling in bone formation were the observations made from loss- or gain-of-function mutations in LRP5. Loss-of-function mutations in LRP5 result in the syndrome, osteoporosis pseudo-glioma (OPPG), characterized by extremely low bone mass (Gong *et al.* 2001); whereas gain-of-function mutations in the same receptor results in a high bone mass phenotype (Boyden *et al.* 2002). A loss-of-function mutation in the co-receptor LRP6 has been linked to a hereditary disorder characterized not only by

osteoporosis, but coronary artery disease (CAD) and metabolic syndrome (based on the presence of diabetes, hypertension and high triglycerides) (Mani *et al.* 2007). Moreover, deletion mutations of sclerostin (Sost), an endogenous inhibitor of the Wnt pathway, lead to osteosclerotic phenotypes characterized by generalized skeletal overgrowth, more pronounced in the skull and mandible (Balemans *et al.* 2001). Finally, Wnt10b signaling has been shown to control osteoblast commitment by inhibiting the expression of adipogenic transcription factors CCAAT/enhancer binding protein-α (C/EBPα) and PPARγ and upregulating the expression of osteoblast-specific transcription factors Runx2, distal-less homeobox 5 (Dlx5) and Osx (Bennett *et al.* 2005).

Figure 1.4 - Schematic representation of the canonical Wnt signaling pathway.

Wnt ligands are a group of secreted glycoproteins that bind to Fz and LRP 5/6 receptors triggering a cascade which results in the accumulation and translocation of β-catenin to the nucleus to regulate gene expression. **Panel A:** In the absence of Wnt ligand binding, β-catenin is hyperphosphorylated and targeted for proteasomal degradation by the APC/Axin/CK1/GSK-3 “destruction” complex. Hyperphosphorylated β-catenin is subject to ubiquitination by β-TrCP and targeted for proteasomal degradation. In the nucleus, TCF molecules are bound by co-repressors that help to shut off expression of Wnt target genes. Wnt ligand binding induces the association of Axin with phosphorylated LRP 5/6 such that parts of the destruction complex dissociate. β-catenin accumulates in the cytoplasm and translocates to the nucleus to induce gene expression. **Panel B:** In an alternative model, the destruction complex resides in the cytoplasm, where it binds, phosphorylates, and promotes the ubiquitination of β-catenin by β-TrCP. However, in this model, Wnt ligand binding induces the association of the completely intact destruction complex with phosphorylated LRP 5/6. The destruction complex still binds and phosphorylates β-catenin but ubiquitination of β-catenin is blocked. Any newly synthesized β-catenin then accumulates in the cytoplasm and translocates to the nucleus to induce gene expression. (Clevers and Nusse 2012;Li *et al.* 2012)



Reprinted from Open Access source. Clevers, H., and Nusse, R. (2012). Wnt/β-catenin signaling and disease. *Cell* **149**, 1192-1205.

GSK-3 Regulation

Unlike most signaling kinases, GSK-3 is active under resting/unstimulated conditions and is inactivated by extracellular signals. GSK-3 regulation appears to require a delicate balance that is achieved by a combination of phosphorylation, localization and sequestration by GSK-3 binding proteins.

GSK-3 Regulation Through Phosphorylation

GSK-3α and GSK-3β activity can be regulated through phosphorylation of its N-terminal serine Ser21/9 residue, respectively (Desbois-Mouthon *et al.* 2001;Ding *et al.* 2005;Holnthoner *et al.* 2002). The crystal structure of GSK-3β reveals a positively charged pocket comprised of Arg96, Arg180 and Lys205. Substrates of GSK-3β bind to this positively charged pocket which serves two functions: (1) to optimize the orientation of a substrate's kinase domain, and (2) to orient the substrate at the correct position within the catalytic groove for phosphorylation by GSK-3β to occur. However, when GSK-3β is phosphorylated at Ser9, it creates a "primed pseudosubstrate" which competitively binds intramolecularly at the positively charged pocket and thus occupies its own active site. This prevents true substrates such as β-catenin from binding and becoming phosphorylated (Doble and Woodgett 2003;ter Haar *et al.* 2001). There are additional phosphorylation sites on GSK-3. Crystal structure studies have also revealed that when GSK-3α and GSK-3β are phosphorylated at Tyr276 and Tyr216, respectively, the kinase undergoes a conformational change which

provides access for substrates to bind the enzyme (Hughes *et al.* 1993;Kaidanovich-Beilin and Woodgett 2011). To summarize, phosphorylation on a tyrosine residue within the kinase domain of GSK-3 is required for GSK-3 activity, whereas, phosphorylation of a conserved serine residue inhibits GSK-3 activity.

In mammals, newly synthesized GSK-3β appears to autophosphorylate its tyrosine-216 residue (Lochhead *et al.* 2006)**.** However, extracellular signals can induce a rapid and reversible increase in serine-9 phosphorylation of GSK-3β to decrease its enzymatic activity. Some of the kinases shown to phosphorylate GSK-3β include p70S6 kinase (Armstrong *et al.* 2001;Krause *et al.* 2002); p90RSK (Ding *et al.* 2005;Eldar-Finkelman *et al.* 1995;Saito *et al.* 1994); Akt (Cross *et al.* 1995;Shaw *et al.* 1997;van Weeren *et al.* 1998); specific isoforms of protein kinase C (PKC) (Ballou *et al.* 2001;Fang *et al.* 2002); protein kinase A (PKA) (Fang *et al.* 2000;Li *et al.* 2000).

Ding *et al*. examined the mechanism by which β-catenin is upregulated in human cancers (Ding *et al.* 2005). They demonstrated that ERK 1/2 can phosphorylate GSK-3β on Thr43 by associating with GSK-3β through a docking motif (^{291}FKFP). This event primes GSK-3β for subsequent phosphorylation by p90RSK at Ser9 which results in the inactivation of GSK-3β and the subsequent accumulation of β-catenin. It is important to note that, in addition to β-catenin, GSK-3β can phosphorylate a wide range of substrates including metabolic and signaling proteins (i.e. eIF2B, glycogen synthase, pyruvate dehydrogenase,

amyloid precursor protein), structural proteins (i.e. tau, dynamin-like proteins) and transcription factors (i.e NFAT, NFκB) (reviewed in (Grimes and Jope 2001). This suggests that the effect(s) of GSK-3β inactivation are diverse and very much dependent on the stimuli and cell type in which signaling occurs. Virtually all previous studies have focused on the regulation and function(s) of GSK-3β and have neglected the potential role of GSK-3α, but it is becoming increasingly clear that GSK-3α may also be involved in Wnt signaling (Doble *et al.* 2007).

GSK-3 Regulation Through Complex Formation

The canonical Wnt signaling pathway uses protein-protein interactions involving a number of distinct proteins (Fz, LRP 5/6, Dvl, Axin, APC, CK1, β-catenin) to regulate GSK-3 activity (see section: *Canonical Wnt/β-catenin Pathway* for complete details on how GSK-3 participates in this complex) (Kaidanovich-Beilin and Woodgett 2011;Wu and Pan 2010). A small fraction of cellular GSK-3 (<5-10%) exists as part of the destruction complex which phosphorylates β-catenin and targets it for ubiquitin-mediated proteasomal degradation thus maintaining β-catenin at low levels in the cytoplasm. However, Wnt ligands have been demonstrated to induce the recruitment of GSK-3/Axin to the LRP 5/6 receptor complex resulting in the dissolution of the destruction complex and accumulation of β-catenin (Kaidanovich-Beilin and Woodgett 2011). It has not yet been clearly demonstrated whether protein complexes are used to regulate GSK-3 activity in other signaling pathways.

GSK-3 Regulation Through Intracellular Localization

Although GSK-3β is traditionally considered a cytoplasmic protein, there is evidence for its subcellular localization in the nucleus and mitochondria. GSK-3β levels fluctuate in the nucleus based on intracellular cues. For example, nuclear GSK-3β levels increase during the S-phase to promote the phosphorylation and nuclear export of cyclin D1 by facilitating its association with nuclear exportin CRM1 (Alt *et al.* 2000;Diehl *et al.* 1998). Apoptotic stimuli can also cause a rapid increase in the nuclear levels of GSK-3β allowing GSK-3β to regulate gene expression by modulating the activity of various transcription factor substrates (Bijur and Jope 2001). The nuclear export of GSK-3β is in part mediated by FRAT-1 (Frequently rearranged in advanced T-cell lymphomas 1) (Franca-Koh *et al.* 2002), but until a recent study published by Meares and Jope (2007), the mechanism for GSK-3 nuclear import was unclear. GSK-3β is now known to contain a bipartite nuclear localization sequence (NLS) between amino acids 85-103 rich in lysines and arginines which are sufficient for nuclear localization. GSK-3β cytosolic protein interactions likely mask the NLS to limit the capability of GSK-3β to transverse the nuclear membrane (Meares and Jope 2007). How does a kinase that is intermediary to so many signaling pathways exhibit cell - and tissue - specific effects? As Kaidanovich-Beilin and Woodgett (2011) stated: “The elegant cellular solution to this is to fractionate GSK-3 between scaffolding proteins or other structures such that each system has its own population of GSK-3 molecules assigned to it.

The mechanisms discussed above provide the means for a delicate balance in GSK-3 activity. Emerging evidence links the dysregulation of these precise control mechanisms to the development and progression of multiple disease states including: cancer, Alzheimer disease, bipolar disorder, cardiac myopathy, and insulin resistance. Various signaling pathways such as MAPK, PI3K/Akt have already been shown to intersect with GSK-3 to regulate its activity. There is now evidence of the ability of endoplasmic reticulum (ER) stress to signal through GSK-3 and ER stress-induced GSK-3 activity has been implicated in the pathogenesis of several diseases (Baltzis *et al.* 2007;McAlpine *et al.* 2012;Qu *et al.* 2004;Song *et al.* 2002).

1.6 Endoplasmic Reticulum Stress

The endoplasmic reticulum (ER) is the organelle that provides a specialized environment to facilitate the proper folding of secreted and transmembrane proteins. The ER is the site for calcium ($Ca^{2+)}$ storage which is essential for the proper function of a number of calcium-dependent chaperone proteins including glucose-regulated protein 78 kDa (Grp78/BiP), Grp94 and calreticulin. In addition, the oxidative environment of the ER allows its resident protein, protein disulphide isomerase (PDI), to facilitate the formation of disulphide bonds (Sundar *et al.* 2007). Various pathological conditions such as the expression of mutant proteins, insufficient ER chaperone protein levels, Ca^{2+} content or ATP status, changes in cellular reduction and oxidation (redox)

capacity, ER phospholipid depletion and cholesterol accumulation can result in an accumulation of misfolded or aggregated proteins (Cnop *et al.* 2012). This loss of ER homeostasis activates an adaptive response system called the Unfolded Protein Response (UPR) in an attempt to alleviate stress placed on the ER.

The Unfolded Protein Response

The UPR signal is mediated by three different membrane-bound proteins: inositol-requiring transmembrane kinase/endonuclease 1 (IRE1), RNA-dependent-protein kinase-like ER kinase (PERK), and activating transcription factor 6 (ATF6) (**Figure 1.5**). Under nonstressed conditions, the transducers of ER stress are kept inactive by their interaction with chaperone protein, Grp78. However, under conditions of ER stress, Grp78 dissociates from IRE1, PERK and ATF6 and binds luminal misfolded proteins to assist in the proper folding of proteins.

Upon dissociation from Grp78, IRE1 is activated through a homodimerization and trans-autophosphorylation mechanism. Once activated, IRE1 cleaves an intronic sequence from the mRNA of the XBP1 transcription factor creating a highly active spliced variant of XBP1 (sXbp1). sXbp1 upregulates the expression of ER chaperone proteins, components of the ER-associated degradation (ERAD) machinery and phospholipid biosynthesis which leads to the expansion of the ER membrane. ERAD is the cellular pathway which targets misfolded proteins of the ER for ubiquitination and subsequent

degradation by proteasomes. IRE1 also retains some non-specific RNAse-activity which allows for degradation of any mRNA that is localized to the ER membrane. Finally, IRE1 can induce pro-apoptotic signals by activating Jun N terminal kinase (JNK) (Cnop *et al.* 2012;Tabas 2010).

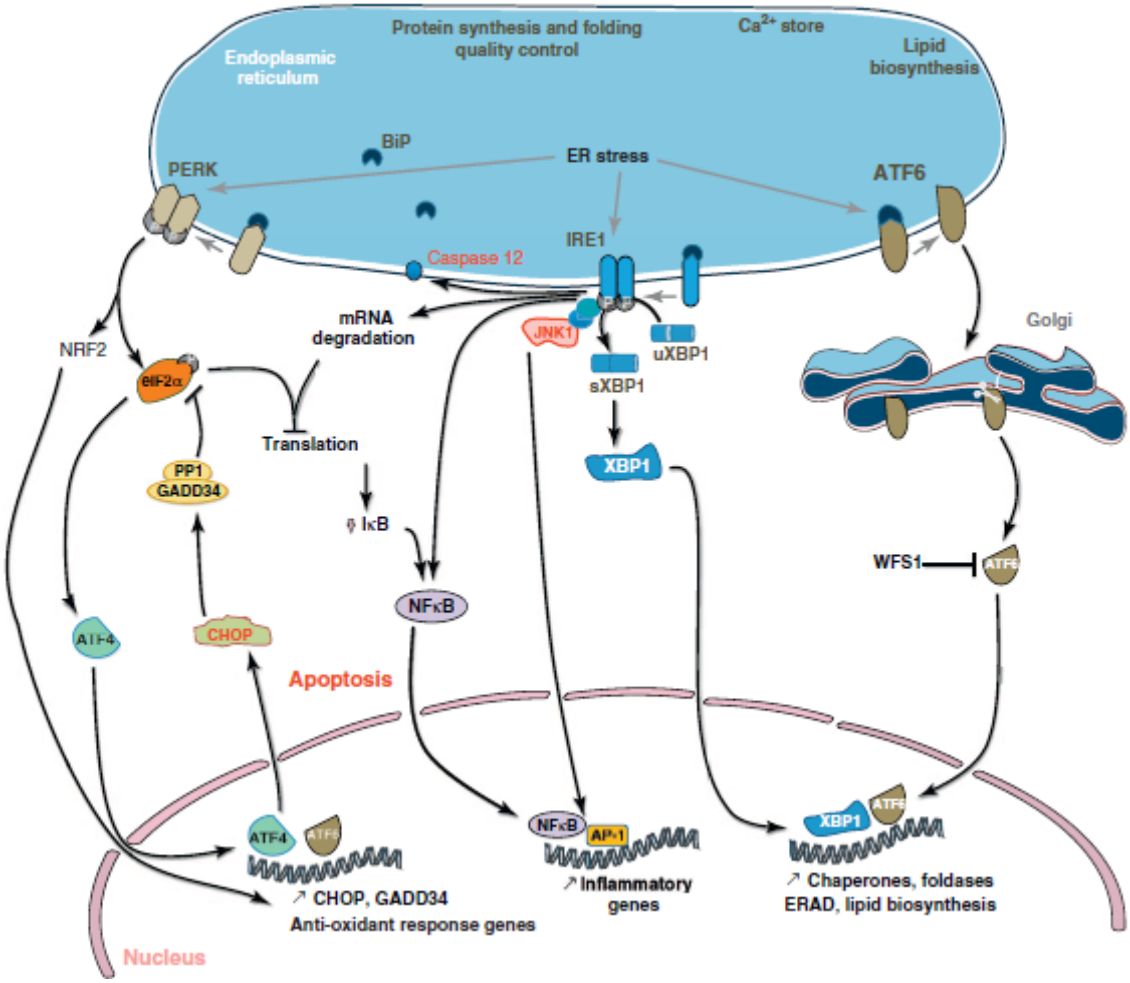
Upon activation, ATF6 translocates to the Golgi where it undergoes proteolytic cleavage by site 1 protease (S1P) and S2P. The cleaved cytoplasmic domain of ATF6 acts as a transcriptional activator of genes involved in ERAD, lipid biosynthesis, and proper protein folding (Cnop *et al.* 2012;Tabas 2010).

PERK signaling attenuates general protein translation by phosphorylation of the α-subunit of eukaryotic translation initiation factor 2 alpha (eIF2α). Phospho-eIF2α promotes the specific translation of ATF4, which induces the expression of ATF3, C/EBP homologous protein (CHOP/GADD153), tribbles homolog 3 (TRB3) and growth arrest and DNA damaged-inducible protein (GADD34) (Cnop *et al.* 2012;Tabas 2010).

UPR signaling can be downregulated via negative feedback signals including GADD34-mediated eIF2α dephosphorylation and Wolfram syndrome 1 (WSF1) - mediated ATF6 degradation. However, under conditions of chronic ER stress, when the UPR cannot restore the imbalance in the ER, the UPR will generate pro-apoptotic signals. These include the upregulation of CHOP by PERK, the activation of JNK and caspase-12 by IRE1 and the interaction of IRE1 with the Bcl-2 family of pro-apoptotic signals (Cnop *et al.* 2012).

Figure 1.5 - The unfolded protein response to endoplasmic reticulum stress.

ER stress causes the activation of membrane-bound proteins: PERK, IRE1 and ATF6 which are present in the ER membrane bound to Grp78/BiP. Increased binding of Grp78 to luminal misfolded proteins and its dissociation from the transducers leads to their activation. PERK phosphorylates and inhibits the activity of eIF2α - an essential factor in general protein translation. ATF4 translation is also facilitated inducing the expression of CHOP and GADD34. GADD34 targets protein phosphatase 1 (PP1) to eIF2α for dephosphorylation, relieving translational inhibition. IRE1 activation results in the splicing of XBP1 mRNA for translation of the transcription factor sXbp1. sXbp1 upregulates ER chaperone proteins, components of the ERAD machinery and phospholipid biosynthesis. IRE1 also degrades mRNAs localized to the ER membrane reducing newly synthesized protein import into the ER lumen. IRE1 activates JNK by recruiting pro-apoptotic factors, tumor necrosis factor receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase (ASK1) and caspase 12. The TRAF2/ASK1 complex can also activate nuclear factor kappa beta (NF-κB) which upregulates expression of inflammatory genes. Activated ATF6 translocates to the Golgi where it is processed by S1P and S2P. The cleaved-off cytoplasmic domain works in concert with sXbp1 to upregulate transcription. ATF6 activity can be inhibited by the WFS1 protein (Cnop *et al.* 2012).



Reprinted with permission from: Cnop, M., Foufelle, F., and Velloso, L. A. (2012). Endoplasmic reticulum stress, obesity and diabetes. *Trends Mol.Med.* **18**, 59-68 (License # 3438401179822).

ER Stress in Smooth Muscle Cells

Endothelial cells, macrophages and VSMCs all play an integral role in the development and progression of atherosclerosis. Less is known about the atherogenic role of ER stress in VSMCs. It has previously been reported that human aortic SMCs treated with 7-ketocholesterol (7-KC) produce ROS, develop signs of ER stress and undergo apoptosis. 7-KC induces apoptosis through the upregulation of the ROS-generating NAD(P)H oxidase subunit, Nox-4; and 7-KC-induced expression of ER stress markers Grp78 and CHOP was dependent on Nox-4 expression. The siRNA-mediated knockdown of Nox-4 in these cells prevented 7-KC-induced CHOP expression and subsequent apoptosis (Pedruzzi *et al.* 2004). In a separate study, SMC foam cell formation and SMC death was caused by loading cells with free cholesterol (FC). FC-overloading compromised both mitochondrial and ER integrity. While FC-overloading was associated with increased expression of the pro-apoptotic protein Bax, and the release of cytochrome c via the mitochondria, impairment of ER morphology was also associated with an increase in the expression of Grp78/94 and pro-apoptotic factor, CHOP (Kedi *et al.* 2009). Homocysteine is another known activator of ER stress and a risk factor for cardiovascular disease. Patients with severe hyperhomocysteinemia display a number of clinical abnormalities including atherosclerosis (Austin *et al.* 2004). In accordance with this, homocysteine-induced ER stress was shown to increase intracellular cholesterol in human aortic SMCs by activating the sterol regulatory element-binding proteins -1 and -2

(SREBP-1 and -2), a group of transcription factors that control lipid biosynthesis and uptake. This led the authors to suggest that SMC-lipid accumulation may promote the development and progression of atherosclerosis (Werstuck *et al.* 2001).

ER Stress in Atherosclerosis

There are several independent risk factors for cardiovascular disease, including hyperglycemia (Werstuck *et al.* 2006), hyperhomocysteinemia (Werstuck *et al.* 2001;Zhou *et al.* 2004), obesity (Ozcan *et al.* 2004), and elevated levels of unesterified cholesterol (Li *et al.* 2005b) or palmitate (Cunha *et al.* 2008), which can promote the activation of the UPR. Furthermore, ER stress can stimulate the key steps in the development and progression of atherosclerosis. These hallmark features include lipid accumulation, inflammation in part by activating the NF-κB transcription factor, and endothelial/macrophage/SMC/ foam cell apoptosis (Ross 1999).

The link between ER stress and pro-atherogenic response has been demonstrated in several experiments using mouse models of atherosclerosis. For example, ApoE-/- mice that were made hyperglycemic, hyperhomocysteinemic or fed a high-fat diet, experienced significantly increased atherosclerotic lesion size at the aortic root and along the entire aorta, and increased levels of necrosis within lesion areas. This was associated with elevated levels of ER stress as measured by the expression of Grp 78/94, PDI and CHOP (McAlpine *et al.* 2012).

Under normoglycemic conditions, 1-3% of total glucose will enter the hexosamine biosynthetic pathway (HBP) and be converted to glucosamine-6-phosphate. Under conditions of hyperglycemia, however, the flux through HBP increases and this has been associated with insulin resistance (Veerababu *et al.* 2000) and atherosclerosis. For example, ApoE$^{-/-}$ mice that were supplemented with 5% glucosamine demonstrated significantly increased vascular ER stress and atherosclerotic lesion size when compared to control mice (Beriault *et al.* 2011). These findings were consistent with a study that used LDLR$^{-/-}$ mice (Tannock *et al.* 2006).

Chemical Chaperones

In addition to its potential effects in the development and progression of atherosclerosis, ER stress has also been identified as an underlying cause in the pathogenesis of several disease states including neurodegenerative diseases (i.e Alzheimer, Parkinson, and Huntington), cancer, and diabetes. There are currently two chemical chaperones, 4-phenylbutyric acid (PBA) and tauroursodeoxycholic acid (TUDCA) that have been approved for use in humans (Engin and Hotamisligil 2010).

PBA is low-molecular weight fatty acid that is currently being used to treat urea-cycle disorders. TUDCA is a derivative of an endogenous bile acid that has previously been used as a heptaoprotective agent in humans with cholestatic liver disease. Both of these chemical chaperones have been employed to

examine effects on ER stress and atherosclerosis *in vitro* as well as in mouse models of atherosclerosis to relieve the load of misfolded proteins (Engin and Hotamisligil 2010). For example, *in vitro,* PBA was shown to relieve palmitate-induced ER stress and apoptosis in macrophages as determined by attenuated phosphorylation of PERK and eIF2α, sXBP-1 and CHOP expression and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining, respectively. More convincingly, this study also demonstrated that *in vivo,* PBA not only suppresses ER stress in macrophages and reduces apoptosis within the atherosclerotic lesions of $ApoE^{-/-}$ mice, but it also attenuates atherosclerosis by 32% when used at 100 mg/kg body weight. PBA's effect on atherosclerosis occurred in the absence of any impact on lipids, lipoprotein profiles, glucose and insulin levels and body weight (Erbay *et al.* 2009). In a study using *ob/ob* mice, PBA and TUDCA both normalized hyperglycemia, restored insulin sensitivity, and enhanced insulin signaling in the liver, muscle and adipose tissue by alleviating ER stress (Ozcan *et al.* 2006). Given that type 2 diabetes is a common feature of obesity and both pathologies are risk factors for cardiovascular disease, uncovering the molecular mechanism behind these disease states opens up the potential for more diverse therapeutic applications. However, several important points must be considered when implementing anti-ER stress therapy. First, aside from being able to downregulate excessive activation of the UPR, PBA exerts other biological effects such as the inhibition of histone deacetylase (HDAC) activity. Moreover, different cells (i.e. SMCs vs. macrophages vs.

endothelial cells) within the atherosclerotic lesion may utilize different branches of the ER-stress signaling pathways suggesting that therapeutic applications need to be more streamlined and specific. Finally, it is important to find a balance between excessive UPR activation and activation of the UPR as an adaptive mechanism (Engin and Hotamisligil 2010).

The Role of GSK-3 in ER Stress

The current literature strongly suggests an ER –stress mediated effect on the development and progression of atherosclerosis. A number of ER stress inducing agents including glucosamine, homocysteine, glucose and specific lipids have been shown to exacerbate lesion size in mouse models of atherosclerosis (Austin *et al.* 2004;Beriault *et al.* 2011;Khan *et al.* 2009;McAlpine *et al.* 2012;Werstuck *et al.* 2006). However, despite the abundance of current research, the exact molecular mechanism that links ER stress to disease development are yet unknown. Several studies have demonstrated that ER stress can signal through the serine/threonine kinase, GSK-3 (Baltzis *et al.* 2007;Qu *et al.* 2004;Song *et al.* 2002). Our lab has previously shown that GSK-3$\alpha^{-/-}$ and GSK-3$\beta^{-/-}$ mouse embryonic fibroblasts (MEFs) are protected from glucosamine-induced lipid accumulation when compared to wildtype MEFs. Similarly, wildtype MEFs were also protected from glucosamine-induced lipid accumulation if they were pretreated with valproate - an inhibitor of GSK-3 activity (Bowes *et al.* 2009). *In vivo,* our lab demonstrated that hyperglycemic, hyperhomocysteinemic,

and dyslipidemic ApoE-/- mice showed signs of ER stress, lipid accumulation, enhanced GSK-3 activity and increased atherosclerotic lesion size when compared with control ApoE-/- mice maintained on a standard chow diet (McAlpine *et al.* 2012). However, valproate supplementation attenuated both GSK-3β activity and atherosclerotic lesion size in these mice without having any effects on levels of ER stress (Bowes *et al.* 2009;McAlpine *et al.* 2012). When taken together, these finding suggest that GSK-3 acts a downstream target of ER stress and may influence the hallmark features of atherosclerosis. Prior to these studies, the potential effects of ER stress signalling through GSK-3 on vascular calcification had not been investigated.

CHAPTER 2: Hypothesis & Objectives

2.1 Hypothesis

We hypothesize that leptin promotes the osteogenic differentiation of VSMCs, and thus vascular calcification, by regulating the activity of GSK-3. We further speculate that in vascular disease, GSK-3 functions as a checkpoint for VSMCs at which point cells commit to: i) de-differentiation, thereby contributing to atherosclerosis, or ii) osteogenic differentiation, thereby contributing to vascular calcification.

2.2 Objectives

The specific objectives are as follows:

1. To determine whether leptin promotes vascular calcification in an ApoE$^{-/-}$ mouse model of atherosclerosis.
2. To investigate leptin's ability to mediate osteoblast differentiation and examine the role of GSK-3β in leptin-mediated osteoblast differentiation of VSMCs.
3. To examine the role of ER stress-mediated GSK-3 activation in the pathogenesis of vascular calcification versus atherosclerosis.

CHAPTER 3: The Effect of Leptin on Vascular Calcification in Apolipoprotein E-Deficient Mice.

Foreword

These studies investigate whether or not leptin can promote vascular calcification in an ApoE$^{-/-}$ mouse model of atherosclerosis. They demonstrate that leptin-treated mice develop extensive vascular calcification at the aortic root. This increase in calcification is associated with an increase in the expression of several osteoblast-specific markers. These studies suggest that leptin mediates the osteogenic differentiation of VSMCs. However, the mechanism by which this occurs is unknown.

This work was published in Arteriosclerosis, Thrombosis and Vascular Biology (Volume 29, pages 2069-2075, September 2009). The authors are the following: Melec Zeadin, Martin Butcher, Geoff Werstuck, Mohammad Khan, Colin K. Yee, Stephen G. Shaughnessy. The corresponding author is Dr. Stephen Shaughnessy. The experiments in this manuscript were performed by Melec Zeadin with assistance from Martin Butcher. The manuscript was written by Melec Zeadin in collaboration with Dr. Stephen Shaughnessy. The content of Chapter 3 is a representation of the above manuscript. The complete reference is as follows: Zeadin, M., Butcher, M., Werstuck, G., Khan, M., Yee, C. K., and Shaughnessy, S. G. (2009). Effect of leptin on vascular calcification in apolipoprotein E-deficient mice. *Arterioscler.Thromb.Vasc.Biol.* **29**, 2069-2075.

3.1 Abstract

Objective **-** The adipocytokine leptin has been proposed to increase cardiovascular risk in both obese and diabetic individuals. In the current study, therefore, we used apoE-deficient mice to examine the effects of leptin on both lesion size and calcification.

Methods and Results - Mice were treated with once daily intraperitoneal injections of leptin (125 µg/mouse/day) for 2 months. The mice were then euthanized, and sections of the aortic root and thoracic aorta analyzed histomorphometrically. Measurements of lesion size and surface area occupied by atherosclerotic lesions did not reveal any differences between non-treated and leptin-treated animals. However, von Kossa staining of the aortic root demonstrated an 8.3±2.0-fold increase in lesion calcification as well as a 2.5±0.6-fold increase in valvular calcification in those animals treated with leptin. In addition, the percent total lesion area demonstrating ALP-positive staining was 5.4±2.1-fold greater in leptin-treated mice when compared to non-treated control mice. This increase in ALP staining was also accompanied by an increase in the expression of the osteoblast-specific markers, osteocalcin, and OPN.

Conclusions - Based on these observations, we conclude that leptin may increase cardiovascular risk by promoting osteogenic differentiation and thus vascular calcification.

3.2 Introduction

Recent studies suggest that factors secreted by adipose tissue may be playing a role in promoting cardiovascular disease (CVD). One such group of factors, referred to as adipocytokines, is known to circulate at elevated levels in obese individuals and to positively correlate with an increased risk of CVD (Kougias *et al.* 2005;Lau *et al.* 2005). In particular, clinical studies have shown that high circulating levels of the adipocytokine leptin can accelerate the atherosclerotic process and is thus predictive of adverse cardiovascular events in both obese and diabetic populations (Reilly *et al.* 2004;Wallace *et al.* 2001). The possible proatherogenic effects of leptin include induction of endothelial cell dysfunction, stimulation of inflammatory processes, increased levels of oxidative stress, and increased migration and proliferation of vascular smooth muscle cells (Konstantinides *et al.* 2001;Loffreda *et al.* 1998;Oda *et al.* 2001;Yamagishi *et al.* 2001).

Leptin has been shown to promote neointimal thickening after chemical or mechanical injury (Schafer *et al.* 2004) and to promote lesion development in apoE-deficient mice (Bodary *et al.* 2005;Chiba *et al.* 2008). In addition, Parhami *et al* demonstrated that leptin can induce vascular smooth muscle cells to undergo a phenotypic transition into bone forming cells, which are capable of mineralization *in vitro* (Parhami *et al.* 2001). When taken together, these findings suggest that leptin may be acting to increase the risk of myocardial infarction by promoting not only lesion development but also vascular calcification *in vivo*.

The clinical consequences of vascular calcification are well documented. For example, arterial calcification is known to increase vessel wall rigidity (Sugioka *et al.* 2002), decrease diastolic coronary perfusion (Ohtsuka *et al.* 1994;Seely 1997), and increase the risk of myocardial infarction or stroke (Arad *et al.* 2000;Iribarren *et al.* 2000;Vliegenthart *et al.* 2002). Furthermore, calcified atherosclerotic plaques are known to be less stable and thus more likely to cause vessel wall dissection during balloon angioplasty or stent placement (Fischman *et al.* 1994;Fitzgerald *et al.* 1992;Haude *et al.* 1991;Virmani *et al.* 1994). Finally, the calcification of cardiac valves can lead to valvular aortic stenosis resulting in a loss of valve mobility (Lindroos *et al.* 1993;Stewart *et al.* 1997).

In the current study, we use an established animal model of atherosclerosis to examine the effect of leptin on vascular calcification. Herein, we report that elevated levels of leptin can promote vascular calcification *in vivo*. In addition, by measuring the expression of osteoblast-specific markers within the lesions of leptin-treated mice, we were able to demonstrate that increased vascular calcification is associated with an increase in osteoblast number.

3.3 Materials and Methods

3.3.1 Materials

Recombinant murine leptin was purchased from R&D Systems. Alkaline phosphatase kits were purchased from Sigma-Aldrich, whereas all antibodies

were obtained from Santa Cruz Biotechnology Inc. Finally, the DIG-RNA Labeling Kit, Blocking Reagent, anti-Digoxigenin-AP antibody, and NBT/BCIP Ready-to-Use tablets were purchased from Roche Diagnostics. All other reagents for in situ hybridization experiments were purchased from Sigma-Aldrich.

3.3.2 Experimental Protocol

Beginning at 8 weeks of age, female apoE-deficient mice were placed on a high-fat Western diet (Harlan Tekland; TD. 88137) and then randomized into 1 of 2 treatment groups. The first group (n=7 animals per group) received daily intraperitoneal (i.p.) injections of recombinant murine leptin at a concentration of 125 µg/mouse, whereas the second (n=7 animals) received an equivalent volume of saline (200 µL i.p./mouse) and acted as the vehicle control. On day 60, all animals were euthanized, and the heart, including the thoracic aorta, was removed for histological evaluation.

3.3.3 Tissue Sample Preparations and Histology

On day 60, all animals were fasted for 4 hours to obtain fasting blood glucose levels using a glucometer (Bayer). Whole blood was also collected into K_2EDTA-coated microtubes (VWR) via cardiac puncture of the right ventricle. Plasma lipid levels and insulin levels were determined using colorimetric diagnostic kits for total cholesterol, total triglycerides and plasma insulin (Crystal Chem Inc). After the animals were euthanized, the intact circulation was flushed with PBS, and the heart, including the thoracic aorta, was removed. The aortic

root was then separated from the aorta and embedded in Optimal Cutting Temperature (OCT) gel before storage at -80°C. Serial cross-sections beginning at the aortic valves were collected and measurements of lesion size were performed at the same point within the aortic root for each animal. Six serial sections per mouse were analyzed. In addition, Sudan IV staining of the thoracic aorta (n=5 per group) was used to assess the total surface area occupied by atherosclerosis.

3.3.4 Quantification of Vascular Calcification

Calcification of the aortic valves and atherosclerotic lesions of the aortic root was detected by von Kossa staining of 7 - µm cryosections as previously described (Phan *et al.* 2005;Phan *et al.* 2008). Briefly, the cryosections were placed in 5% silver nitrate solution for 30 minutes in the absence of light. The sections were then treated with film developer (Ilford Ilfosol S) for 5 minutes and finally fixed in 5% sodium-thiolsulphate. Calcification was visualized as distinct black deposits of calcium using an epifluorescent microscope (Zeiss Axioscope 2, Carl Zeiss) coupled to an IBM computer (Hewlett Packard). All images were captured using a 3CCD video camera module and analyzed using the Northern Eclipse imaging software system (Empix Imaging Inc). Measurements of calcified area were made by measuring calcification within the atherosclerotic lesions themselves and are expressed as a percentage of total lesion size. Similarly, measurements of valvular calcification were made by quantitating the amount of

calcification tracking along the valvular surface and are expressed as a percentage of valvular surface area. All measurements of calcification and either lesion size or valvular surface area were made on the same sections.

3.3.5 Alkaline Phosphatase Activity

Measurements of ALP activity were used to quantify osteoblast differentiation in primary cultures of bovine aortic smooth muscle cells (BASMCs) (Bear *et al.* 2008;Yang *et al.* 2005). Briefly, BASMCs were obtained from explants cultures as described previously (Bear *et al.* 2008;Yang *et al.* 2005). They were then seeded into 24 well plates, at a concentration of 5 x 10^4 cells per well and cultured for up to 6 days in DMEM containing 10% FBS and increasing concentrations of leptin. In some experiments, the cells were also cultured in the presence of the MEK inhibitor, PD098059, before treatment with leptin. At various times thereafter, the cells were harvested, lysed with 1% Triton X-100, and ALP activity assessed at 405 nm using a p-nitrophenol phosphate substrate kit (Sigma Chemical Co). ALP values (U/mg) were normalized to protein using the Bio-Rad DC protein assay (Bio-Rad, Hercules).

Alkaline phosphatase staining of the aortic root was also used to identify osteoblast-like cells present in the atherosclerotic lesions of leptin-treated and non-treated mice. Briefly, slides were fixed in citrate buffered acetone for 30 seconds and then rinsed in deionized water. The slides were subsequently incubated in an alkaline-dye mixture (consisting of Fast Blue RR Salt and Napthol

AS-MX Phosphate Alkaline Solution) at room temperature for 30 minutes. The percentage of total lesion area staining positive for alkaline phosphatase was then quantified using an epifluorescent microscope as described above.

3.3.6 Preparation of Cell-Free Lysates

Vascular smooth muscle cells were cultured to 70% confluence in DMEM containing 10% FBS before being stimulated with 2 µg/mL leptin. At various times thereafter, cell-free lysates were prepared and run on SDS-PAGE as described previously (Bear *et al.* 2008). Immunoblots were then performed using either an anti-Erk 1/2 antibody or an anti–P-Erk antibody and a horseradish peroxidase-conjugated secondary antibody.

3.3.7 Osteocalcin and Osteopontin Immunofluorescence

To confirm the presence of osteoblast-differentiation markers within the aortic root, osteocalcin goat anti-mouse polyclonal IgG and osteopontin goat anti-mouse polyclonal IgG primary antibodies were used. Briefly, cryosections were fixed in ice-cold acetone, washed in PBS, and blocked with 5% normal donkey serum and 2% BSA for 1 hour. Sections were incubated in primary antibody (4 µg/mL) overnight at 4°C before being incubated with AlexaFluor 594 at a 1:200 dilution (2 mg/mL, Invitrogen) for 1 hour. The sections were then visualized under oil immersion using the Zeiss Axioscope 2 (Carl Zeiss).

3.3.8 Preparation of DIG-Labeled Riboprobes and In Situ Hybridization

Total RNA was extracted from primary murine calvaria cells using an RNeasy RNA mini kit (Qiagen) before being transcribed into single-stranded cDNA. Double-stranded cDNA was generated by polymerase chain reaction (PCR) using specific sense and antisense primers for osteopontin and osteocalcin that were designed to include the T7 RNA polymerase binding sequence on their 5' ends. PCR products were run on a 1% agarose gel, and the positively identified DNA fragments were excised for subsequent purification using a QIAquick Gel Extraction Kit (Qiagen). DIG-labeling of the sense and antisense probes was performed using a DIG-RNA Labeling Kit and T7 RNA polymerase (Roche Diagnostics). The DIG-labeled sense and antisense riboprobes (50 ng/mL) were then used to perform in situ hybridizations on proteinase K–treated cryosections as described previously (Bidder *et al.* 1998).

3.3.9 Statistical Analysis

Analysis of variance was used to compare the results in the experimental group with those in the control. A significance of differences was determined using unpaired Student *t* tests with a Bonferroni correction for multiple comparisons. All data are expressed as a mean ± standard error (SEM).

3.4 Results

3.4.1 Effect of Leptin on Body Weight and Plasma Lipid Levels

Elevated leptin levels have been reported to cause weight loss and to increase lesion size in apoE-deficient mice (Bodary *et al.* 2005;Chiba *et al.* 2008). We therefore measured body weight and lipid levels in leptin-treated and non-treated apoE-deficient mice after a 2-month period. As seen in the **Table 3**, leptin-treated mice weighed significantly ($P<0.05$) less than did age-matched vehicle control mice (19.5±0.3 g versus 20.7±0.5 g). In contrast, whereas plasma triglyceride and cholesterol levels were slightly higher in leptin-treated animals, this difference was not statistically significant when compared to age-matched vehicle control mice (1.59±0.07 mmol/L versus 1.47±0.12 mmol/L; $P>0.05$) and (122±6.4 mmol/L versus 106±10.7 mmol/L; $>P0.05$, respectively; Table). Similarly, while blood glucose levels also tended to be higher in leptin-treated mice (10.84±0.84 mmol/L versus 9.31±0.57 mmol/L), this difference too was not statistically significant ($P>0.05$; **Table 3**). However, when fasting plasma insulin levels were measured, insulin levels were found to be significantly lower in the leptin-treated group (0.33±0.02 ng/ml versus 0.89±0.25 ng/ml; $P<0.02$).

Table 3. The effects of leptin on body weight and plasma biochemistry.

	Vehicle Control	Leptin Treated
Body weight, g	20.7 ± 0.5	19.5 ± 0.3*
Blood glucose, mmol/L	9.31 ± 0.57	10.84 ± 0.84
Plasma triglycerides, mmol/L	1.47 ± 0.12	1.59 ± 0.07
Plasma cholesterol, mmol/L	106 ± 10.7	122 ± 6.4
Plasma insulin, ng/ml	0.89 ± 0.25	0.33 ± 0.02*

Data are expressed as a mean ± SEM. *P<0.05 when compared to vehicle control.

3.4.2 Effect of Leptin on the Calcification of Atherosclerotic Lesions

Although several studies have suggested that leptin promotes atherosclerosis in apoE-deficient mice, to date none have examined the effect of leptin on vascular calcification *in vivo*. We therefore examined the effect of leptin on both lesion size and calcification using ApoE-deficient mice. As can be seen in **Figure 3.1A**, lesion size within the aortic root of leptin-treated and non-treated animals did not differ significantly (9.5±1.5 x 10^4 μm^2 versus 10.6±2.0 x 10^4 μm^2, respectively; $P>0.05$). Similarly, no significant difference ($P>0.2$) in the percentage of surface area covered by lesion was found when the thoracic aorta of leptin-treated and non-treated animals was examined after lipid staining with Sudan IV (**Figure 3.1B**).

Next, to determine the effect of leptin on vascular calcification, we stained the aortic root of leptin-treated and non-treated apoE-deficient mice with von Kossa (**Figure 3.2A** versus **Figure 3.2B**). As seen in **Figure 3.2B**, the lesions found within the aortic root of leptin-treated animals demonstrated both large and small punctate areas of calcification which stained black. As compared to non-treated animals, the lesions found within leptin-treated animals demonstrated significantly ($P<0.001$) more calcification (**Figure 3.2C**). Thus, those animals which received leptin demonstrated an 8.3±2.0-fold increase in the proportion of lesion area that was calcified as compared to non-treated animals (9.02±2.18% versus 1.09±0.20%; $P<0.001$; **Figure 3.2C**).

Figure 3.1 - Effect of leptin on lesion size and surface area covered by atherosclerotic lesions.

Average lesion size within the aortic root (A) and total surface area within the thoracic aorta covered by atherosclerosis (B) of leptin-treated and non-treated animals was quantified as described in Material and Methods. Data are represented as a mean ± SEM.

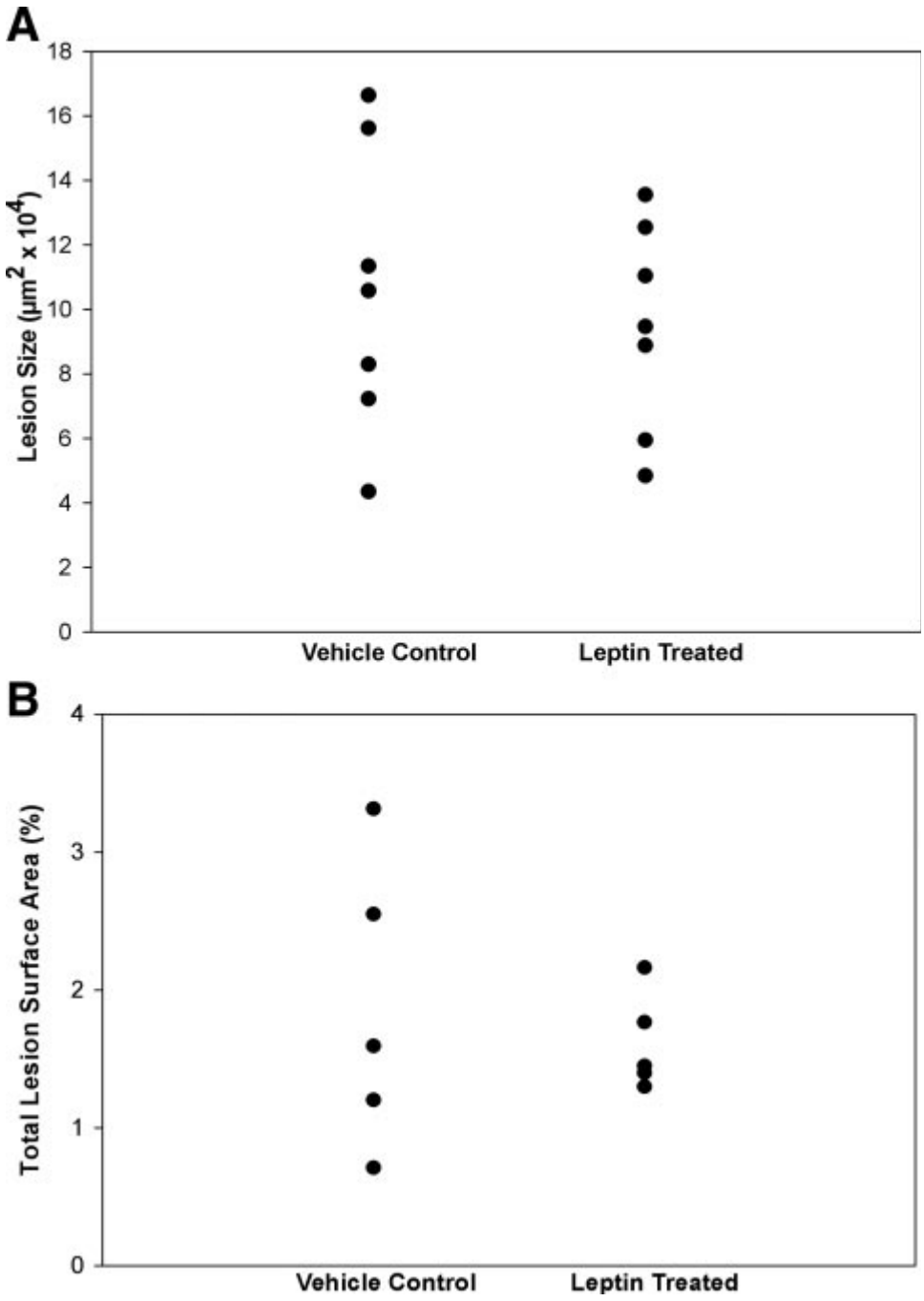
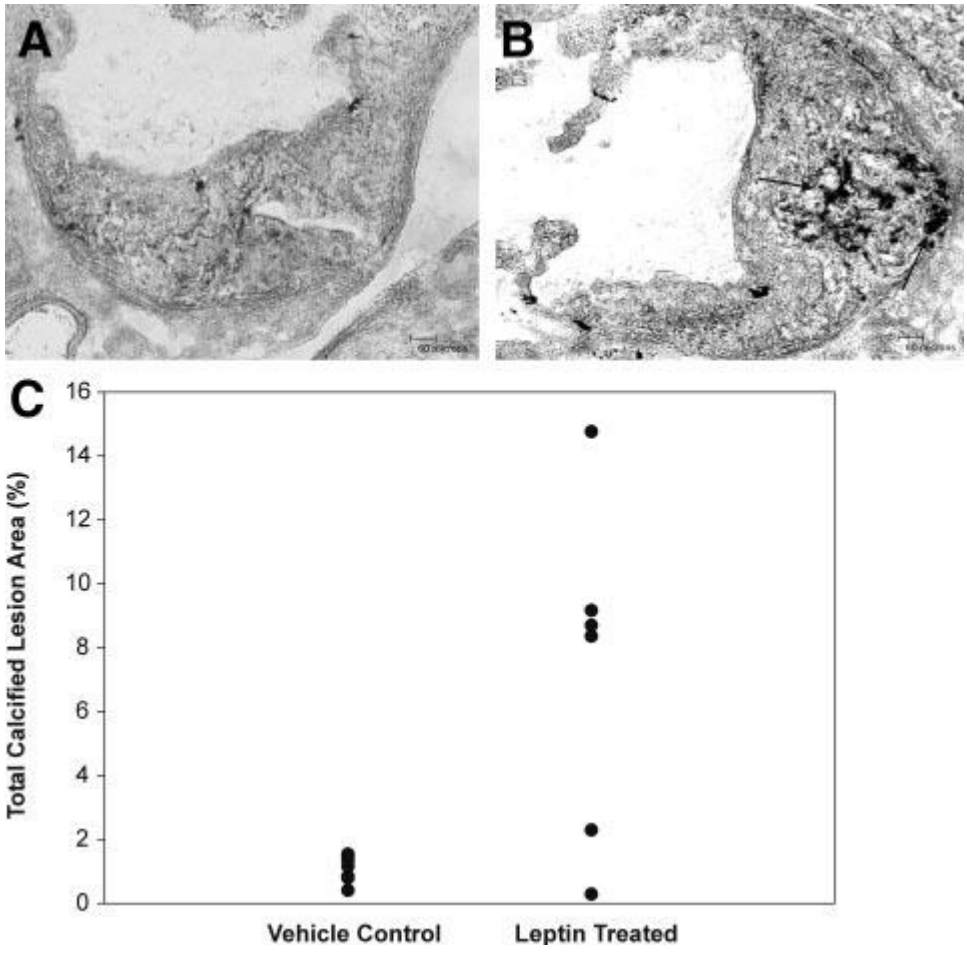

A
Lesion Size (μm² x 10⁴)
0
2
4
6
8
10
12
14
16
18
Vehicle Control
Leptin Treated
B
Total Lesion Surface Area (%)
0
1
2
3
4
Vehicle Control
Leptin Treated

Figure 3.2 - Effect of leptin on the calcification of atherosclerotic lesions.

Vascular calcification in non-treated (A) and leptin-treated animals (B) was detected by von Kossa staining. Calcified lesion area is expressed as a percentage of total lesion size (C). Data are represented as a mean ± SEM. *P<0.001 when compared to non-treated animals.

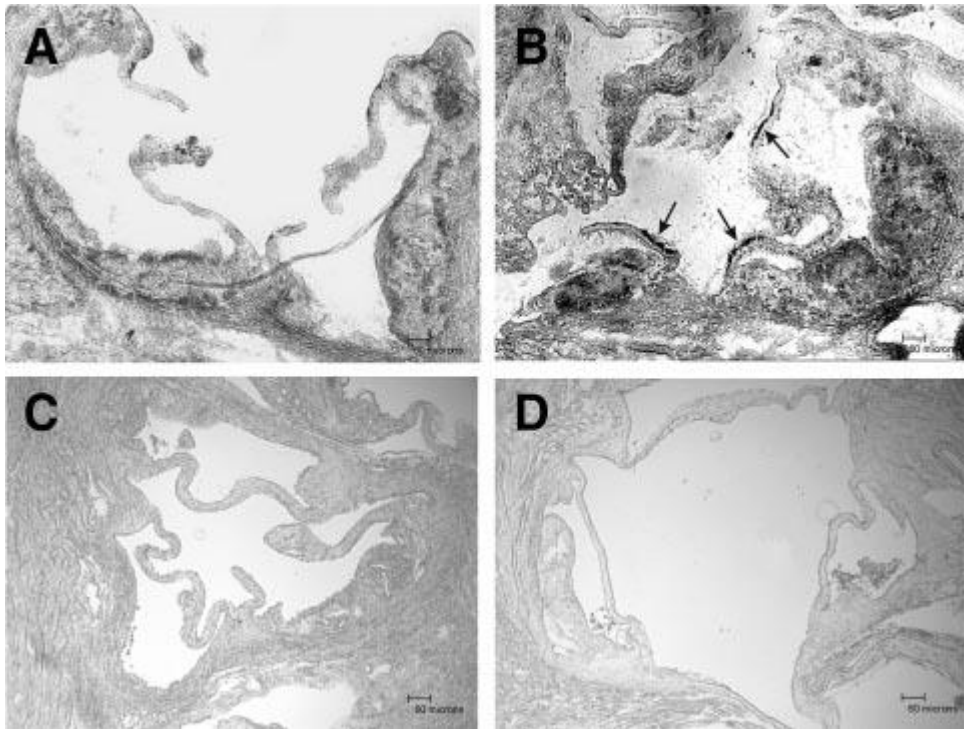

A
B
C
Total Calcified Lesion Area (%)
16
14
12
10
8
6
4
2
0
Vehicle Control
Leptin Treated

3.4.3 Effect of Leptin on Valvular Calcification

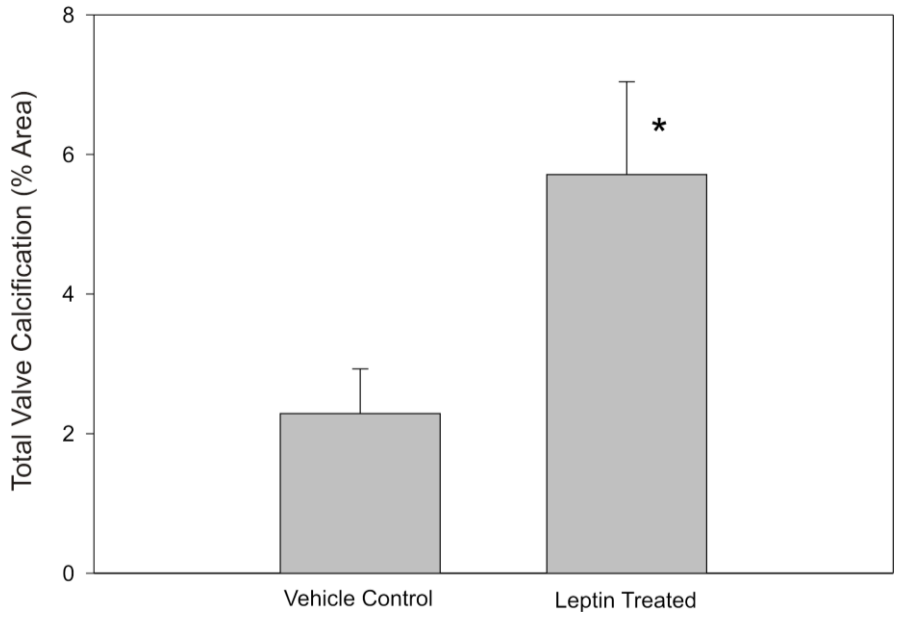
In addition to promoting the calcification of atherosclerotic lesions, leptin was also found to promote the calcification of the aortic valves. Thus, black punctate deposits denoting calcification were readily observable tracking along the length of the aortic valves in those animals treated with leptin (**Figure 3.3B**). Little or no staining was observed in those sections that were either obtained from non-leptin treated animals (**Figure 3.3A**) or left unstained (**Figure 3.3C** and **Figure 3.3D**). When quantified, leptin-treated animals showed a 2.5±0.6-fold increase in valvular calcification as compared to non-treated animals (5.71±1.33% versus 2.29±0.64%; $P<0.05$).

Figure 3.3 - Effect of leptin on valvular calcification.

Sections of the aortic root were obtained from non-treated (A and C) and leptin-treated animals (B and D) and either subjected to von Kossa staining to detect valvular calcification (A and B) or left unstained (C and D). Von kossa staining along aortic valves was quantified and expressed as a percentage of total valve area (E). *P<0.05 when compared to vehicle control group.


A
B
C
D

E


Total Valve Calcification (% Area)
0
2
4
6
8
*
Vehicle Control
Leptin Treated

3.4.4 Effect of Leptin on Osteoblast Differentiation in Primary Cultures of Bovine Aortic Smooth Muscle Cells

Because leptin was found to promote the calcification of atherosclerotic lesions, we next decided to verify an earlier report of leptin inducing the osteogenic differentiation of vascular smooth muscle cells (Parhami *et al.* 2001). Primary BASMCs were cultured in the presence or absence of leptin for increasing periods of time and the effect on BASMC differentiation determined by quantifying the amount of ALP activity in our vascular smooth muscle cell cultures. As seen in **Figure 3.4A**, ALP activity was significantly (P<0.01) and dose dependently increased when BASMCs were cultured in the presence of increasing concentrations of leptin. Maximum ALP activity was seen when the BASMCs were cultured for 6 days in the presence of 2 µg/mL leptin (434±46 U/mg versus 151±12 U/mg; P<0.01).

In an effort to determine how leptin was acting to increase osteoblast differentiation in primary BASMC cultures, we next decided to examine the effect of leptin on extracellular signal-related kinase (Erk) activation. Primary BASMCs were treated with leptin for increasing periods of time before lysing the cells and immunoblotting for either Erk 1 & 2 or p-Erk 1 & 2. As seen in **Figure 3.4B**, leptin treatment of BASMCs significantly increased Erk 1 & 2 phosphorylation in a time-dependent manner (4.5-fold, P<0.05). Maximal stimulation of Erk was obtained when BASMCs were treated for 20 minutes with 2 µg/mL leptin and was sustained for at least 2 hours before returning to control levels (data not shown).

Finally, to determine whether Erk 1 & 2 activation by leptin was responsible for the ability of leptin to induce osteoblast differentiation in primary BASMC cultures, we treated BASMCs with leptin and increasing concentrations of the MEK inhibitor PD098059. Six days later, the cells were lysed and alkaline phosphatase activity determined as an index of osteoblast differentiation. As shown in **Figure 3.4C**, significant alkaline phosphatase activity was found when BASMCs were cultured in the presence of 2 µg/mL leptin. However, when the cells were also cultured in the presence of PD098059, the ability of leptin to induce ALP activity was significantly diminished.

3.4.5 Effect of Leptin on Markers of Osteoblast Differentiation *In Vivo*

Because we were able to confirm the ability of leptin to induce osteoblast differentiation *in vitro*, we next decided to look for evidence of osteoblast-like cells within the calcified lesions of animals treated with leptin. As seen in **Figure 3.5A**, areas staining positive for ALP were observed within the atherosclerotic lesions and to a lesser extent the medial layer of leptin-treated animals. When quantified, the percent total lesion area demonstrating ALP positive staining was 5.4±2.1-fold (P<0.05) greater in leptin-treated mice as compared to non-treated age-matched control mice (1.72±0.69% versus 0.32±0.22%, respectively). Only half of the animals from the vehicle control group stained positive for ALP, whereas 6 of the 7 animals showed ALP staining in the leptin-treated group (data not shown).

To confirm our ALP findings we next immunostained the lesions of leptin-treated and non-treated animals for several other osteoblast-specific markers. As seen in **Figure 3.5B**, the atherosclerotic lesions of our leptin-treated mice stained more intensely for the osteoblast-specific markers osteocalcin and osteopontin than did the lesions of non-treated animals. In addition, the expression of both osteocalcin and osteopontin was detected by in situ hybridization within the atherosclerotic lesions of leptin-treated animals (**Figure 3.5C**) and to a lesser extent in non-treated animals.

Figure 3.4 - Effect of leptin on osteoblast differentiation in primary cultures of BASMCs.

Primary BASMCs were cultured in the absence or presence of leptin (A and B) or leptin and the MEK inhibitor, PD098059 (C). The effect on either alkaline phosphatase activity (A and C) or Erk 1 & 2 activation (B) was then determined as described in the Materials and Methods section. Data are represented as a mean ± SEM.

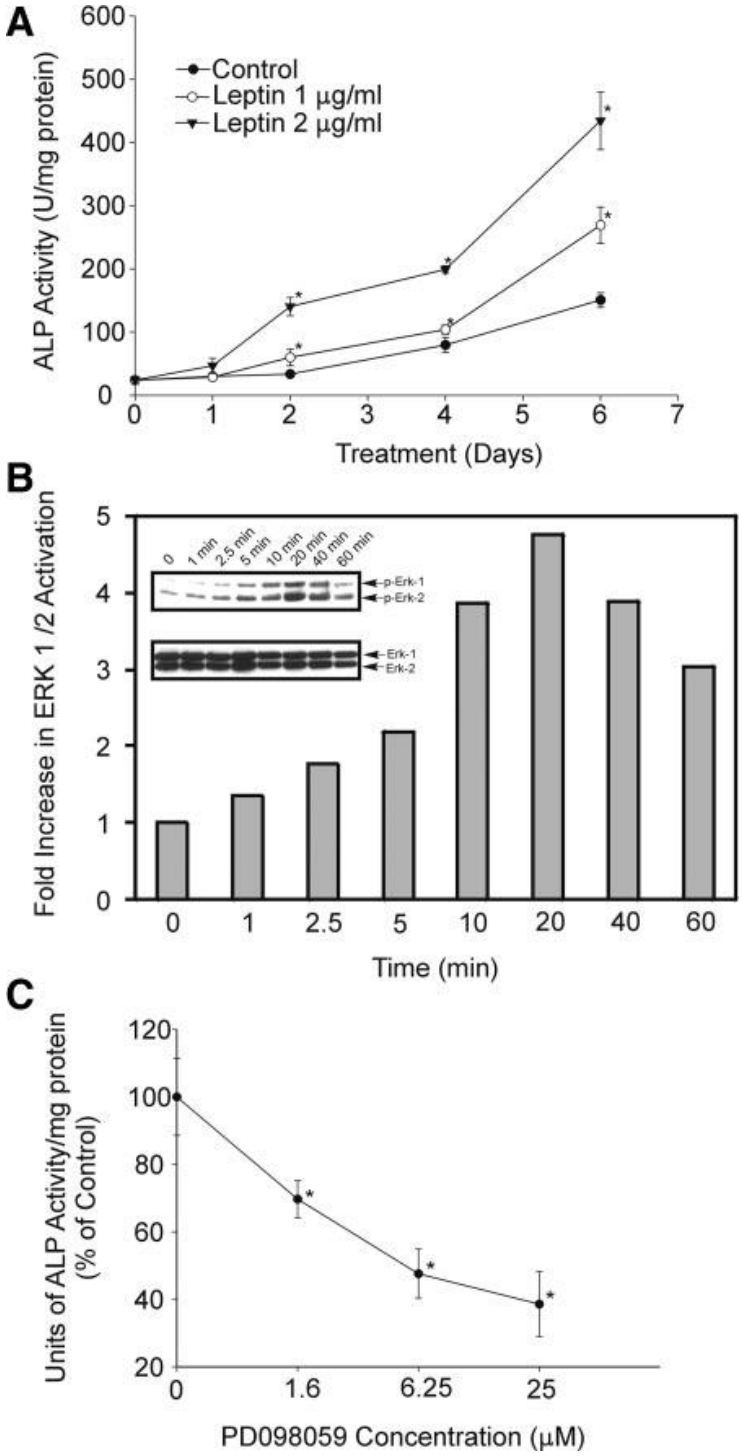
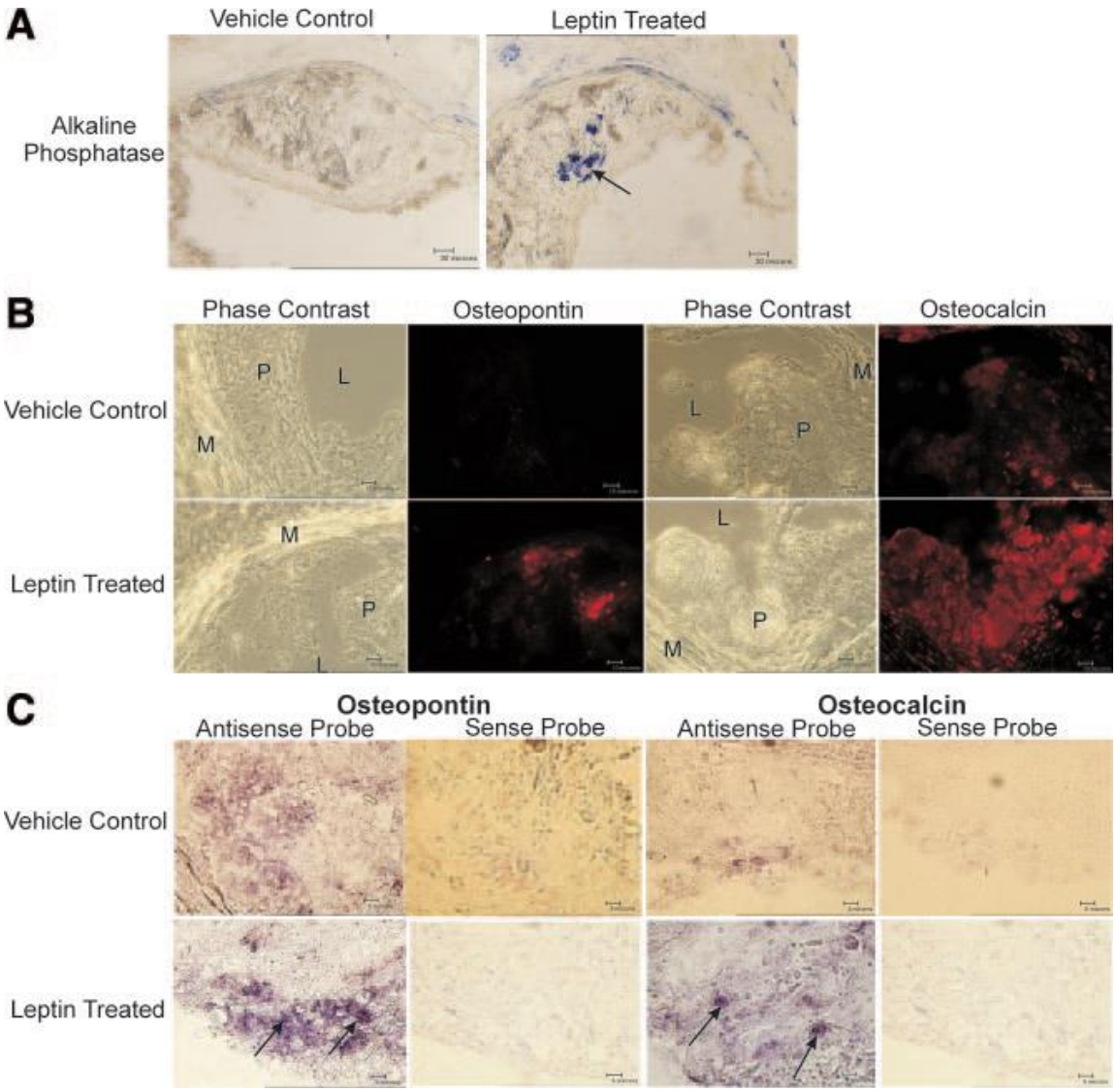

A
ALP Activity (U/mg protein)
Control
Leptin 1 µg/ml
Leptin 2 µg/ml
Treatment (Days)
B
Fold Increase in ERK 1 /2 Activation
0 1 min 2.5 min 5 min 10 min 20 min 40 min 60 min
p-Erk-1
p-Erk-2
Erk-1
Erk-2
Time (min)
C
Units of ALP Activity/mg protein (% of Control)
PD098059 Concentration (µM)

Figure 3.5 - Effect of leptin on markers of osteoblast differentiation.

Lesions of non-treated and leptin-treated animals were either stained for alkaline phosphatase activity (A), immunostained for osteocalcin and osteopontin (B), or probed for osteocalcin and osteopontin expression by in situ hybridization (C). M indicates medial layer; P, plaque; L, lumen.


A
Vehicle Control
Leptin Treated
Alkaline Phosphatase
B
Phase Contrast
Osteopontin
Phase Contrast
Osteocalcin
Vehicle Control
Leptin Treated
P
L
M
C
Osteopontin
Osteocalcin
Antisense Probe
Sense Probe
Antisense Probe
Sense Probe
Vehicle Control
Leptin Treated

3.5 Discussion

By using a well-defined animal model of atherosclerosis, we have shown that the adipocytokine leptin promotes the calcification of atherosclerotic lesions *in vivo*. In addition, by measuring the expression of osteoblast-specific markers within the lesions of leptin-treated mice, we were able to demonstrate that this increase in vascular calcification is associated with an increase in osteoblast number. To our knowledge, this is the first report of leptin increasing vascular calcification in an *in vivo* animal model.

Several working hypotheses have been advanced in an attempt to explain how vascular calcification occurs. These include the presence of osteoblasts (bone-forming cells) within the vessel wall (Bostrom *et al.* 1993;Dhore *et al.* 2001;Shanahan *et al.* 1994;Tyson *et al.* 2003), the loss of specific calcification inhibitors such as matrix Gla protein (Luo *et al.* 1997;Price *et al.* 2002b;Schinke and Karsenty 2000), and finally the nonspecific entrapment of calcium (Bucay *et al.* 1998;Simonet *et al.* 1997). Based on the findings of the current study, however, we believe that vascular calcification results from the localization of osteoblast-like cells within the vessel wall. Thus, in the current study we were able to demonstrate that leptin not only promotes vascular calcification but that the increase in vascular calcification is associated with an increase in osteoblast-specific markers within the vessel wall (**Figure 3.5**). Indeed several investigators have also localized osteoblast-specific markers to the calcified atherosclerotic lesions of human vessels (Bostrom *et al.* 1993;Dhore *et al.* 2001;Shanahan *et al.*

1994;Tyson *et al.* 2003). How leptin increases the expression of osteoblast-specific markers within the vessel wall is unknown. However, in the current study, we demonstrate that leptin can induce osteoblast differentiation in primary cultures of vascular smooth muscle cells (**Figure 3.4A**). In addition, we demonstrate that this effect is Erk1 & 2 dependent (**Figure 3.4B** and **3.4C**). Thus, it is possible that leptin is promoting osteoblast differentiation within the atherosclerotic lesions of our apoE-deficient mice and that this is occurring in an Erk 1 & 2-dependent manner. Alternatively, leptin has been shown to increase true osteoblast differentiation within the bone marrow space (Gordeladze *et al.* 2002;Thomas *et al.* 1999), and thus it is possible that osteoblasts can, in low numbers, migrate within the circulation and thereby localize within the vessel wall at sites of atherosclerosis.

In the current study, leptin was found to not only promote the calcification of atherosclerotic lesions but also valvular calcification within the aortic root. Whether the process of vascular calcification at these 2 distinct sites occurs by similar mechanisms is not known. However, we could not unequivocally observe osteoblast-specific markers lining the valve surface. Interestingly, a number of studies have suggested an association between valvular aortic stenosis and high circulating levels of leptin (Glader *et al.* 2003). Although postinflammatory scarring can account for aortic stenosis, by far the most common cause of aortic stenosis is senile calcification of the heart valve(s) (Boudoulas *et al.* 1994;Passik *et al.* 1987).

Leptin-treated mice were found to weigh slightly less than age-matched vehicle control mice (19.5±0.3 versus 20.7±0.5 g; $P<0.05$). A one gram difference in body weight (19.5±0.3 versus 20.7±0.5 g) represents an approximate 5% decrease in overall weight. Whether such a minor drop in body weight affected any of the other outcomes that we measured is unknown. Leptin-treated mice did show improved insulin sensitivity when compared to non-treated mice. However, whether this occurred in response to the decrease in body weight or because of some yet to be defined mechanism is unknown. Interestingly, neither glucose levels nor plasma lipid levels showed any improvement when the animals, which were maintained on a high-fat diet, were also treated with leptin.

Our findings failed to show an increase in lesion size or total surface area occupied by lesions after the administration of leptin to apoE-deficient mice (**Figure 3.1**). At first glance, these findings appear to be at odds with previous reports that show leptin having proatherogenic effects (Bodary *et al.* 2005;Chiba *et al.* 2008). Thus, prior studies have shown that leptin increases total lesion area within the thoracic aorta of leptin-treated animals (Bodary *et al.* 2005;Chiba *et al.* 2008). However, when we examined the thoracic aorta of leptin-treated and non-treated apoE-deficient mice, we found no difference in the surface area occupied by lesions (**Figure 3.1B**). Why our findings are at odds with previous studies is not clear. However, in previous studies only male mice were used, whereas in our study only female apoE-deficient mice were used. Whether this accounts for the observed discrepancy is not known.

In addition to examining the effect of leptin within the thoracic aorta, we also examined its effects on lesion size within the aortic root. Again no effect on lesion size was observed. In previous studies, the proatherogenic effects of leptin were only examined within the carotid and brachiocephalic arteries or the thoracic aorta and not within the aortic root. Thus, in addition to gender differences, it is possible that the proatherogenic effects of leptin are site-specific and that lesion size is not increased by leptin within the aortic root regardless of gender. In support of this conclusion, Bodary *et al* (2005) did not find a significant increase in total lesion area when examining lesions within the carotid artery of leptin-treated mice, even though the same animals showed a significant increase in total lesion area within the brachiocephalic artery and thoracic aorta. When taken together, our findings suggest that the effect(s) of leptin on vascular calcification are independent of any proatherogenic effects.

Our findings are supported by several clinical trials that have shown a positive correlation between circulating levels of plasma leptin and coronary artery calcification (CAC). Thus in a cross-sectional study conducted by Reilly *et al* (2004) a positive correlation between plasma leptin levels and CAC was found in 200 type 2 diabetic subjects, even after controlling for other traditional risk factors such as body mass index, waist circumference, and C-reactive protein levels. Similar findings were reported in a separate study by Qasim *et al* (2008) who examined CAC in 860 asymptomatic nondiabetic individuals. Thus, Qasim *et*

al (2008) examined the association of several plasma cytokines with CAC and found that only leptin was a significant independent predictor.

In summary, by treating apoE-deficient mice with exogenous leptin, we found that leptin promotes the calcification of atherosclerotic lesions without increasing lesion size, and that this is associated with an increase in osteoblast-specific markers found within the calcified lesions of leptin-treated mice. In addition, leptin treatment also appears to increase valvular calcification by a yet to be determined mechanism. When taken together, these findings may help to explain why individuals with high leptin levels have a higher incidence of CAC.

CHAPTER 4: Leptin Promotes Osteoblast Differentiation and Mineralization of Primary Cultures of Vascular Smooth Muscle Cells by Inhibiting Glycogen Synthase Kinase (GSK) -3β.

Foreword

We have previously demonstrated that the adipocytokine, leptin, promotes vascular calcification in ApoE - deficient mice and that this increase in calcification is associated with an increase in the expression of several osteoblast-specific markers within the vessel wall. In the following study, we begin to investigate the mechanism(s) by which leptin mediates osteoblast differentiation of VSMCs with a particular emphasis on the role that GSK-3 may play in leptin-mediated osteoblast differentiation. It is well-established that leptin promotes osteoblast differentiation of primary cultures of BASMCs. Here, we demonstrate that leptin regulates the expression of genes involved in osteoblast differentiation and induces GSK-3β phosphorylation/inactivation. Furthermore, we show for the first time that constitutively active GSK-3β attenuates leptin-induced osteoblast differentiation suggesting that leptin signals through GSK-3β to regulate osteoblast differentiation.

This work was published in Biochemical and Biophysical Research Communication (Volume 425, Issue 4, pages 924-930, August 2012). The authors are the following: Melec G. Zeadin, Martin K. Butcher, Stephen G. Shaughnessy and Geoff H. Werstuck. The corresponding author is Dr. Werstuck.

The experiments in this manuscript were performed by Melec Zeadin. The manuscript was written by Melec Zeadin and Dr. Werstuck. Chapter 4 has been reprinted with permission (under license#34384102200968). The complete reference is as follows: Zeadin, M.G, Butcher, M.K, Shaughnessy, S.G. and Werstuck, G. (2012). Leptin promotes osteoblast differentiation and mineralization of primary cultures of vascular smooth muscle cells by inhibiting glycogen synthase kinase (GSK)-3β. *Biochemical and Biophysical Research Communication* **425**, 924-930. Note: For the purpose of maintaining consistency throughout this dissertation, the Results and Discussion section are separated. In the published manuscript, these sections are combined.

4.1 Abstract

In this study, we begin to investigate the underlying mechanism of leptin-induced calcification. We found that treatment of cultured bovine aortic smooth muscle cells (BASMCs) with leptin (0.5-4 μg/mL) induced osteoblast differentiation in a dose-dependent manner. Furthermore, we found that leptin significantly increased the mRNA expression of osteopontin and bone sialoprotein, while down-regulating matrix gla protein (MGP) expression in BASMCs. Key factors implicated in osteoblast differentiation, including members of the Wnt signaling pathway, were examined. Exposure to leptin enhanced phosphorylation of GSK-3β on serine-9 thereby inhibiting activity and promoting the nuclear accumulation of β-catenin. Transfection of BASMCs with an adenovirus that expressed constitutively active GSK-3β (Ad-GSK-3β S9A) resulted in a > 2-fold increase in GSK-3β activity and a significant decrease in leptin-induced alkaline phosphatase (ALP) activity. In addition, qRT-PCR analysis showed that GSK-3β activation resulted in a significant decrease in the expression of osteopontin and bone sialoprotein, but a marked increase in MGP mRNA expression. When taken together, our results suggest a mechanism by which leptin promotes osteoblast differentiation and vascular calcification *in vivo.*

4.2 Introduction

Obesity is recognized as a significant independent risk factor for cardiovascular disease and associated morbidity and mortality (Bray 2004;Yusuf *et al.* 2004). We now know that adipose tissue can actively secrete adipocytokines, including leptin, resistin, and adiponectin which may play direct and indirect roles in the development of cardiovascular disease (CVD). Furthermore, specific adipocytokines are known to circulate at elevated levels in obese individuals and to correlate positively with the risk for CVD (Azuma *et al.* 2003;Degawa-Yamauchi *et al.* 2003;Lee *et al.* 2009;Reilly *et al.* 2005;Romero-Corral *et al.* 2008;Wallace *et al.* 2001).

Leptin is a secreted protein of approximately 16 kDa that plays a pivotal role in the hypothalamus to regulate energy intake and expenditure (Myers, Jr. 2004;Tartaglia 1997). Leptin also has proatherogenic effects that have been well described in various mouse models (Bodary *et al.* 2005;Loffreda *et al.* 1998;Oda *et al.* 2001;Schafer *et al.* 2004). Recently, our lab demonstrated that leptin can promote the calcification of atherosclerotic lesions in ApoE - deficient mice and that this process of leptin-mediated vascular calcification is associated with an increase in the expression of osteoblast - specific markers, including alkaline phosphatase, osteopontin and osteocalcin, within the vessel wall (Zeadin *et al.* 2009).

There are several clinical consequences associated with the presence of vascular calcification (Johnson *et al.* 2006). For example, the calcification of

coronary arteries contributes to an increased risk of plaque rupture and as such the possibility of a myocardial infarction/stroke (Bobryshev *et al.* 2008;Vliegenthart *et al.* 2002); in addition to an increased risk of vessel wall dissection during balloon angioplasty or stent placement (Fitzgerald *et al.* 1992). The calcification of cardiac valves can also lead to valvular stenosis resulting in the loss of valve mobility which is a major mechanism for valve failure (Stewart *et al.* 1997).

Previous findings support a role for “osteoblast-like” cells within calcified vessel walls. Indeed, our lab and others have demonstrated that cultured vascular smooth muscle cells (VSMCs) can be induced to undergo a phenotypic transition into osteoblast-like cells (Bear *et al.* 2008;Steitz *et al.* 2001). However, the mechanism by which this may be occurring is still largely unknown. There are several signal transduction pathways which are thought to be important in the process of osteoblast differentiation. One such pathway is the canonical Wnt signaling pathway. This pathway becomes activated when Wnt ligands (i.e. Wnt1, Wnt3a) bind the cell-surface Frizzled (Fz) receptor that in turn complexes with co-receptors low-density-lipoprotein-receptor-related protein 5/6 (LRP 5/6). The activation of the cell surface Fz/LRP5/6 receptor complex mediates the inactivation of GSK-3β. Although active GSK-3β can phosphorylate β-catenin targeting this substrate for ubiquitination and proteasomal degradation, when GSK-3β is inactivated, β-catenin escapes degradation, accumulating instead in the cytoplasm and eventually translocating to the nucleus. In the nucleus, β-

catenin can heterodimerize with members of the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors to induce the expression of various genes (Baron and Rawadi 2007;Glass and Karsenty 2007). The ability of leptin to modulate Wnt-signaling pathways is supported by studies in various cell types including breast cancer cells (Garza *et al.* 2011;Yan *et al.* 2012), but to date no one has investigated the effect of leptin on Wnt signaling in a model for osteogenic differentiation. In this study, we propose a mechanism by which leptin is promoting osteogenic differentiation and thus vascular calcification *in vivo.*

4.3 Materials and Methods

4.3.1 Materials

Recombinant human leptin and *p*-nitrophenol phosphate substrate kit used to assess ALP activity were purchased from Sigma Chemical Co. (St.Louis, MO, USA). Antibodies for phospho-GSK-3β (Ser9), total GSK-3β, β-catenin and Lamin A/C were obtained from Cell Signaling (Beverly, MA, USA). Ad-CMV-Null and Ad-GSK-3β S9A constructs were purchased from Vector Biolabs (Philadelphia, PA, USA).

4.3.2 Isolation of Vascular Smooth Muscle Cells

VSMCs were isolated from fresh bovine aortas that were obtained from a local slaughter house (Highland Packers LTD, Hamilton, ON). Fresh aortas were rinsed twice in 100% ethanol and transported on ice in 1X sterile Hanks solution

(pH 7.1). The aortas were then denuded of the endothelial layer to expose the underlying medial layer containing smooth muscle cells. The medial layer was cut into several 1-2 mm^2 pieces and arranged in a circular pattern on a 150 mm x 25 mm dish. The cells were cultured in Dulbecco's Modified Eagle Media (DMEM, Invitrogen, Burlington, ON) containing 20% FBS, 100 U/ml penicillin/streptomycin and essential amino acids. An additional 5 ml of 20% DMEM was added after 5-7 days to keep tissues hydrated. When sufficient outgrowth was observed, tissues were carefully removed and media was changed every 3-4 days. Once confluent, the cells were harvested and stored in liquid nitrogen.

4.3.3 Measurements of Alkaline Phosphatase Activity

To assess the extent of osteoblast differentiation, BASMCs were seeded onto 24-well plates at a concentration of 2.5 x 10^4 cells/well and then cultured for 8 days in differentiating media (DMEM containing 10% FBS, 10 mM β-glycerophosphate and 0.5 mM ascorbic acid) in the absence or presence of 2 µg/ml leptin. In some experiments, cells were infected with either 10 MOI of a control adenovirus (Ad-CMV-Null) or an adenovirus that constitutively expressed active GSK-3β by substitution of the serine residue with an alanine residue at position 9 (Ad-GSK-3β S9A). Cells were infected for 24 hrs before being cultured in the absence or presence of 2 µg/ml leptin. Cells were harvested by being lysed in 1% Triton X-100, 0.9% NaCl for one hour on ice. ALP activity was assessed at 405 nm using a *p*-nitrophenol phosphate substrate kit. Bio-Rad DC protein assay

(Bio-Rad, Hercules, CA) was used to quantify protein concentrations and ALP values were normalized to protein (U/mg of protein).

4.3.4 qRT-PCR

BASMCs were plated onto 60 mm dishes at a concentration of 2.5 x 10^5 cells/plate. When cells reached 70% confluency, they were allowed to continue differentiating in 10% FBS, 10 mM β-glycerophosphate and 0.5 mM ascorbic acid in either the absence or presence of leptin (2µg/ml) for 8 days in order to measure mRNA expression levels of osteopontin, bone sialoprotein and MGP. Media was changed every 3-4 days for up to 8 days. Briefly, cellular RNA was isolated using an RNeasy mini kit (Qiagen, Mississauga, ON, Canada). RNA was quantified by measuring the absorbance at 260 nm, while purity was verified by calculating the ratio of the absorbance at 260 nm and 280 nm (A_{260}/A_{280}). cDNA was then reverse transcribed from 4 µg of RNA using Superscript II Reverse Transcriptase (Invitrogen). Real-time PCR analysis was performed on the ABI PRISM 7300 (Applied Biosystems, USA) using iQ SYBR Green Supermix (BioRad), 4 µg cDNA and 500 nM foward and reverse primers. Denaturation took place at 94°C for 15 sec, annealing reactions were carried out for 30 sec at primer-specific temperatures followed by extension at 72°C for 34 sec, for a total of 40 cycles. GAPDH was used as the internal control. See **Table 4** for specific primers and protocols.

Table 4. Primers and protocols for qRT-PCR.

Genes	Primer Sequence	Annealing Temperature (°C)
Osteopontin	FWD: CCG CCGCAGACCAAGGAAAAAT REV: AACTGGAAGGGCGGAGGCAAT	60
Bone Sialoprotein	FWD: ATGGGACTACCCCACCACCGT REV: CCCCACGAGGATCTCCGTTCTCA	62
Matrix Gla Protein	FWD: GAGCTCAACCGGGAAGCTTGTG REV: CCTCGGCGCTGCCGGAAATAA	61
GAPDH	FWD: ATGGCCTTCCGCGTCCCCACTCC REV: AGCCAAATTCATTGTCGTACC	60-62

4.3.5 Von Kossa Staining

BASMCs were plated onto coverslips at a concentration of 5 x 10^4 cells in a 24-well plate. Cells were cultured in DMEM supplemented with 10% FBS, 10 mM β-glycerophosphate and 0.5 mM ascorbic acid in the absence or presence of leptin (0.5-2 µg/ml). Media was changed every 3-4 days for up to 12 days. Cells were then fixed in 10% formalin and mineralization was detected by von Kossa staining. Briefly, cells were incubated in 1% silver nitrate under UV light before being washed in 2.5% sodium thiosulphate. Mineralized surface was quantified under low power light microscopy (200X magnification).

4.3.6 Preparation of Cell-Free Lysates and Nuclear Extracts

BASMCs were plated onto 60 mm dishes at a density of 2.5 x 10^5 cells/plate and then cultured in DMEM containing 10% FBS. Once cell cultures reached 70% confluency, cells were washed twice in 1X phosphate buffered saline (PBS) and cultured in DMEM containing 0.5% FBS for an additional 24 hrs before being treated for various periods of time with leptin (2µg/ml). Cells were then rinsed twice with ice-cold 1X PBS before being lysed in lysis buffer (1% sodium deoxycholate, 1% triton X-100, 50 mM Tris HCl pH7.2, 0.25 EDTA, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 1 µg/ml pepstatin A, 1 mM PMSF, 0.1 mM Na_3VO_4) and isolated using a cell scraper. To obtain nuclear extracts, cells were first resuspended in a hypotonic buffer [10mM Hepes-KOH (pH 7.9) containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1 mM Na_3VO_4, 0.5 mM PMSF, 10

μg/ml aprotonin, 2 μg/ml pepstatin A and 2 μg/ml leupeptin] to release the cytoplasmic proteins. To then isolate nuclear proteins, the pellet was resuspended in 20 mM Hepes-KOH (pH 7.9) containing 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF 10 μg/ml aprotonin, 2 μg/ml pepstatin A, 2 μg/ml leupeptin and 25% glycerol. Bio-Rad DC protein assay was used to determine protein concentrations.

4.3.7 Immunoblot Analysis

Cell-free lysates or nuclear extracts were prepared as previously described. Samples were then loaded onto a 10% SDS-PAGE and run at 4°C and 120 V before being transferred onto a nitrocellulose membrane using the semi-dry transfer cell. The membrane was washed with TBST [50 mM Tris (pH 7.4), 150 mM NaCl and 0.05% Tween-20] before being blocked in 5% non-fat skim milk containing 50 mM Tris (pH 8.0), 150 mM NaCl and 0.05% Tween-20 for one hour at 37°C and incubated with primary antibody at 4°C overnight. The next day, membranes were washed and incubated with the corresponding secondary antibody for one hour at room temperature. Protein bands were visualized by an enhanced chemiluminescent system. Results were quantified using ImageJ version 1.32j software (Wayne Rasband, National Institute of Health, USA).

4.3.8 GSK-3β Activity Assay

BASMCs were infected with either 10 MOI Ad-CMV-Null or Ad-GSK-3β S9A. To measure GSK-3β activity, whole cell lysates were collected in GSK-3

lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 10 mM DTT, 1 mM benzamidine, 0.1X Roche PhosSTOP, 1 mM Na_3VO_4, and 1 mM PMSF] on day 3, 5 and 7 and then immunoprecipitated overnight with 0.625 µg purified mouse anti-GSK-3β antibody (BD Biosciences, Mississauga, ON). GSK-3 activity was then assayed as previously described by Bowes *at al* (Bowes *et al.* 2009).

4.3.9 Statistical Analysis

Data are expressed as mean ± standard error (SEM) of at least three or four independent experiments. A significance of differences was determined using an unpaired student's t-test of equal variance. qRT-PCR data are expressed with adjusted p-values using the Benjamin-Hochberg False Discovery Rate.

4.4 Results

4.4.1 Effect of Leptin on Osteoblast Differentiation and Mineralization

Our lab and others have previously demonstrated that primary cultures of BASMCs can be induced to undergo a phenotypic transition into "osteoblast-like cells" (Bear *et al.* 2008;Shioi *et al.* 1995;Yang *et al.* 2005). Furthermore, Parhami *et al* (Parhami *et al.* 2001) demonstrate that leptin can enhance the calcification of a subpopulation of vascular cells (referred to as "calcifying vascular cells") to undergo osteoblastic differentiation and mineralization *in vitro.* To determine the

ability of leptin to promote osteoblast differentiation in primary cultures of BASMCs, we assessed ALP activity as an index of osteoblast differentiation. BASMCs were cultured in the absence or presence of increasing concentrations of leptin (0.5-4 μg/ml) for 8 days. As seen in **Figure 4.1A**, ALP activity was significantly, and dose-dependently increased when BASMCs were cultured in the presence of increasing concentrations of leptin with a 4.4 ± 0.2-fold ($P<0.001$) increase in ALP activity at 4 μg/ml. Similar results were found when we used qRT-PCR to examine the effect of leptin on several other osteoblast-specific markers (**Figure 4.1B**). We observed a significant increase in the expression of both osteopontin and bone sialoprotein expression but a significant decrease in the mRNA levels of the calcification inhibitor, MGP. To determine if leptin has the same effect on mineralization, BASMCs were cultured in the absence or presence of increasing doses of leptin (0.5, 1, and 2 μg/ml) for 12 days. Von Kossa staining was used to assess the extent of mineralization (**Figure 4.2A**). As seen in **Figure 4.2B**, there was a dose-dependent increase in the degree of mineralization (2-fold at the highest concentration tested). When taken together, these findings confirm leptin's ability to promote the osteogenic differentiation and mineralization of vascular smooth muscle cells (VSMCs).

4.4.2 Effect of Leptin on GSK-3β Phosphorylation and β-catenin Nuclear Accumulation

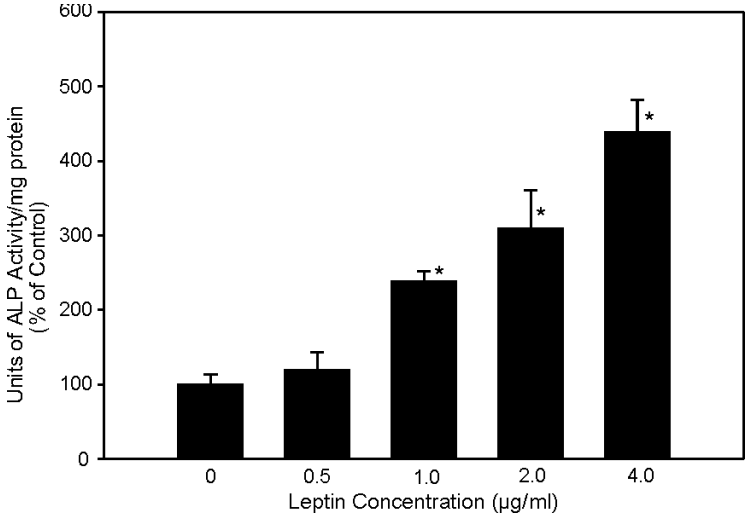
To begin to delineate the mechanism by which leptin may be promoting the osteogenic differentiation of BASMCs, we examined its effects on the canonical Wnt signaling pathway. BASMCs were treated with leptin for increasing periods of time and analysed by western blot to determine the effect on GSK-3β. As shown in **Figure 4.3A**, leptin induced the phosphorylation of GSK-3β at serine 9 by 3.2 ± 0.63 - fold ($P<0.05$) at 80 min when compared to the untreated controls. Leptin-induced phosphorylation of GSK-3β on serine 9 was transient, peaking at 80 minutes and returning to control levels after 100 min (data not shown). In a dose response experiment, maximal GSK-3β phosphorylation was observed when BASMCs were treated with 2 µg/ml leptin (**Figure 4.3B**). This dose was used in all subsequent experiments to evaluate the role of leptin in Wnt signaling. The inactivation of GSK-3β by the canonical Wnt ligands is associated with the cytosolic accumulation and subsequent nuclear translocation of β-catenin. To determine whether in our model of osteoblast differentiation, leptin's ability to promote GSK-3β phosphorylation also results in the nuclear accumulation of β-catenin, we performed immunoblot analysis on nuclear extracts of BASMCs treated with leptin for up to 12 hrs. As shown in **Figure 4.3C**, treatment with leptin resulted in an increase in the nuclear accumulation of β-catenin. These findings suggest that leptin may be inducing its osteogenic effects by acting on key

players of the canonical Wnt signaling pathway and mimicking the effects of Wnt ligands.

Figure 4.1 - The effect of leptin on osteoblast differentiation in primary cultures of BASMCs.

Panel A: BASMCs were cultured in the presence of 0 to 4 µg/ml leptin for 8 days before assessing ALP activity as an index of osteoblast differentiation. **Panel B:** Expression of osteoblast-specific markers was analyzed by qRT-PCR in BASMCs cultured in the presence of 2 µg/ml leptin for 8 days. *P<0.001 compared to BASMCs cultured in the absence leptin.

A


Units of ALP Activity/mg protein
(% of Control)
600
500
400
300
200
100
0
0
0.5
1.0
2.0
4.0
Leptin Concentration (µg/ml)
*
*
*

B

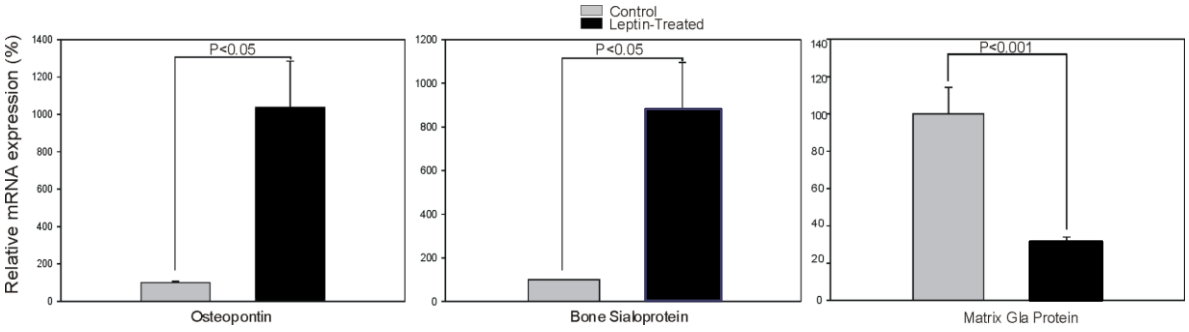
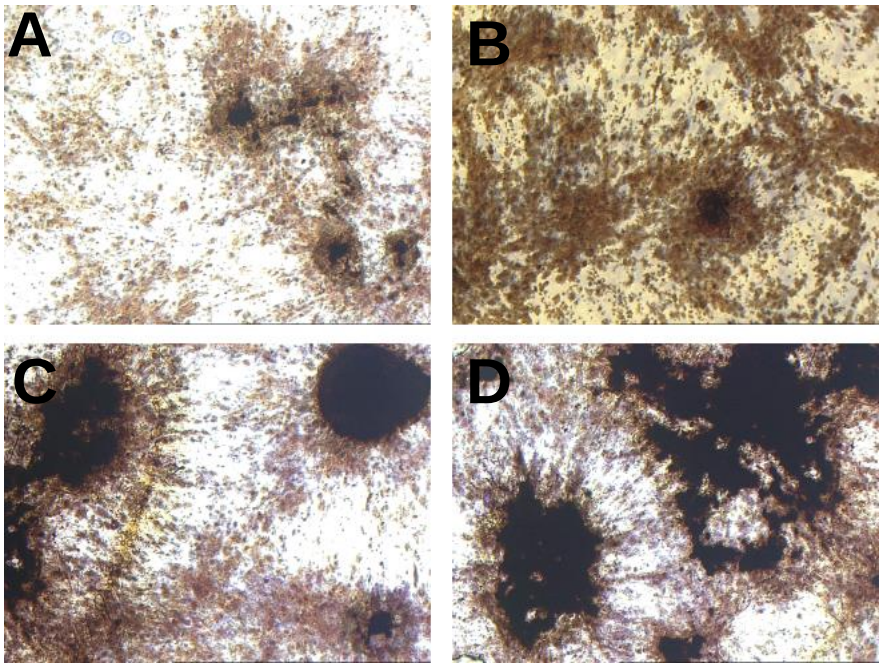

Control
Leptin-Treated
Relative mRNA expression (%)
P<0.05
P<0.05
P<0.001
Osteopontin
Bone Sialoprotein
Matrix Gla Protein

Figure 4.2 - The effect of leptin on mineralization of primary cultures of BASMCs.

Panel A-D: BASMCs were cultured in the absence or presence of 0.5, 1.0 or 2.0 µg/ml leptin, respectively. Twelve days later, the cells were stained by von Kossa to assess the degree of mineralization (**Panel E**). *P<0.05 compared to BASMCs cultured in the absence of leptin.


A
B
C
D

D

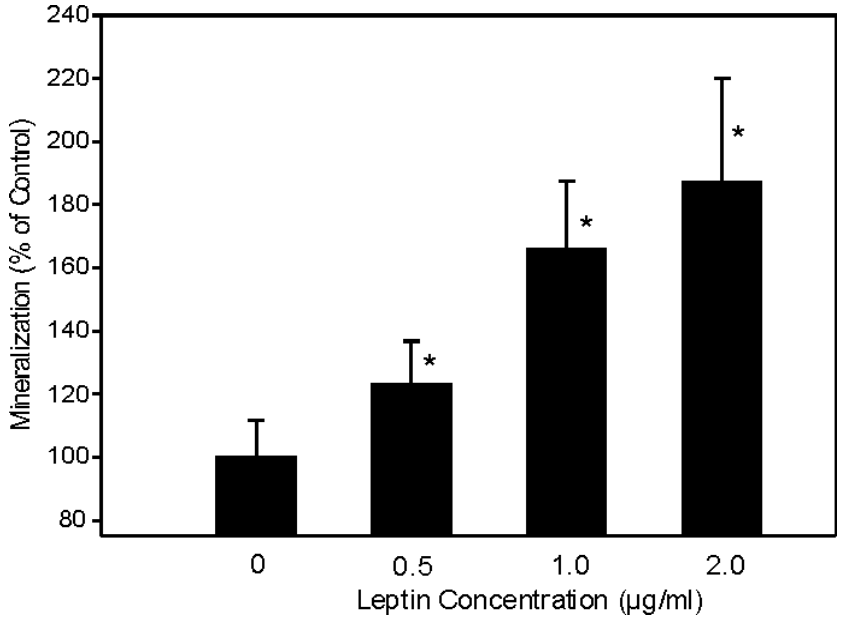
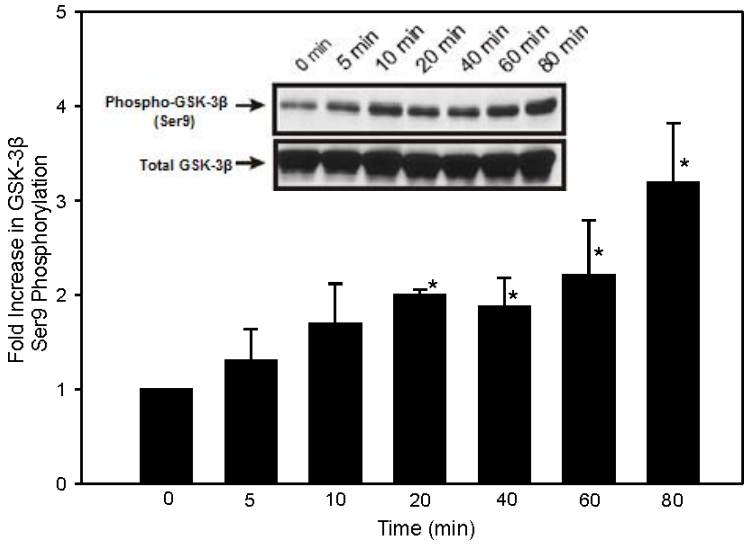

240
220
200
180
160
140
120
100
80
Mineralization (% of Control)
*
*
*
0
0.5
1.0
2.0
Leptin Concentration (µg/ml)

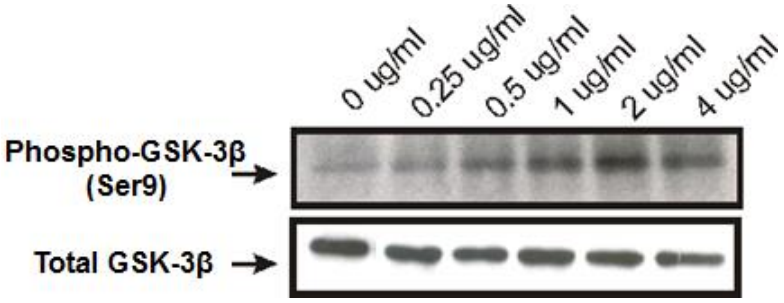
Figure 4.3 - The effect of leptin on GSK-3β phosphorylation and β-catenin accumulation in primary cultures of BASMCs.

Panel A: BASMCs were cultured in the presence of 2 μg/ml leptin for increasing periods of time up to 80 min. **Panel B:** BASMCs were cultured in either the absence or presence of 0.25, 0.5, 1, 2 or 4 μg/ml leptin for 80 min. Cell lysates were prepared and immunoblotted for either phospho-GSK-3β (Ser9) or total GSK-3β. **Panel C:** BASMCs were cultured in the presence of leptin for 3, 9 or 12 hrs before nuclear extracts were isolated and immunoblotted for β-catenin or Lamin A/C. $P < 0.05$ compared to BASMCs cultured in the absence of leptin.

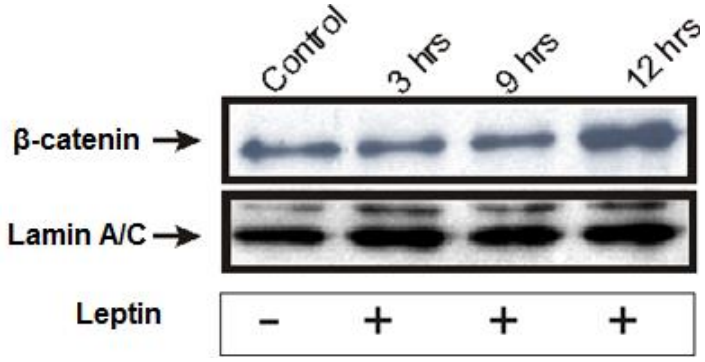
A


0 min
5 min
10 min
20 min
40 min
60 min
80 min
Phospho-GSK-3β (Ser9)
Total GSK-3β
Fold Increase in GSK-3β Ser9 Phosphorylation
0
1
2
3
4
5
*
*
*
*
0
5
10
20
40
60
80
Time (min)

B


0 ug/ml
0.25 ug/ml
0.5 ug/ml
1 ug/ml
2 ug/ml
4 ug/ml
Phospho-GSK-3β (Ser9)
Total GSK-3β

C


Control
3 hrs
9 hrs
12 hrs
β-catenin
Lamin A/C
Leptin
-
+
+
+

4.4.3 Role of GSK-3β in Leptin-Mediated Osteoblast Differentiation

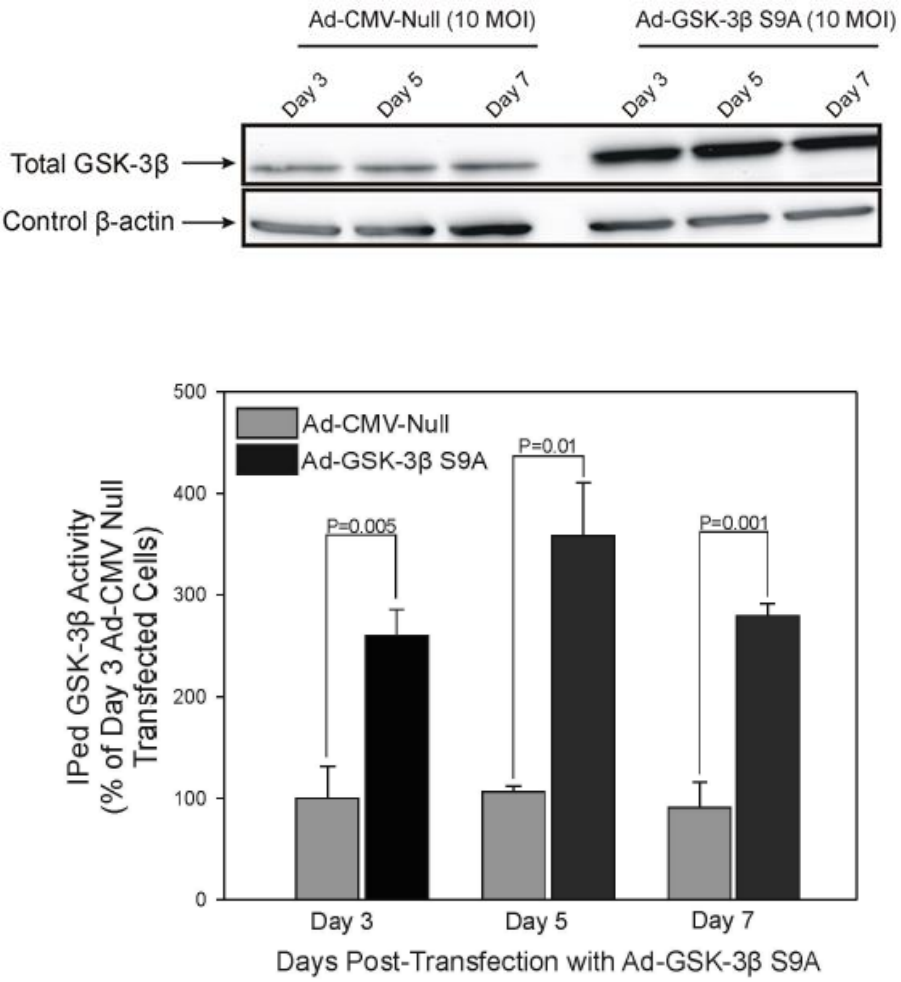
To determine if GSK-3β plays a direct role in osteoblast differentiation, primary BASMCs were infected with an adenovirus expressing constitutively active GSK-3β (Ad-GSK-3β-S9A) or an empty control adenovirus (Ad-CMV-Null). In this cell culture system, total GSK-3β over expression was achieved and sustained for up to 7 days post infection when compared to cells infected with a control adenovirus (**Figure 4.4A**). GSK-3β activity was increased more than 2-fold at 3, 5 and 7 days post infection, compared with cells that were infected with the control adenovirus (**Figure 4.4A**). We then examined the effect of constitutive GSK-3β activity on the mRNA expression of osteoblast-specific markers after 8 days. Constitutive GSK-3β expression significantly decreased osteopontin and bone sialoprotein expression while increasing MGP expression (**Figure 4.4B**). These results suggest that GSK-3β plays a role in osteoblast differentiation. Next, we employed the adenovirus system in an ALP assay (**Figure 4.4C**). BASMCs were once again infected with either Ad-CMV-Null or Ad-GSK-3β S9A and 24 hrs later, cells were cultured in the absence or presence of 2 µg/ml leptin for 8 days. Cells infected with Ad-CMV-Null and treated with leptin showed a 4.8 - fold increase in ALP activity compared to cells cultured in the absence of leptin (63.9 ± 3.43 U/mg vs. 13.2 ± 1.63 U/mg). However, cells infected with Ad-GSK-3β S9A and also treated with leptin did not show the same increase in ALP activity as Ad-CMV Null-infected and leptin-treated BASMCs. Ad-GSK-3β S9A-infected and leptin-treated BASMCs showed only a 3.0 - fold increase in ALP activity

compared to the cells cultured in the absence of leptin (50.6 ± 2.63 U/mg vs. 17.1 ± 0.84 U/mg). Cells that were infected with Ad-GSK-3β S9A and treated with leptin had a significantly attenuated increase in ALP activity compared to cells infected with a control adenovirus and also treated with leptin (P=0.01). These results suggest that GSK-3β also plays a role in leptin-mediated osteoblast differentiation.

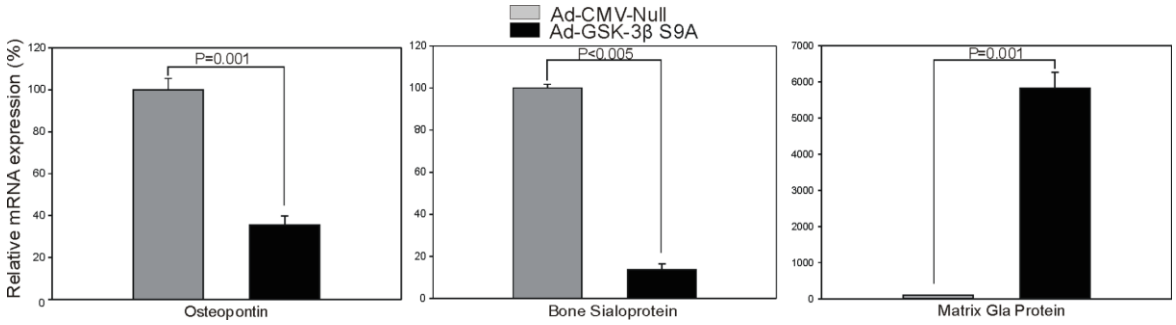
Figure 4.4 - Transfection of primary cultures of BASMCs with adenovirus that constitutively expresses active GSK-3β.

Panel A: BASMCs were transfected with 10 MOI of either an empty adenovirus (Ad-CMV-Null) or an adenovirus that constitutively expresses active GSK-3β (Ad-GSK-3β S9A) for up to 7 days. Immunoblots were probed with total GSK-β antibody to verify adenovirus expression and GSK-3β activity was measured by ^{32}-P incorporation onto phospho-glycogen synthase peptide-2 substrate. **Panel B:** Expression of osteoblast-specific markers was analyzed by qRT-PCR in BASMCs transfected Ad-CMV-Null or Ad-GSK-3β S9A. **Panel C:** ALP activity was measured as an index of osteoblast differentiation in BASMCs transfected with Ad-CMV-Null or Ad-GSK-3β S9A and cultured in the absence or presence of 2 µg/ml leptin for 8 days.

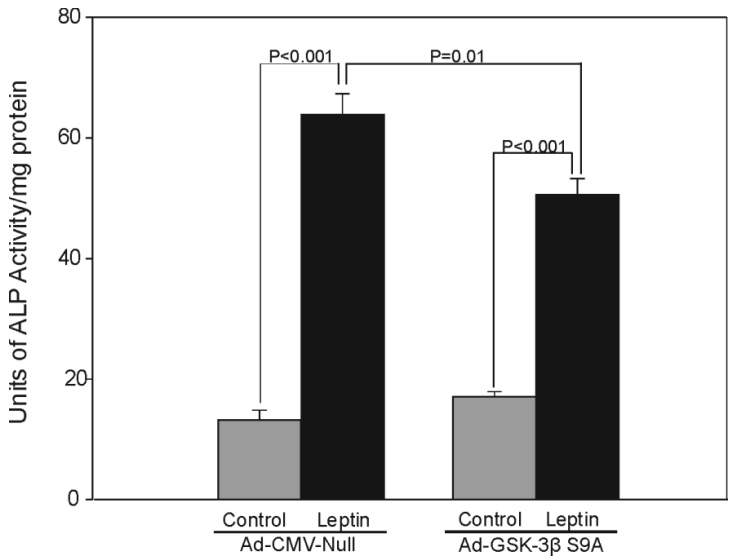
A


Ad-CMV-Null (10 MOI)
Ad-GSK-3β S9A (10 MOI)
Day 3
Day 5
Day 7
Day 3
Day 5
Day 7
Total GSK-3β
Control β-actin
IPed GSK-3β Activity (% of Day 3 Ad-CMV Null Transfected Cells)
Ad-CMV-Null
Ad-GSK-3β S9A
P=0.005
P=0.01
P=0.001
0
100
200
300
400
500
Day 3
Day 5
Day 7
Days Post-Transfection with Ad-GSK-3β S9A

B


Ad-CMV-Null
Ad-GSK-3β S9A
Relative mRNA expression (%)
P=0.001
P<0.005
P=0.001
Osteopontin
Bone Sialoprotein
Matrix Gla Protein

C


80
60
40
20
0
Units of ALP Activity/mg protein
P<0.001
P=0.01
P<0.001
Control
Leptin
Ad-CMV-Null
Control
Leptin
Ad-GSK-3β S9A

4.5 Discussion

We have previously shown that the adipocytokine, leptin, can promote the vascular calcification of atherosclerotic lesions in ApoE - deficient mice. We demonstrated that the increase in vascular calcification was associated with an increase in the presence of osteoblast-specific markers (e.g. alkaline phosphatase, osteocalcin and osteopontin) within the vessel wall (Zeadin *et al.* 2009). Vascular calcification is a tightly-regulated, cell-mediated process that recapitulates the progression of bone mineralization. Several theories exist to describe the process by which vascular calcification may be occurring (Johnson *et al.* 2006). Vascular calcification may result from the loss of calcification inhibitors such as MGP (Luo *et al.* 1997;Price *et al.* 2002b), the nonspecific entrapment of nucleation complexes that arise due to bone resorption (Bucay *et al.* 1998;Min *et al.* 2000) or the phenotypic transition of a subpopulation of VSMCs into bone-forming (osteoblast-like) cells (Bear *et al.* 2008;Dhore *et al.* 2001;Steitz *et al.* 2001). Our *in vivo* findings suggest that leptin enhances the expression of osteoblast-specific markers within the vessel wall by inducing the differentiation of VSMCs into osteoblast-like cells. How leptin is exerting these effect is not known.

In the current study, we used primary cultures of BASMCs to investigate the mechanism by which leptin induces vascular calcification. Our lab and others have previously demonstrated that BASMCs can undergo a phenotypic transition, losing their smooth muscle phenotype and becoming “osteoblast-like” (Reseland

et al. 2001;Shioi *et al.* 1995;Taylor *et al.* 2011;Yang *et al.* 2005). Bear et al. (Bear *et al.* 2008) showed that oxidized-LDL can promote the differentiation of BASMCs and upregulate the expression of the osteoblast-specific transcription factor, osterix (Osx). Other *in vitro* evidence suggests that leptin can also promote both osteoblast differentiation and mineralization in various cell types (Parhami *et al.* 2001;Reseland *et al.* 2001;Thomas *et al.* 1999). For example, in the presence of leptin, the bipotential human marrow stromal cell line, hMS2-12, showed a dose- and time-dependent increase in ALP activity, type I collagen and osteocalcin mRNA and protein expression thus favouring osteogenic differentiation over adipocyte differentiation (Thomas *et al.* 1999). Consistent with these findings, we demonstrated that leptin can induce osteogenic differentiation and mineralization of primary cultures of BASMCs (**Figure. 4.1** and **Figure 4.2**).

Since vascular calcification shares many similarities with the process of bone formation, we examined the role of Wnt signaling in our *in vitro* model of osteogenic differentiation. We show that constitutive GSK-3β expression significantly attenuates osteopontin and bone sialoprotein expression while increasing MGP expression in the absence of leptin (**Figure 4.4B**). This suggested to us that GSK-3β may be playing a role in the osteoblast differentiation of our primary cultures of VSMCs. One mechanism by which the activity of GSK-3β is inhibited is by phosphorylation of its serine residue at position 9. Inactive GSK-3β can no longer phosphorylate β-catenin. Therefore, β-catenin accumulates in the cytoplasm and excess β-catenin translocates to the

nucleus and heterodimerizes with members of the LEF/TCF family of transcription factors to regulate the expression of various genes including those involved in osteoblast differentiation (Baron and Rawadi 2007;Glass and Karsenty 2007). In the current study, we show that leptin signaling promotes the phosphorylation of GSK-3β on serine 9 (**Figure 4.3A** and **Figure 4.3B**) and that the phosphorylation of GSK-3β is associated with the nuclear accumulation of β-catenin (**Figure 4.3C**). The *in vitro* activation of Wnt signaling has previously been shown to upregulate the osteogenic differentiation of mesenchymal stem cells (Day *et al.* 2005;de Boer *et al.* 2004) and the Wnt signaling pathway has been shown to be active in the calcified vasculature of diabetic low density lipoprotein receptor – deficient ($LDLR^{-/-}$) mice (Shao *et al.* 2005). We demonstrate that Ad-GSK-3β S9A-infected, leptin-treated BASMCs have a significantly attenuated increase in ALP activity compared to Ad-CMV Null-infected, leptin-treated cells which also suggests that GSK-3β plays a role in leptin-mediated osteoblast differentiation.

The mechanism(s) by which leptin modulates GSK-3β phosphorylation/activity to promote the osteogenic differentiation of VSMCs remains to be determined. It is well established that leptin can regulate many signaling pathways including JAK/STAT, mitogen-activated protein kinases (MAPK), suppressors of cytokine signaling (SOCS) and insulin receptor substrate/phosphatidylinositol-3-kinase (IRS/PI3K) (Sweeney 2002). More recently, Yan et al. (Yan *et al.* 2012) demonstrated that leptin induces epithelial-

mesenchymal transition in breast cancer cells by modulating Akt/GSK-3β and MTA1/Wnt1 axes to mediate β-catenin activation. Alternatively, in hepatocellular carcinomas, it has been shown that extracellular-regulated kinase (ERK) acts to prime GSK-3β for subsequent phosphorylation at serine 9 by p90RSK. This event results in the inactivation of GSK-3β and the subsequent accumulation of β-catenin (Ding *et al.* 2005). Our lab has previously shown that the treatment of cells with PD98059 significantly and dose-dependently attenuates ALP activity by up to 60% suggesting that leptin's ability to promote the osteoblast differentiation of primary BASMCs is, at least in part, ERK 1/2 – dependent (Zeadin *et al.* 2009). Thus it is possible that leptin induces the phenotypic transition of smooth muscle cells by modulating ERK/GSK-3β to mediate β-catenin activation (see **Figure 4.5** for a working model of leptin-induced osteoblast differentiation). Leptin has been shown to signal through STAT3 and/or STAT5 in other cell types, however not in VSMCs (Bodary *et al.* 2007). Therefore, leptin-mediated STAT signaling was not considered to play a role in our model of leptin-induced vascular calcification.

We observed that leptin significantly upregulated the expression of various pro-osteogenic factors including, osteopontin, and bone sialoprotein while downregulating the expression of MGP – a key calcification inhibitor. Future studies should also aim to investigate which homeodomain-containing proteins (Msx2, Dlx3, Dlx5, ATF4, Runx2 and Osx) are playing a role in leptin-mediated osteoblast differentiation. Runx2 and Osx are widely recognized as master osteogenic transcription factors since Runx2-deficient and Osx-deficient mice

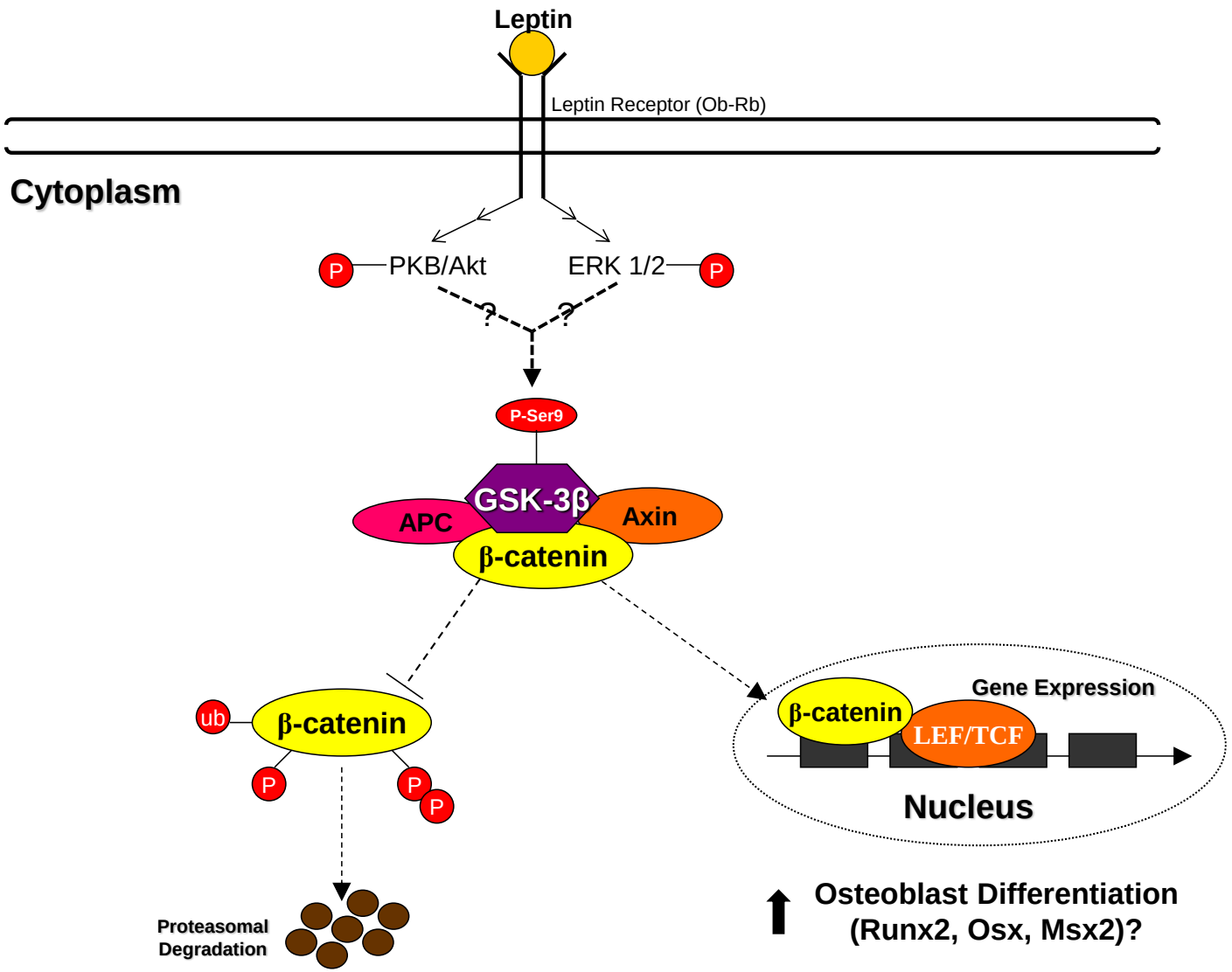
lack functional osteoblasts and do not exhibit a mineralized cartilaginous skeleton (Komori 2005;Nakashima *et al.* 2002). Osx can be regulated by Runx2 - dependent and - independent mechanisms involving Dlx5 or Msx2 (Matsubara *et al.* 2008;Ulsamer *et al.* 2008). In line with these findings, Runx2, Dlx5 and Msx2 promoter regions are reported to contain Wnt and BMP-responsive regions (Rodriguez-Carballo *et al.* 2011). Runx2 is a positive regulator of various bone matrix proteins including osteocalcin, osteopontin, bone sialoprotein, type I collagen and osteoprotegrin. However, it is reported that Msx2 can suppress osteocalcin expression though chromatin interaction but in addition to Msx2, Dlx3 and Dlx5 are also variably expressed to regulate osteocalcin expression among other osteoblast-specific markers (Rodriguez-Carballo *et al.* 2011). These observations suggest that osteoblast differentiation is a rather complex process that depends on temporal expression of various factors and is highly influenced by protein-protein and protein-DNA interactions, thus requiring further research.

Clinical trials support the current research by demonstrating a positive correlation between circulating plasma leptin levels and CVD. The five year prospective West of Scotland Coronary Prevention Study (WOSCOPS) (Wallace *et al.* 2001) demonstrated that leptin is a significant risk factor for CVD while a separate finding concluded that plasma leptin levels are a strong predictor for the risk of an acute myocardial infarction (Soderberg *et al.* 1999). Other studies have shown an association between leptin and the risk of coronary artery calcification in a population of type 2 diabetic and non-diabetic individuals (Qasim *et al.*

2008;Reilly *et al.* 2004). Our findings provide a novel mechanism by which elevated leptin levels, associated with obesity, promote osteoblast differentiation and vascular calcification *in vivo* by regulating GSK-3β activity in VSMCs.

Figure 4.5 - Our working model of leptin-induced osteoblast differentiation.

The binding of leptin to its receptor on the surface of VSMCs promotes the downstream phosphorylation/activation of kinases, ERK 1/2 and Akt. ERK 1/2 and Akt inactivate GSK-3β through phosphorylation of the serine 9 residue. Inactivated GSK-3 cannot promote the phosphorylation and subsequent degradation of β-catenin. Instead, β-catenin accumulates in the cytoplasm and translocates to the nucleus where it promotes osteoblast differentiation by upregulating the expression of osteoblast-specific transcription factors in an Akt/ERK 1/2-dependent manner. This model predicts that Akt, ERK 1/2 inhibition or constitutively active GSK-3 will attenuate leptin-induced osteoblast differentiation and that a dominant negative isoform of GSK-3 will promote osteoblast differentiation, even in the absence of leptin signaling.


Leptin
Leptin Receptor (Ob-Rb)
Cytoplasm
P
PKB/Akt
ERK 1/2
P
?
?
P-Ser9
GSK-3β
APC
Axin
β-catenin
ub
β-catenin
P
P
P
Proteasomal Degradation
Gene Expression
β-catenin
LEF/TCF
Nucleus
Osteoblast Differentiation
(Runx2, Osx, Msx2)?

CHAPTER 5.0: Investigating the Role of ER stress and GSK-3 in the Osteogenic Differentiation of the MOVAS Cell Line.

Foreword

For these studies, we use a novel murine vascular smooth muscle cell line (MOVAS) as our model of vascular calcification. MOVAS cells were originally isolated in 1999, and have been specifically evaluated as an *in vitro* model of vascular calcification (Mackenzie *et al.* 2011). The objective of our current study was to investigate the effect of modulating GSK-3, using agents and conditions that promote or attenuate ER stress, on the differentiation of VSMCs.

We show that ER stressor, glucosamine, and ER stress modulator, PBA, promote the osteogenic differentiation of MOVAS cells by different mechanisms which appear to be independent of their effect on ER stress. This is supported by the results of our ALP assays with ER stress-inducing agents, tunicamycin and A23187. While both tunicamycin and A23187 attenuate ALP activity, only tunicamycin increases the expression of markers of the UPR. The increase in ER stress which we observe in our cell culture model is likely a consequence of the osteogenic differentiation that VSMCs are undergoing. During osteoblast differentiation, the UPR acts to expand the capacity of the ER to manage the increase in the production of extracellular matrix proteins and to avoid apoptosis induced by ER overload. The current ER stress tools display multiple and sometimes conflicting effects. This makes it a challenge to investigate the effect

of the ER stress - GSK-3 axis on osteogenic differentiation in VSMCs. We propose that future studies will need to focus on (1) delineating the mechanisms of various ER stressors/attenuators independently of one another; (2) using consistent cell culture models; and (3) repeating these experiments in various mouse models to determine if they in fact promote vascular calcification under physiological conditions.

This work will be submitted for publication in the fall of 2014. The authors are the following: Melec G. Zeadin and Geoff H. Werstuck. The experiments in this chapter were performed by Melec Zeadin. The manuscript was written by Melec Zeadin in collaboration with Dr. Geoff Werstuck.

5.1 Abstract

In these studies, we utilize a novel murine vascular smooth muscle cell line (MOVAS) as our *in vitro* model for vascular calcification. Our aim was to investigate the effect(s) of modulating GSK-3 activity on the differentiation of vascular smooth muscle cells (VSMCs) using agents and conditions that promote or attenuate ER stress. We show that chronic exposure to glucosamine promotes the osteogenic differentiation of MOVAS cells by a mechanism which is likely independent of GSK-3. Suprisingly, the chemical chaperone, PBA, also promotes the osteogenic differentiation of MOVAS cells and significantly attenuates GSK-3 activity. However, both glucosamine and PBA, promote ER stress in our *in vitro* model of vascular calcification. We speculate that the mechanisms of glucosamine - and PBA – mediated osteogenic differentiation may be independent of the ER stress – GSK-3 signaling axis. This is supported by the results of our ALP assays with ER stress-inducing agents, tunicamycin and A23187. While both tunicamycin and A23187 attenuate ALP activity, only tunicamycin increases the expression of markers of the unfolded protein response (UPR). Thus, the currently available tools to modulate ER stress display multiple, and sometimes conflicting, effects that involve regulation through multiple pathways. This makes it a challenge to investigate the direct effect of the ER stress - GSK-3 axis on osteogenic differentiation in VSMCs. We propose that future studies will need to focus on (1) delineating the mechanisms of various ER stressors/attenuators independently of one another; (2) using consistent cell

culture models; and (3) repeating these experiments in various mouse models to determine if they in fact promote vascular calcification under physiological conditions.

5.2 Introduction

Vascular calcification, an important complication of cardiovascular disease, is a tightly regulated process within the artery wall that shares many fundamental characteristics with the process of true bone formation. Vascular calcification is associated with significantly impaired cardiovascular hemodynamics resulting in an increased risk of cardiovascular events and all-cause mortality (Bobryshev *et al.* 2008;Detrano *et al.* 2008;Rennenberg *et al.* 2009;Vliegenthart *et al.* 2002). Although the clinical implications are well recognized, the mechanism(s) driving the development and progression of vascular calcification are still being investigated. *In vivo* and *in vitro* studies have linked several negative and positive regulators, including serum phosphate, lipid-derived molecules (oxysterol and oxidized phospholipids), and hormonal factors (bone morphogenetic protein-2, matrix gla protein, and vitamin D) to the pathogenesis of vascular calcification (Demer and Tintut 2011;Johnson *et al.* 2006). Our lab has previously shown that elevated levels of circulating leptin, which are associated with obesity, promote osteoblast differentiation and vascular calcification by down-regulating the activity of glycogen synthase

kinase–3 α/β (GSK-3) in vascular smooth muscle cells (VSMCs) (Zeadin *et al.* 2009;Zeadin *et al.* 2012).

GSK-3 is a multifunctional serine/threonine kinase now known to be involved in a variety of cellular processes including signal transduction, protein synthesis, cell migration, mitosis, apoptosis and cell fate determination. Dysregulated GSK-3 activity is implicated in a number of human diseases including, Alzheimer's, cancer, bone abnormalities, diabetes and atherosclerosis (Luo *et al.* 2007). For example, hyperglycemic apolipoprotein E-deficient ($ApoE^{-/-}$) mice supplemented with either valproate or lithium chloride, both inhibitors of GSK-3, develop significantly smaller lesions than non-supplemented control mice with hyperglycemia. More recently, our lab has shown that $LDLR^{-/-}$/GSK-$3\alpha^{-/-}$ mice fed a high fat diet develop significantly smaller atherosclerotic lesions when compared to $LDLR^{-/-}$/GSK-$3\alpha^{+/+}$ control mice (Banko *et al.* 2014, in press).

There is evidence that supports a causative role for endoplasmic reticulum (ER) stress and/or activation of the unfolded protein response (UPR) in the activation of GSK-3 activity. The UPR is activated in response to the accumulation of unfolded or misfolded proteins within the lumen of the ER. Traditional ER stress-inducing agents disrupt protein folding by interfering with disulphide bond formation (dithiothreitol (DTT)) (Jamsa *et al.* 1994), ER Ca^{2+} balance (A23187, thapsagargin) (Li *et al.* 1993), ER membrane structure (palmitate, unesterified cholesterol) (Borradaile *et al.* 2006;DeVries-Seimon *et al.* 2005) or by blocking protein N-glycosylation (tunicamycin) (Feige and Scheffler

1987). Conditions of ER stress have been shown to increase the activity of GSK-3 in cultured cells (Bowes *et al.* 2009).

Several independent risk factors for cardiovascular disease including, hyperglycemia (Werstuck *et al.* 2006), hyperhomocysteinemia (Werstuck *et al.* 2001;Zhou *et al.* 2004), elevated levels of unesterified cholesterol (Li *et al.* 2005b) or palmitate (Cunha *et al.* 2008) and obesity (Ozcan *et al.* 2004), can also promote the activation of the UPR. Furthermore, our lab has shown that the pro-atherogenic effects of ER stress may also be mediated through the activation of GSK-3 and that inhibiting GSK-3 attenuates atherosclerosis in hyperglycemic, hyperhomocysteinemic, and dysplipidemic ApoE$^{-/-}$ mouse models (Bowes *et al.* 2009;McAlpine *et al.* 2012).

Thus, we have shown that the adipocytokine, leptin, promotes the osteogenic differentiation of VSMCs by inhibiting the activity of GSK-3β, and yet, cardiovascular risk factors appear to accelerate atherosclerosis by a mechanism involving ER stress-induced GSK-3 activity. Knowing that the plasticity and phenotypic modulation of VSMCs plays a pivotal role in the pathogenesis of cardiovascular disease led us to hypothesize that GSK-3 may function as a checkpoint at which VSMCs commit toward an osteogenic or 'synthetic' phenotype in the pathogenesis of vascular disease (**Figure 5.1**).

VSMCs are known to have the capability of displaying phenotypic diversity (**Figure 5.2**). Normally, in the medial layer of the artery wall, VSMCs are responsible for maintaining vascular tone and responsiveness to vasoconstrictive

(e.g. endothelin-1, serotonin, thromboxanes) or vasodilative (e.g. prostacyclin, nitric oxide) signals. These VSMCs are characterized by a mature differentiated 'contractile' phenotype and the expression of proteins involved in the maintenance of myofilament structure and function. The proteins that characterize a contractile SMC phenotype include: smooth muscle α-actin (αSM-actin; gene: *ACTA2*), smooth muscle 22 alpha (SM22α; gene: *TAGLN)*; and smooth muscle – myosin heavy chain (SM-MHC; gene: *MYH11)* (Beamish *et al.* 2010) . VSMCs can undergo a phenotypic switch in a process called de-differentiation to become 'synthetic' VMSCs which are associated with vascular disease or injury. This synthetic phenotype is characterized by an increase in the expression of markers such as vimentin (gene: *VIM*), tropomyosin 4 (gene: *TPM4*) and collagen, type III, alpha 1 (type III collagen; gene: *COL3A1*) (Beamish *et al.* 2010). VSMCs likely exist in a differentiated contractile state in the medial layer, de-differentiating into a synthetic phenotype as they migrate into the intimal layer in response to arterial injury. This acquired ability to migrate, proliferate and secrete an extracellular matrix is essential for vascular repair.

Vascular calcification is also a prominent feature of (1) atherosclerosis and (2) Mönckeberg's sclerosis. Mönckeberg's sclerosis (or medial calcific sclerosis) is associated with diabetes which is characterized by calcification of the medial layer. It is evident that local factors in a pathological environment can induce VSMCs to undergo osteogenic differentiation. Specific markers are up-regulated under conditions of calcification including runt-related transcription factor 2

(Runx2), osterix (Osx), osteocalcin and alkaline phosphatase (ALP) (Iyemere *et al.* 2006). Runx2 and Osx are widely recognized as master osteogenic transcription factors since Runx2-deficient and Osx-deficient mice lack functional osteoblasts and do not exhibit a mineralized cartilaginous skeleton (Komori 2005;Nakashima *et al.* 2002). ALP activity is essential to bone mineralization. A missense mutation in the human tissue nonspecific ALP was identified in a rare heritable form of rickets referred to as hypophosphatasia (Henthorn and Whyte 1992). Several *in vitro* and *in vivo* studies have demonstrated that ALP expressed in vascular cells plays an important role in vascular calcification (Levy *et al.* 1991;Maranto and Schoen 1988;Narisawa *et al.* 2007;Shanahan *et al.* 1999;Shioi *et al.* 1995;Shioi *et al.* 2002).

For these studies, a novel murine vascular smooth muscle cell line (MOVAS) was used as our *in vitro* model for vascular calcification. MOVAS cells were originally isolated in 1999, and have been specifically evaluated as an *in vitro* model of vascular calcification. The same study determined that this cell line offers a reliable and economical alternative to primary cells while also reducing experimental variation and allowing stable transformation (Mackenzie *et al.* 2011). The objective of our current study was to investigate the effect of modulating GSK-3, using agents and conditions that promote or attenuate ER stress, on the differentiation of VSMCs.

Figure 5.1 - Proposed model: GSK-3 modulates the pathogenesis of vascular calcification/atherosclerosis.

We have previously shown that increased leptin levels which are associated with obesity can promote osteoblast differentiation and thus vascular calcification by a mechanism which involves the **inhibition** of GSK-3β activity. ER stress has been shown to promote the accelerated development of atherosclerosis by a mechanism which involves the **activation** of GSK-3β. The inhibition of GSK-3β activity using valproate (VPA) significantly attenuates atherosclerosis. **Therefore, we hypothesize that increasing GSK-3 activity cause a phenotypic switch in VSMCs toward a pro-atherogenic pathway. We propose to examine the physiological role that GSK-3 plays in modulating the pathogenesis of vascular calcification/atherosclerosis by using ER stress inducing agents.**

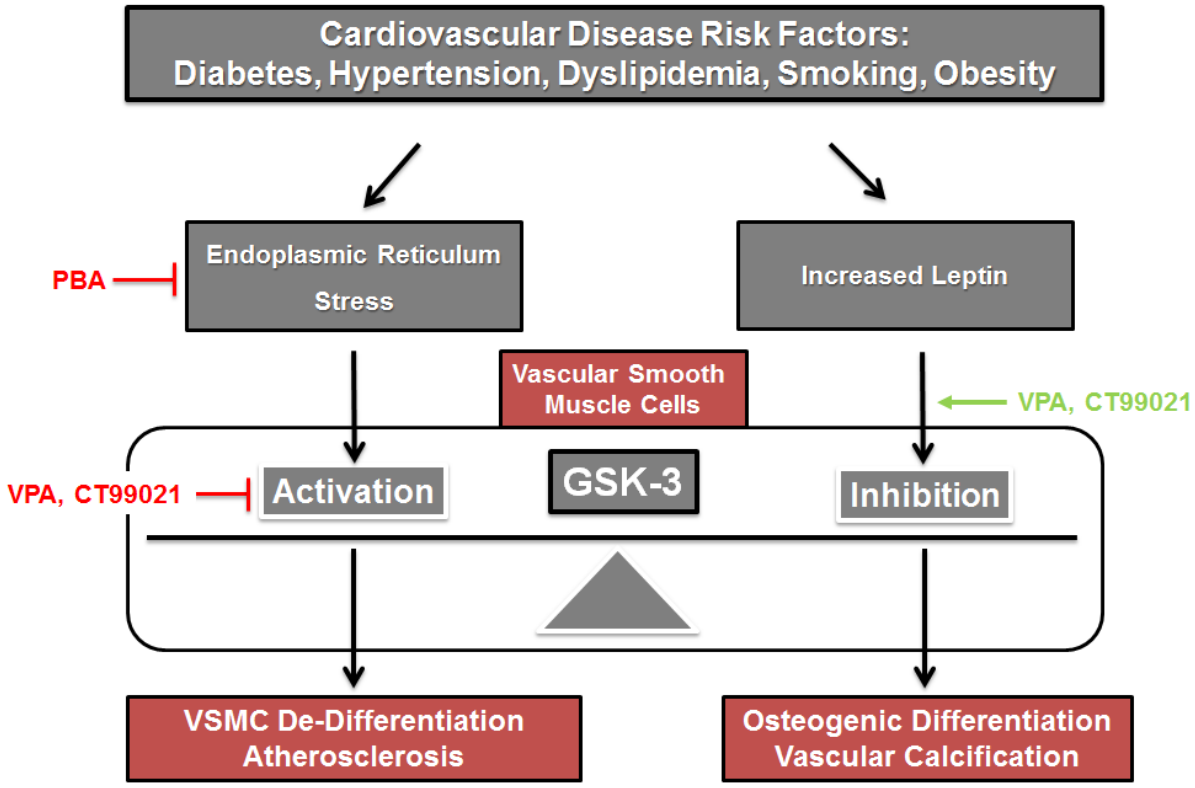
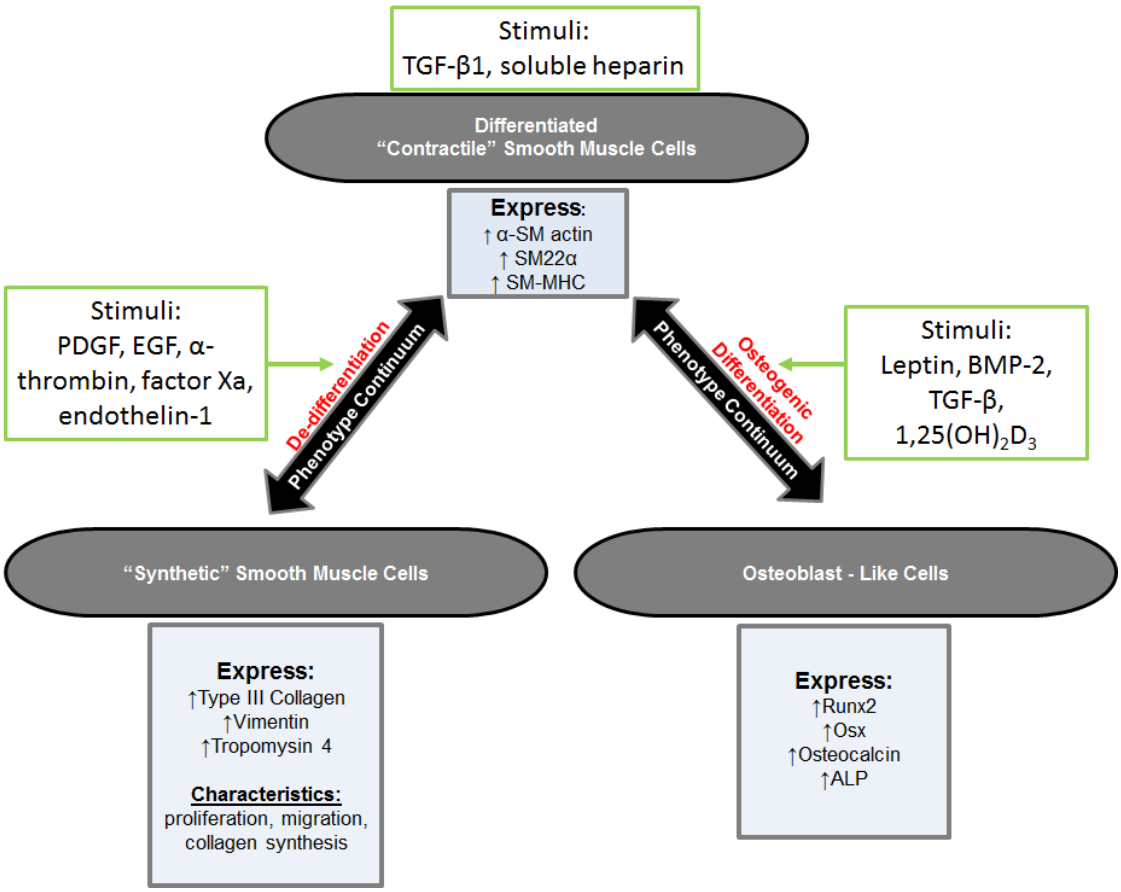

Cardiovascular Disease Risk Factors:
Diabetes, Hypertension, Dyslipidemia, Smoking, Obesity
PBA
Endoplasmic Reticulum Stress
Increased Leptin
Vascular Smooth Muscle Cells
VPA, CT99021
VPA, CT99021
Activation
GSK-3
Inhibition
VSMC De-Differentiation
Atherosclerosis
Osteogenic Differentiation
Vascular Calcification

Figure 5.2 - Summary of the phenotype characteristics of VSMCs and osteoblast - like cells.

Normally, in the medial layer, VSMCs are responsible for maintaining vascular tone and exhibit a mature differentiated ‘contractile’ phenotype. This phenotype is characterized by the expression of smooth muscle α-actin (αSM-actin; gene: ACTA2), smooth muscle 22 alpha (SM22α; gene: TAGLN); and smooth muscle – myosin heavy chain (SM-MHC; gene: MYH11). VSMCs can undergo a phenotypic switch in a process called de-differentiation to become ‘synthetic’ VMSCs which are associated with vascular disease or injury. This synthetic phenotype is characterized by an increase in the expression of markers such as vimentin (gene: VIM), tropomyosin 4 (gene: TPM4) and type III collagen (gene: Col3A1). VSMCs can also be prompted to undergo osteogenic differentiation. Specific markers are up-regulated under conditions of calcification including runt-related transcription factor 2 (Runx2), osterix (Osx), osteocalcin and alkaline phosphatase (ALP). The position along the phenotypic continuum is modulated by a variety of extracellular signals.


Stimuli:
TGF-β1, soluble heparin
Differentiated
"Contractile" Smooth Muscle Cells
Express:
↑ α-SM actin
↑ SM22α
↑ SM-MHC
Stimuli:
PDGF, EGF, α-thrombin, factor Xa, endothelin-1
De-differentiation
Phenotype Continuum
Osteogenic Differentiation
Phenotype Continuum
Stimuli:
Leptin, BMP-2, TGF-β, 1,25(OH)2D3
"Synthetic" Smooth Muscle Cells
Osteoblast - Like Cells
Express:
↑Type III Collagen
↑Vimentin
↑Tropomysin 4
Characteristics:
proliferation, migration, collagen synthesis
Express:
↑Runx2
↑Osx
↑Osteocalcin
↑ALP

5.2 Materials and Methods

5.2.1 Materials

MOVAS cell line (ATCC Cat. No CRL-2797), high glucose (4.5 g/L) Dulbecco's Modified Eagle's Medium (DMEM; ATCC Cat. No 30-2002), fetal bovine serum (FBS; ATCC – tested Cat. No 30-2020), geneticin antibiotic (Cat. No 10131035) and 0.25% Trypsin/0.53 mM EDTA (Cat. No 30-2101) were purchased from Cedarlane (Burlington, ON). Low glucose DMEM (1 g/L; Invitrogen, Burlington, ON) was purchased from Invitrogen (Burlington, ON). Glucosamine (Cat. No G4875), valproic acid (Cat. No P4543), tunicamycin (Cat No. T7765), A23187 (Cat No. C7522), Alizarin Red S (Cat No. A5533) and SIGMA FAST *p*-nitrophenyl phosphate tablets used to assess ALP activity were purchased from Sigma-Aldrich (St.Louis, MO; Cat. No N2770). GSK-3 inhibitor, CT99021 was obtained from Selleckchem.com (Houston, TX; Cat. No S1263). 4-Phenylbutyric acid (PBA) was purchased from Sciencelab.com, Inc (Houston, TX; SLP2254); anti-KDEL (Grp78/94), GADD153/CHOP, and β-actin antibodies were purchased from Assay Designs (Farmingdale, NY; Cat. No SPA-827), Santa Cruz Biotechnology (Santa Cruz, CA; Cat. No sc-7351), and Sigma-Aldrich (Cat. No A3854), respectively. Finally, Albumin Bovine Serum Fraction V, Fatty Acid Poor, Endotoxin Free (Cat. No 125579) was obtained from Calbiochem (La Jolla, CA) and Bovine Serum Albumin, Fatty Acid Free, Low Endotoxin (Cat. No A8806) was purchased from Sigma-Aldrich.

5.2.2 MOVAS Cell Culture

MOVAS cells were grown in T-75 flasks with 0.2mg/ml geneticin and 10% FBS supplemented DMEM (4.5g/L glucose) at 37°C and 5% CO_2. Once cells reached confluency and were ready to be seeded, MOVAS cells were briefly rinsed with 0.25%Trypsin/0.53 mM EDTA to remove all traces of serum. Cells were then dispersed with a fresh solution of Trypsin/EDTA. For all experiments, cells were seeded at a density of 0.5 x 10^4 cells/cm^2 and maintained in low glucose DMEM (1g/L glucose; Invitrogen, Burlington, ON) unless specified otherwise. Medium was changed every third day. Cells were not passaged.

5.2.3 Preparation of Glucosamine and PBA

Glucosamine was prepared to a concentration of 100 mM by dissolving in low glucose DMEM. PBA was prepared to a concentration of 200 mM by dissolving in 70% ethanol (12.5% total volume) before adding low glucose DMEM to make up the final volume. pH of PBA solution was equilibrated with 10N NaOH. All solutions were filter sterilized using a 0.2 µM syringe filter before treating cells.

5.2.4 Immunoblot Analysis

Cells were plated in 6-well dishes for immunoblot analysis. At confluence, MOVAS cells were treated with increasing concentrations of ER stress modulating agents (glucosamine, tunicamyin, A23187 and/or PBA) for 18 hrs. Whole cell extracts were harvested and solubilized in GSK-3 buffer containing 50

mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 1 mM benzamidine, 0.1X Roche PhosSTOP, 1 mM Na_3VO_4, and 1 mM PMSF. Samples were then loaded onto a 10% SDS-PAGE and run at 4°C and 120 V before being transferred onto a nitrocellulose membrane using the semi-dry transfer cell. The membrane was washed with TBST [50 mM Tris (pH 7.4), 150 mM NaCl and 0.05% Tween-20] before being blocked in 5% non-fat skim milk containing 50 mM Tris (pH 8.0), 150 mM NaCl and 0.05% Tween-20 for one hour at 37°C and incubated with primary antibody at 4°C overnight. The next day, membranes were washed and incubated with the corresponding secondary antibody for one hour at room temperature. Protein bands were developed using the Immobilon Western Chemiluminescent Horseradish Peroxidase Substrate (Millipore, Billerica, MA) and visualized with the ChemiDoc XRS System (BioRad). Results were quantified using Image Lab Software 4.1.

5.2.5 Measurement of Alkaline Phosphatase Activity

To assess the extent of osteoblast differentiation, MOVAS cells were seeded in 24-well plates and cultured for 7, 14 or 21 days. Cells were then treated with a combination of increasing concentrations of glucosamine in the absence or presence of PBA. In some experiments, cells were cultured in the presence of increasing concentrations of the GSK-3 inhibitors, valproate or CT99021 (high glucose DMEM supplemented with β-glycerophosphate). Extracts were lysed in 1% Triton X-100/0.9% NaCl for one hour on ice and centrifuged at

12,000 x g for 15 min at 4°C. The supernatant was assayed for protein content using Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) and ALP activity was assessed by measuring the cleavage of *p*-nitrophenyl phosphate (pNPP) at 405 nM. ALP values were normalized to protein (U/mg of protein).

5.2.6 Alizarin Red Staining

MOVAS cells were plated in 6-well dishes. Calcium deposition was detected by staining with Alizarin Red S as previously outlined ((Mackenzie *et al.* 2011). Briefly, cells were washed twice with 1X phosphate buffered saline (PBS, pH 7.4), fixed in 4% paraformaldehyde/PBS for 5 min at 4°C before being washed again in 1X PBS and distilled water. Finally, cells were stained with 2% Alizarin Red S (pH 4.2) made fresh for 5 min at room temperature with gentle rocking, rinsed several times with distilled water and visualized under a 4 × objective (N.A. 0.13) with an Olympus CKX41 microscope and Infinity1-3C camera and saved using Infinity Analyze software (Lumenera Corp., Ottawa, ON).

5.2.7 Total GSK-3 Activity Assay

MOVAS cells were cultured in 60 mm plates in the absence or presence of glucosamine and/or PBA for 18hrs. To measure total GSK-3 activity, whole cell lysates were collected in GSK-3 lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 10 mM DTT, 1 mM benzamidine, 0.1X Roche PhosSTOP, 1 mM Na_3VO_4, and 1 mM PMSF]. Equal

amounts of protein were prepared in 4X Reaction Buffer [8 mM MOPS (pH7.4), 0.2 mM EDTA, 10 mM Mg acetate]. In these experiments, cell lysates were not immunoprecipitated with GSK-3β antibody. Total GSK-3 kinase activity was measured by monitoring the incorporation of ^{32}P on to substrate using the Liquid Scintillation Analyzer TriCarb 2910 TR (Perkin Elmer) as described previously (Bowes *et al.* 2009). 1 µM CT99021 was used to obtain a background value for each experiment which was then subtracted from total CPM and reported as the difference.

5.2.8 qRT-PCR

MOVAS cells were plated onto 60 mm dishes and cultured in the absence or presence of and/or PBA for 18hrs. Cellular RNA was isolated using an RNeasy mini kit (Qiagen, Mississauga, ON, Canada). RNA was quantified by measuring the absorbance at 260 nm, while purity was verified by calculating the ratio of the absorbance at 260 nm and 280 nm (A_{260}/A_{280}). cDNA was then reverse transcribed from 2 µg of RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR analysis was performed on the StepOnePlus Real-Time PCR System (Applied Biosystems) using SYBR Green Master Mix (Applied Biosystems), 2 µg cDNA and 500 nM forward and reverse primers. Annealing temperature was set to 60°C. β-actin was used as the internal control. See **Table 5** for specific primer sequences.

Table 5. Primer sequences for qRT-PCR

Protein Name	Primer Sequence
αSM-actin	FWD: TGC TCC AGC TAT GTG TGA AGA G REV: GAT GGA TGG GAA AAC AGC CCT
SM22α	FWD: TGA GCC AAG CAG ACT TCC AT REV: GGA GAG TAG CTT CGG TGT CTG
SM-MHC	FWD: GTGTGGTGGTCAACCCCTAC REV: GATGTGAGGCGGCATCTCAT
Tropomyosin 4	FWD: ATAGAGAACCGAGCCATGAAAGA REV: CCTCCTCATACTTGCGGTCG
Vimentin	FWD: CGTCCACACGCACCTACAG REV: GGGGGATGAGGAATAGAGGCT
Type III Collagen	FWD: AAGGCTGCAAGATGGATGCT REV: GTGCTTACGTGGGACAGTCA
Runx2	FWD: CCG GTC TCC TTC CAG GAT REV: GGG AAC TGC TGT GGC TTC
Osterix	FWD: CGC CTT TGC GGG AAC AGG GT REV: ATG CGC ACG CCC TCC ACA AA
Osteocalcin	FWD: CCG GGA GCA GTG TGA GCT TA REV: TAG ATG CGT TTG TAG GCG GTC
Grp78	FWD: ACC TGG GTG GGG AAG ACT TT REV: TCT TCA AAT TTG GCC CGA GT
Grp94	FWD: ACC GAA AAG GAC TTG CGA CT REV: CTC TGA CGA ACC CGA AGG TC
CHOP	FWD: GTC CCT AGC TTG GCT GAC AGA REV: TGG AGA GCG AGG GCT TTG
β-actin	FWD: GGG GTG TTG AAG GTC TCA AAC REV: GGC ACC ACA CCT TCT ACA ATG

5.2.9 Statistical Analysis

All data are expressed as the mean ± standard deviation. Statistical analysis was performed using SigmaPlot 11.0. A one-way analysis of variance (ANOVA) with Tukey's post-hoc was used to compare differences between multiple groups. When comparing two groups, a significance of differences was determined using an unpaired student's t-test of equal variance. $P<0.05$ was considered statistically significant. All experiments were performed at least in triplicate ($N\geq3$).

5.3 Results

5.3.1 Inhibiting GSK-3 Activity Promotes Osteoblast Differentiation in MOVAS Cells

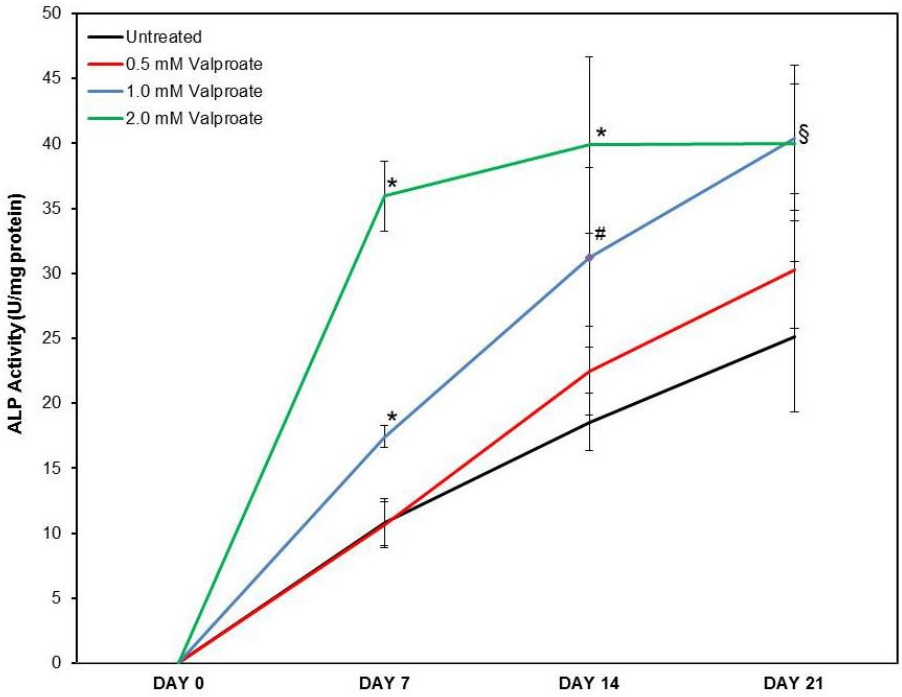
We have previously shown that the adipocytokine leptin promotes osteoblast differentiation by inhibiting GSK-3β activity (Zeadin *et al.* 2012). To confirm that the direct manipulation of GSK-3 promotes osteoblast differentiation in MOVAS cell, we cultured cells in the absence or presence of increasing concentrations of two different GSK-3 inhibitors, valproate (0.5 – 2.0 mM) and CT99021 (1.0 – 4.0 μM) for up to 21 days. ALP activity was used as an indicator of osteoblast differentiation. Valproate promoted a dose-dependent increase in ALP activity (**Figure 5.3A**). At day 14, ALP activity was significantly increased 1.2-fold (22.5 ± 3.4 U/mg), 1.7-fold (31.21 ± 6.91 U/mg) and 2.0-fold (39.87 ±6.81

U/mg) in the presence of 0.5, 1.0, and 2.0 mM valproate respectively, when compared to untreated cells (18.56 ± 2.18 U/mg). CT99021 is the most potent and specific GSK-3 inhibitor currently available. At a concentrations ≤2 μM, it does not significantly affect other protein kinases (Bain *et al.* 2007). In our experiments, treatment with CT99021 also induced a dose-dependent increase in ALP activity when used at micro molar concentrations (**Figure 5.3B**). At day 14, ALP activity was significantly increased 1.3-fold (33.47 ± 2.26 U/mg), 2.2-fold (42.27 ± 3.07 U/mg) and 2.8-fold (52.19 ± 5.93 U/mg) in the presence of 1.0, 2.0, and 4.0 μM CT99021 respectively, when compared to untreated cells (25.70 ± 2.7 U/mg). Dimethyl sulfoxide (DMSO), used as a (vehicle) control, did not significantly affect ALP activity when compared to untreated cell culture conditions (28.85 ± 1.17 U/mg vs. 25.70 ± 2.7 U/mg). These results show that MOVAS cells respond in a similar manner to primary bovine VSMCs and support our previous findings that inhibiting GSK-3 commits SMCs toward a pro-osteogenic phenotype.

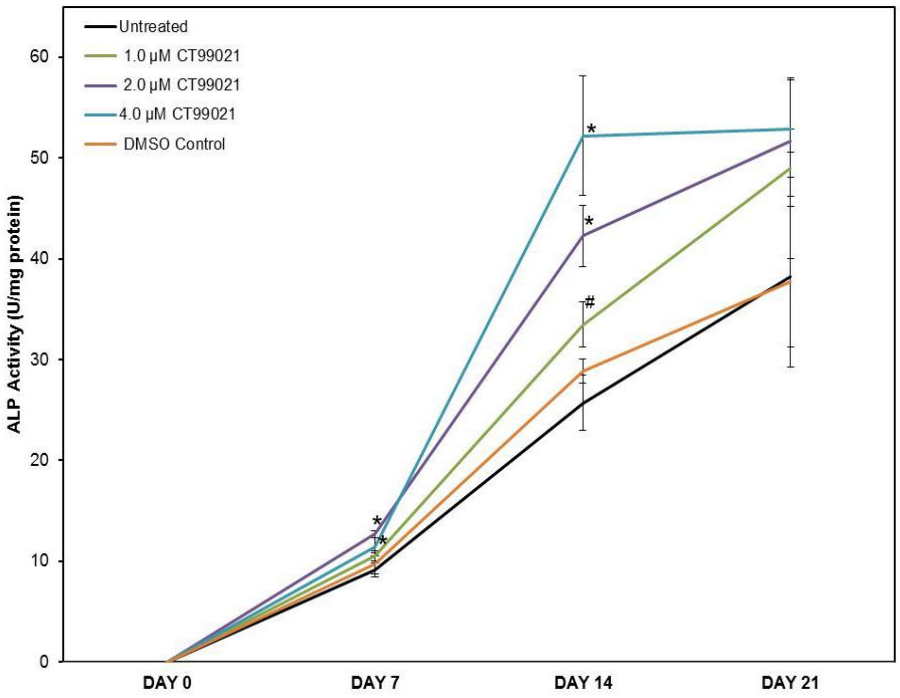
Figure 5.3 - Effect of GSK-3 inhibitors, Valproate and CT99021, on ALP activity.

MOVAS cells were cultured in the presence of increasing concentrations of either valproate **(Panel A)** or CT99021 **(Panel B)** for up to 21 days. Cell lysates were harvested and levels of ALP activity were determined by measuring the cleavage of *p*-nitrophenyl phosphate at 405 nM. All ALP values were normalized to total protein. *P≤0.001; #P<0.05 when compared to untreated cell culture conditions.

A


Untreated
0.5 mM Valproate
1.0 mM Valproate
2.0 mM Valproate
ALP Activity (U/mg protein)
50
45
40
35
30
25
20
15
10
5
0
DAY 0
DAY 7
DAY 14
DAY 21
*
*
*
#
§

B


Untreated
1.0 µM CT99021
2.0 µM CT99021
4.0 µM CT99021
DMSO Control
ALP Activity (U/mg protein)
60
50
40
30
20
10
0
DAY 0
DAY 7
DAY 14
DAY 21
*
*
*
#

5.3.2 Effect of Glucosamine and PBA on GSK-3 Activity and Osteoblast Differentiation

Our next objective was to find a means by which we could activate GSK-3 and examine downstream effects on osteoblast differentiation. Previous studies from our lab, and others, have shown that ER stress can increase GSK-3 activity (McAlpine *et al.* 2012). Therefore, we treated MOVAS cells with 5 mM glucosamine and then determined its effect on total GSK-3 activity by measuring the incorporation of [γ^{32}P] ATP onto a phospho-glycogen synthase substrate. We further hypothesized that by using the chemical chaperone, PBA, to attenuate ER stress we could decrease total GSK-3 activity and thus promote the osteogenic differentiation of VSMCs.

Our results indicated that glucosamine only moderately enhances GSK-3 activity 1.1-fold in MOVAS cells (**Figure 5.4**). We did, however, significantly attenuate total GSK-3 by 53% using 5 mM PBA. PBA also attenuated glucosamine-induced GSK-3 activity by 47% when compared to cells treated with glucosamine alone (**Figure 5.4**). Although these results were unexpected based on our model (**Figure 5.1**), we predicted, therefore, that glucosamine would not affect VSMCs de-differentiation or osteogenic differentiation. We did still expect PBA to promote osteoblast differentiation based on our observation that PBA down-regulates GSK-3 activity.

We next, investigated the effect of glucosamine on the mRNA expression of markers for VMSCs and osteoblast differentiation (**Figure 5.5**). Surprisingly,

glucosamine up-regulated the mRNA expression of Runx2 ($P<0.01$) and Osx ($P=0.001$), two key transcription factors involved in osteoblast differentiation as well as osteocalcin ($P<0.005$) (**Figure 5.5A**). This increase in the expression of markers of osteoblast differentiation was associated with a decrease in the expression of contractile SMC marker, αSM-actin (**Figure 5.5B**). Glucosamine did, however, increase the expression of SM22α ($P<0.005$) and had no significant effect on SM-MHC expression (**Figure 5.5B**). In addition, glucosamine decreased the expression of synthetic SMC markers, tropomyosin 4 ($P<0.05$) but had no significant effect on vimentin or type III collagen mRNA expression (**Figure 5.5B**).

In a separate series of experiments, we examined the effect of glucosamine in the absence and presence of PBA on markers of VSMCs and osteogenic differentiation. Glucosamine exhibited similar effects in these experiments as was previously noted for **Figure 5.5** except for tropomyosin 4 expression which remained unchanged in this series of experiments. PBA also up-regulated the expression of Runx2 (**Figure 5.6A**) and osteocalcin (**Figure 5.6C**) in the absence and presence of glucosamine but had no significant effect on Osx expression (**Figure 5.6C**). In addition, PBA did significantly down-regulate αSM-actin (**Figure 5.6D**) expression while increasing SM22α (**Figure 5.6E**) and SM-MHC expression (**Figure 5.6F**). However, as predicted, PBA did not have any effects on the expression of synthetic SMC markers, tropomyosin 4 (**Figure 5.6G**) and vimentin (**Figure 5.6H**), but PBA did decrease type III collagen (**Figure 5.6I**) expression in the absence of glucosamine.

Consistent with these qRT-PCR findings, 5mM glucosamine promoted ALP activity at day 14 ($P=0.045$) and day 21 ($P=0.033$) with no differences observed at the earlier day 7 time point (**Figure 5.7A**). PBA also increased ALP activity by 13.7-fold ($P<0.001$) when compared to untreated cell culture conditions as early as day 7. Cells cultured in the presence of PBA and either 2 mM or 5 mM glucosamine showed a 10.8-fold and 10.9-fold increase, respectively, in ALP activity compared to cells treated with glucosamine alone (**Figure 5.7B**). This was also confirmed by Alizarin Red staining, where PBA demonstrated an increase in calcium accumulation (**Figure 5.7C**).

Figure 5.4 - Effect of glucosamine and PBA on GSK-3 activity.

MOVAS cells were cultured in the presence of 5 mM glucosamine, 5 mM PBA or both for 18 hrs. Total GSK-3 activity was measured by 32P incorporation onto phospho-glycogen synthase peptide-2 substrate. Activities are represented relative to untreated cell culture conditions.

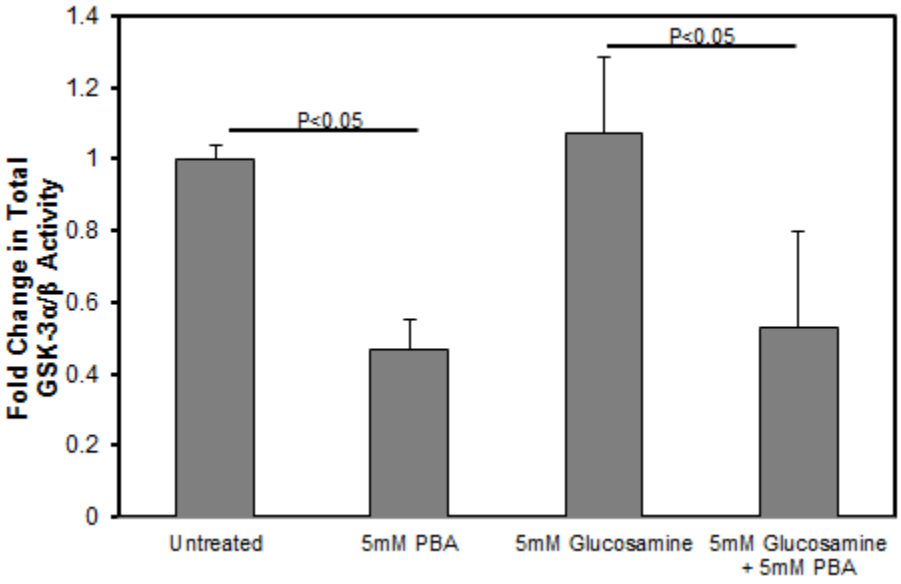
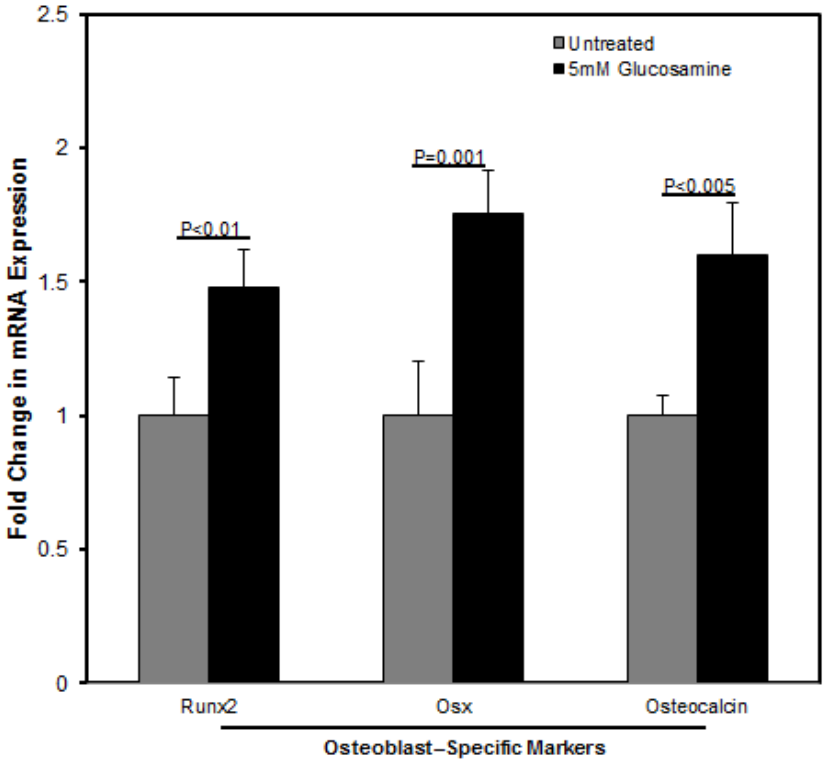

Fold Change in Total GSK-3α/β Activity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
P<0.05
P<0.05
Untreated
5mM PBA
5mM Glucosamine
5mM Glucosamine + 5mM PBA

Figure 5.5 - Effect of glucosamine on the mRNA expression of smooth muscle cell - specific and osteoblast - specific differentiation markers.

MOVAS cells were cultured in the presence of 5 mM glucosamine for 18 hrs. mRNA expression of osteoblast-specific markers **(Panel A)**; differentiated contractile SMC markers and de-differentiated synthetic SMC markers **(Panel B)** was analyzed by qRT-PCR.

A


Untreated
5mM Glucosamine
Fold Change in mRNA Expression
2.5
2
1.5
1
0.5
0
P<0.01
P=0.001
P<0.005
Runx2
Osx
Osteocalcin
Osteoblast–Specific Markers

B

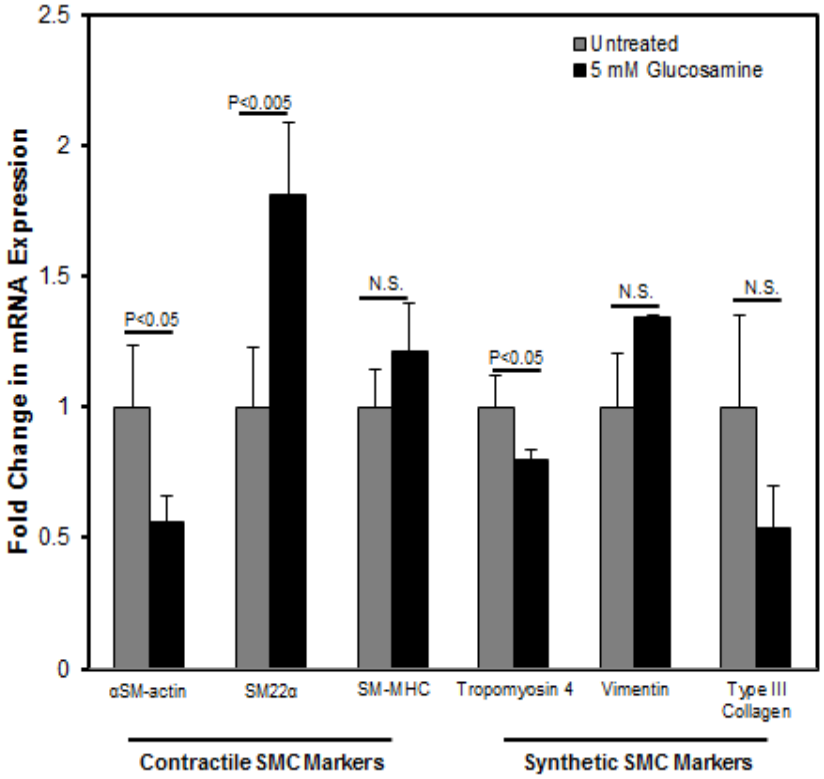
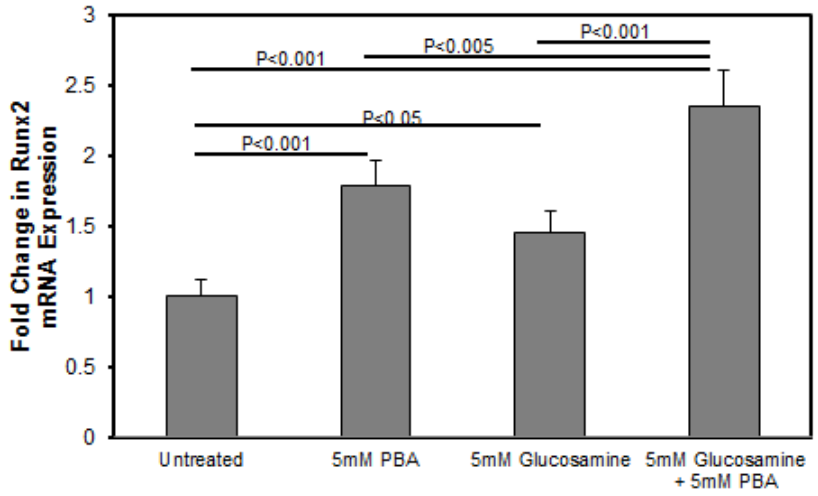

Untreated
5 mM Glucosamine
Fold Change in mRNA Expression
2.5
2
1.5
1
0.5
0
P<0.05
P<0.005
N.S.
P<0.05
N.S.
N.S.
αSM-actin
SM22α
SM-MHC
Tropomyosin 4
Vimentin
Type III Collagen
Contractile SMC Markers
Synthetic SMC Markers

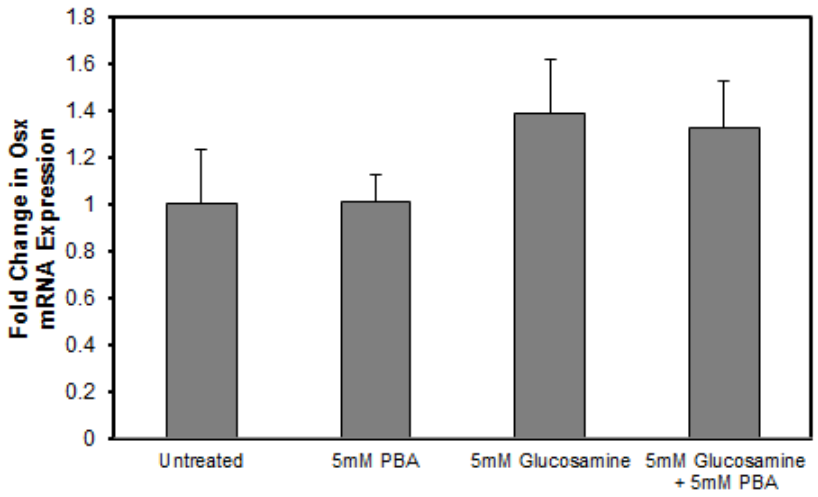
Figure 5.6 - Effect of PBA on the mRNA expression of smooth muscle cell - specific and osteoblast - specific differentiation markers.

MOVAS cells were cultured in the presence of 5 mM glucosamine, 5 mM PBA or both for 18 hrs. mRNA expression of osteoblast-specific markers **(Panels A-C)**; differentiated contractile SMC markers **(Panels D-F)**; and de-differentiated synthetic SMC markers **(Panels G-I)** was analyzed by qRT-PCR.

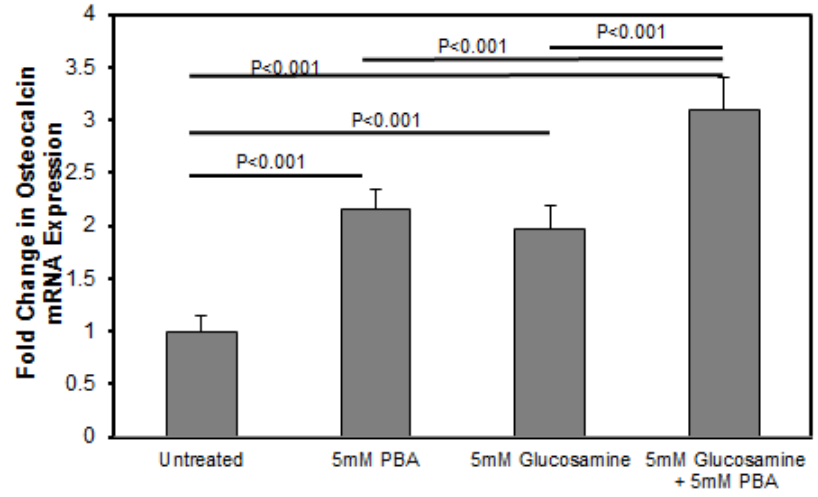
A


Fold Change in Runx2 mRNA Expression
3
2.5
2
1.5
1
0.5
0
P<0.001
P<0.005
P<0.001
P<0.001
P<0.05
P<0.001
Untreated
5mM PBA
5mM Glucosamine
5mM Glucosamine + 5mM PBA

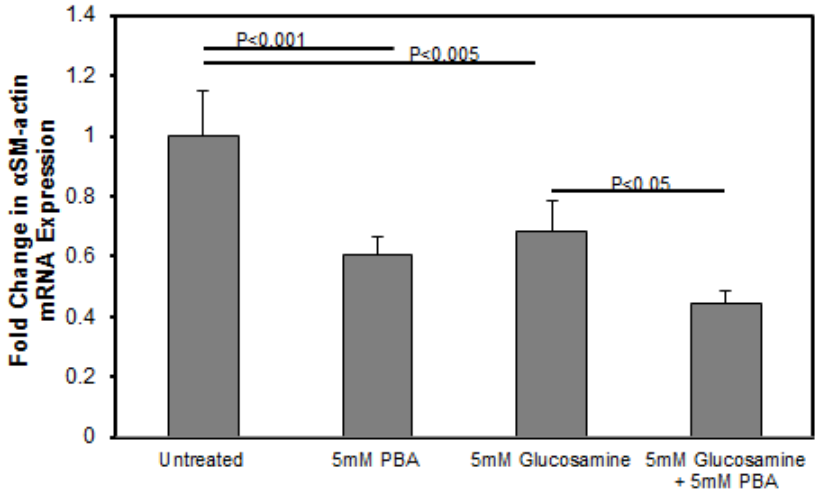
B


Fold Change in Osx mRNA Expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Untreated
5mM PBA
5mM Glucosamine
5mM Glucosamine + 5mM PBA

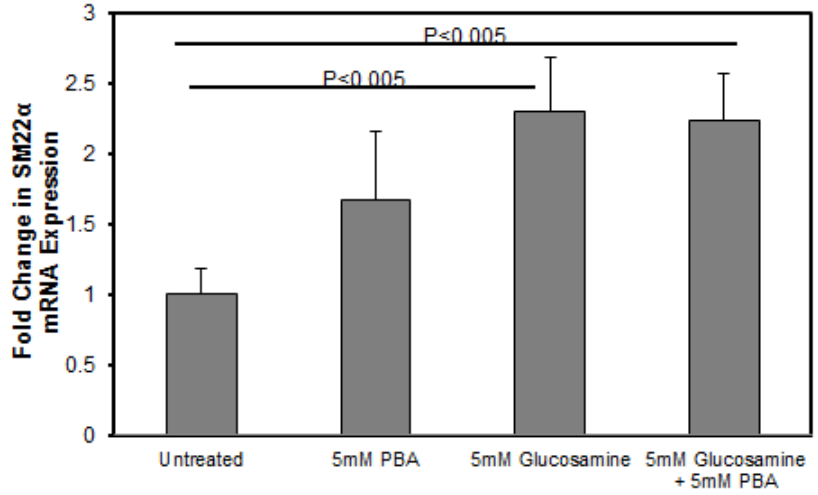
C


Fold Change in Osteocalcin mRNA Expression
4
3.5
3
2.5
2
1.5
1
0.5
0
P<0.001
P<0.001
P<0.001
P<0.001
P<0.001
Untreated
5mM PBA
5mM Glucosamine
5mM Glucosamine + 5mM PBA

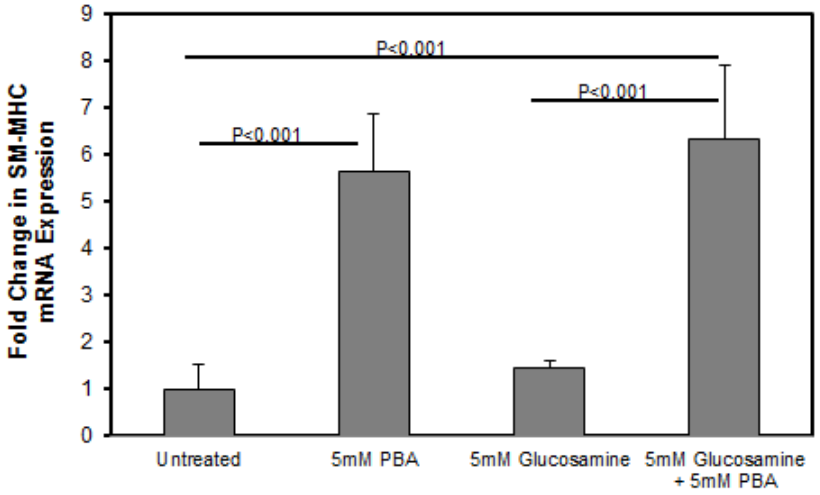
D


Fold Change in αSM-actin mRNA Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
P<0.001
P<0.005
P<0.05
Untreated
5mM PBA
5mM Glucosamine
5mM Glucosamine + 5mM PBA

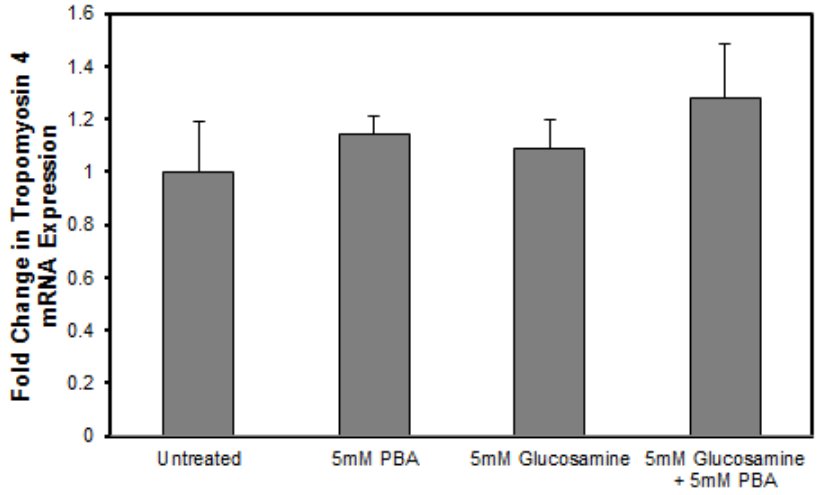
E


Fold Change in SM22α mRNA Expression
3
2.5
2
1.5
1
0.5
0
P<0.005
P<0.005
Untreated
5mM PBA
5mM Glucosamine
5mM Glucosamine + 5mM PBA

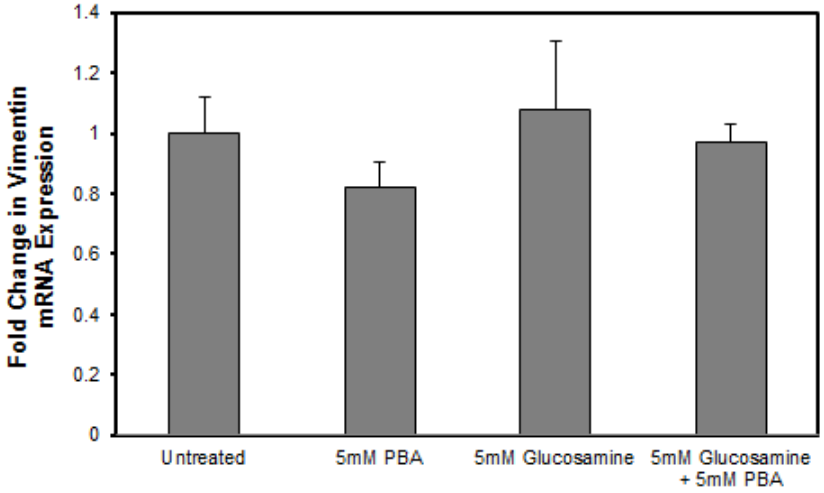
F


Fold Change in SM-MHC mRNA Expression
9
8
7
6
5
4
3
2
1
0
P<0.001
P<0.001
P<0.001
Untreated
5mM PBA
5mM Glucosamine
5mM Glucosamine + 5mM PBA

G


Fold Change in Tropomyosin 4 mRNA Expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Untreated
5mM PBA
5mM Glucosamine
5mM Glucosamine + 5mM PBA

H


Fold Change in Vimentin mRNA Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Untreated
5mM PBA
5mM Glucosamine
5mM Glucosamine + 5mM PBA

I

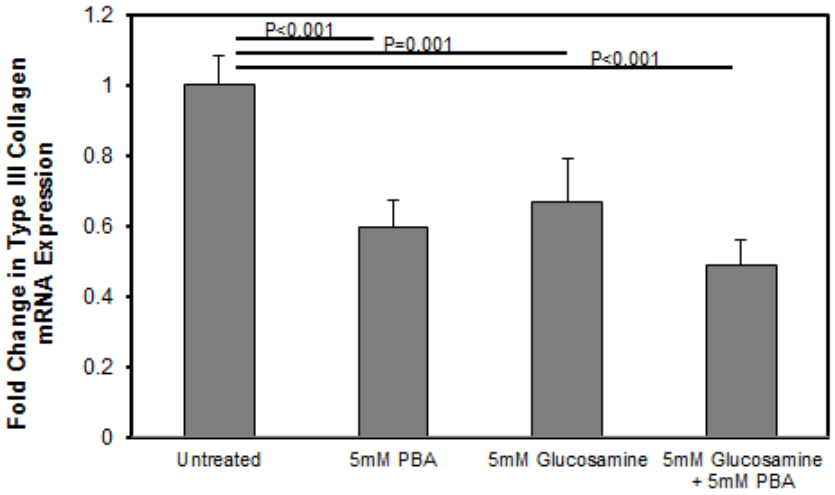
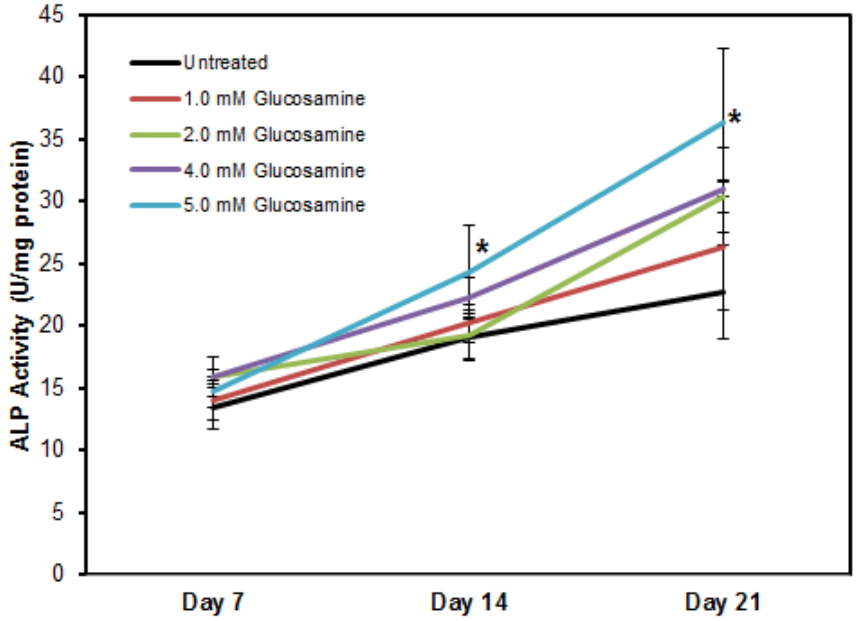

Fold Change in Type III Collagen mRNA Expression
P<0.001
P=0.001
P<0.001
1.2
1
0.8
0.6
0.4
0.2
0
Untreated
5mM PBA
5mM Glucosamine
5mM Glucosamine + 5mM PBA

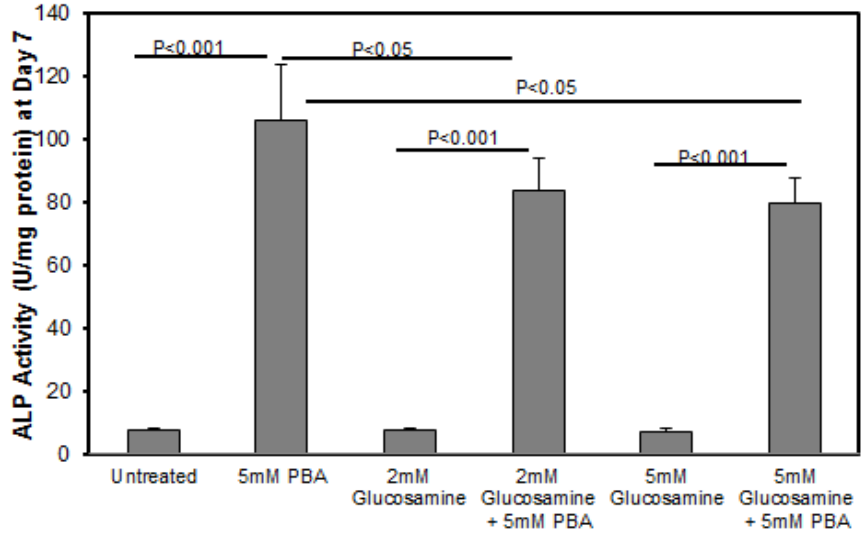
Figure 5.7 - Effect of glucosamine and PBA on osteoblast differentiation.

ALP activity was used as an index of osteoblast differentiation. **Panel A:** MOVAS cells were cultured in the presence of increasing concentrations (1.0 – 5.0 mM) of glucosamine for 7, 14 or 21 days. **Panel B:** Alternatively, MOVAS cells were cultured in the presence of 5 mM glucosamine, 5 mM PBA or both for 7 days. All ALP values were normalized to total protein. **Panel C:** MOVAS cells cultured in the presence of 2 mM PBA for 14 days were also subject to Alizarin Red S staining for calcium deposition. *P<0.05 when compared to untreated cell culture conditions.

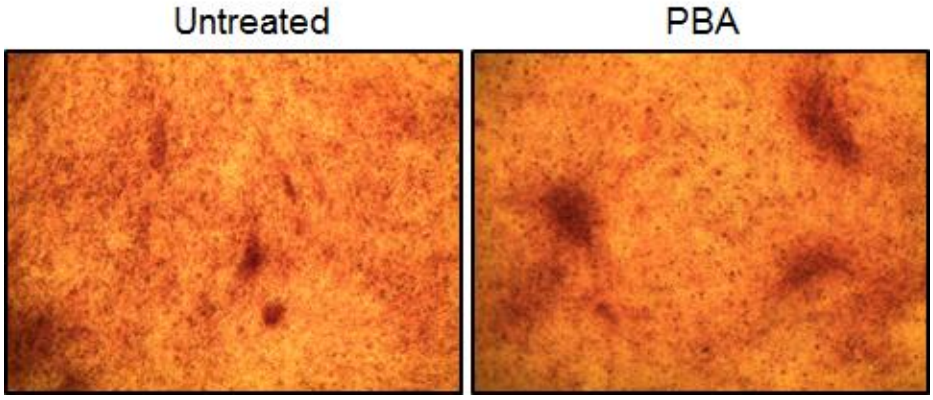
A


Untreated
1.0 mM Glucosamine
2.0 mM Glucosamine
4.0 mM Glucosamine
5.0 mM Glucosamine
ALP Activity (U/mg protein)
0
5
10
15
20
25
30
35
40
45
Day 7
Day 14
Day 21
*
*

B


ALP Activity (U/mg protein) at Day 7
0
20
40
60
80
100
120
140
P<0.001
P<0.05
P<0.05
P<0.001
P<0.001
Untreated
5mM PBA
2mM Glucosamine
2mM Glucosamine + 5mM PBA
5mM Glucosamine
5mM Glucosamine + 5mM PBA

C


Untreated
PBA

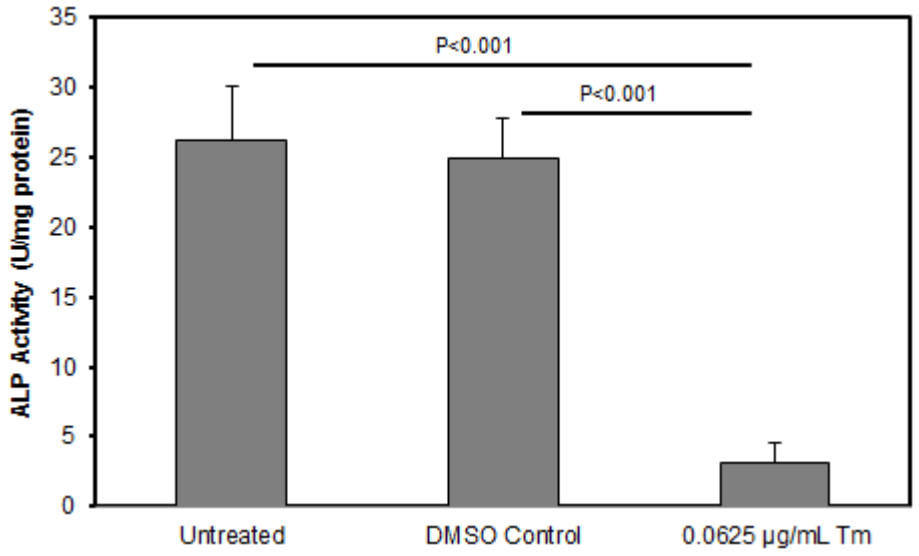
5.3.3 Effect of Other ER Stress Inducers on ALP Activity

Given our findings with glucosamine, we decided to investigate the effect of two other ER stress-inducing agents on osteoblast differentiation. Tunicamycin promotes ER stress by blocking protein N-glycosylation (Feige and Scheffler 1987). A23187 is a calcium ionophore that disrupts Ca^{2+} homeostasis in the ER (Li *et al.* 1993). The tunicamycin and A23187 concentrations used in these ALP assays were relatively low compared to what is commonly cited in the literature. However, we found that 0.0625 µg/mL tunicamyin and 0.25 µM A23187 were the highest concentrations that could be used on MOVAS cells for our long-term ALP assays without causing significant cell death. Even at this concentration, however, tunicamycin significantly attenuated ALP activity by 8.3-fold (26.24 ± 3.81 U/mg vs. 3.15 ± 1.36 U/mg, $P<0.001$) (**Figure 5.8A**). A23187 had a similar effect, attenuating ALP activity by 4.3-fold when compared to untreated cells (25.87 ± 2.39 U/mg vs. 5.95 ± 0.95 U/mg, $P<0.001$) and 3.2-fold when compared to cells cultured in the presence of DMSO (vehicle control) (18.89 ± 2.7 U/mg vs. 5.95 ± 0.95 U/mg, $P<0.005$) (**Figure 5.8B**).

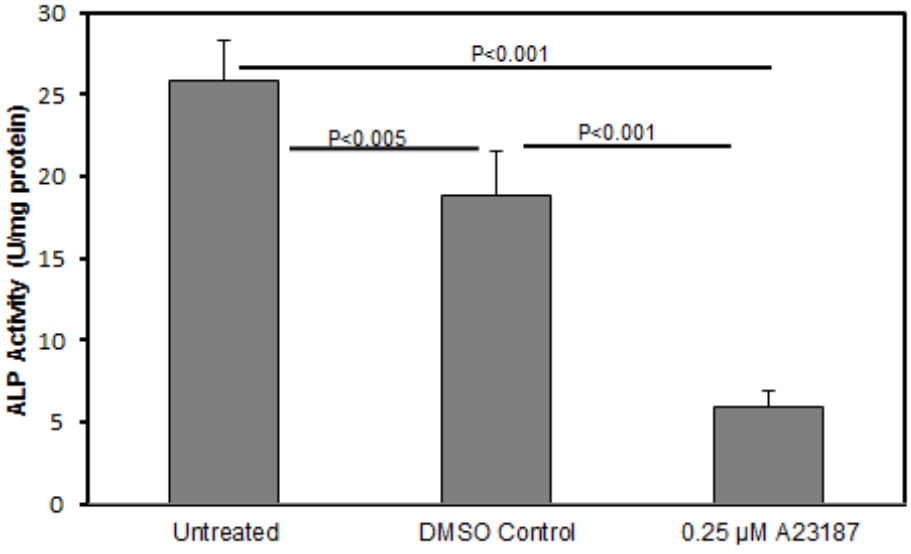
Figure 5.8 - Effect of ER stress inducers, Tunicamycin and A23187, on ALP activity.

MOVAS cells were cultured in the absence or presence of 0.0625 µg/mL tunicamycin **(Panel A)** or 0.25 µM A23187 **(Panel B)** for 14 days before cell lysates were harvested and subject to an ALP assay. All ALP values were normalized to total protein. DMSO was used as a vehicle control. (Tm = tunicamycin)

A


35
30
25
20
15
10
5
0
ALP Activity (U/mg protein)
P<0.001
P<0.001
Untreated
DMSO Control
0.0625 µg/mL Tm

B


30
25
20
15
10
5
0
ALP Activity (U/mg protein)
P<0.001
P<0.005
P<0.001
Untreated
DMSO Control
0.25 µM A23187

5.3.4 Examining the Role of ER Stress in Osteoblast Differentiation

Therefore, of the four ER stress-modulating agents tested, glucosamine and the chemical chaperone PBA enhance ALP activity, while tunicamycin and A23187 inhibit ALP activity. Clearly, the potential link(s) between ER stress and vascular calcification is not as direct as our hypothesis predicted (**Figure 5.1**). In addition, the tools that we have to modulate ER stress appear to have effects on calcification that are independent of their effects on the ER. Based on our observations thus far, it is possible that we see an increase in the activation of the UPR in order to expand the capacity of the ER to cope with an increased demand for protein synthesis.

Glucosamine has previously been shown to activate the UPR in various cells types such as human aortic smooth muscle cells, monocyte-derived macrophages and HepG2 cells (Hossain *et al.* 2003;Khan *et al.* 2009;Kim *et al.* 2005;Werstuck *et al.* 2006). We treated MOVAS cells with increasing concentrations of glucosamine (0.5 – 5 mM) before lysing cells and immunoblotting for the expression of ER chaperone proteins, Grp78, Grp94 and pro-apoptotic factor, GADD153/CHOP (**Figure 5.9A-D**). As depicted in Figure 5.9, the highest concentration of glucosamine significantly increased the mRNA expression of Grp78 (>23-fold) (**Figure 5.9E**), Grp94 (7-fold) (**Figure 5.9F**), and CHOP (6-fold) (**Figure 5.9G**). Higher glucose concentrations do not appear to have an effect on the expression of any of the ER stress markers that were examined.

We next investigated the effect of PBA on markers of the UPR system. Surprisingly, PBA significantly increased Grp78 mRNA expression even in the absence of glucosamine. In fact, PBA did not attenuate glucosamine-induced mRNA expression of Grp78, Grp94 or CHOP but instead significantly increased expression of the UPR markers (**Figure 5.9**). To confirm the effects we observed on the mRNA expression of Grp78, Grp94 and CHOP with PBA, we used Thioflavin T (ThT) fluorescence. Our lab has previously demonstrated that ThT-fluorescence correlates with established indicators of UPR activation and can thus be used to detect and quantify ER stress in live cells (Beriault and Werstuck 2013). In this previously published report, PBA was used to attenuate ER stress in thapsigargin - and glucosamine – treated mouse embryonic fibroblasts (MEFs). We confirmed these findings by culturing MEFs in the absence or presence of glucosamine and/or PBA (**Figure 5.10**). However, when these experiments were repeated in MOVAS cells, we found that cells treated with PBA showed an increase in ThT fluorescence in the absence and presence of glucosamine (**Figure 5.10**).

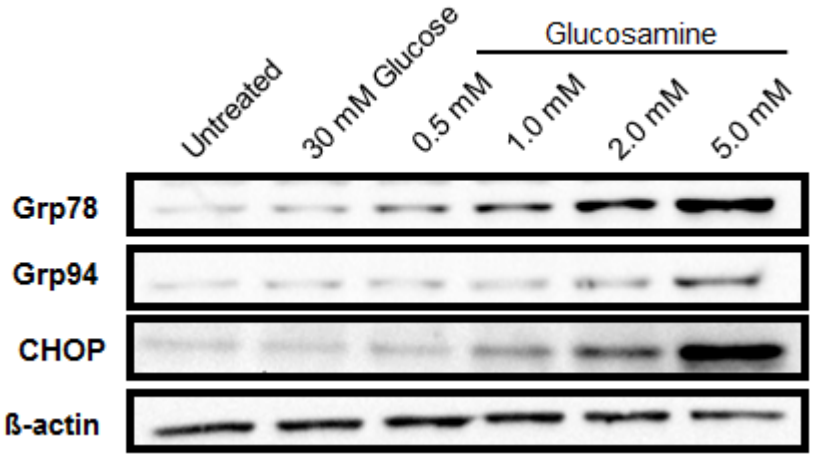
The effect of tunicamyin and A23187 on ER stress was also examined. Although tunicamycin increased Grp78, Grp94 and CHOP protein expression, A21387 did not induce the expression of UPR markers at 18hrs (**Figure 5.11**). These findings suggest that; i) ER stress inducing agents can affect MOVAS cell differentiation independent of causing ER stress, and ii) ER stress does not directly induce VSMC differentiation. In fact, ER stress and/or UPR activation

may be a consequence of the osteogenic differentiation VSMCs undergo when exposed to glucosamine and/or PBA.

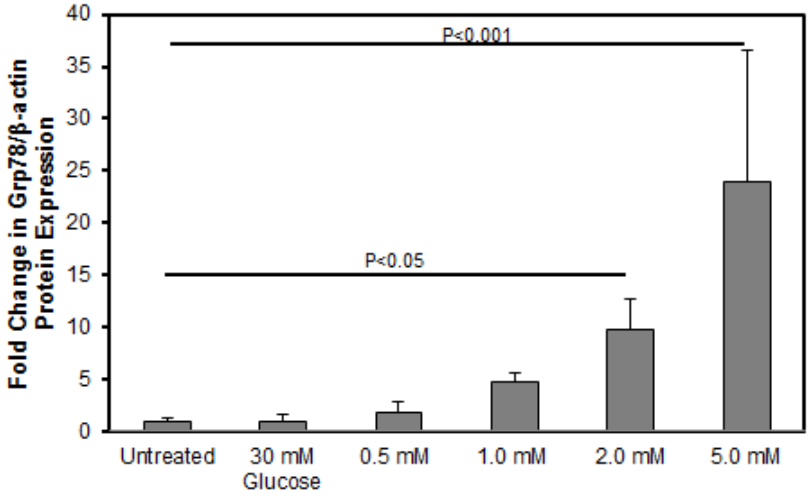
Figure 5.9 - Effect of glucosamine on UPR activation.

Panel A: MOVAS cells were cultured in the presence of 30 mM glucose and increasing concentrations of glucosamine for 18 hrs. Equal amounts of total cell lysates were run on an SDS-PAGE and immunoblots were probed for the expression ER stress markers, Grp78, Grp94 and CHOP. **Panels B-D:** Immunoblots were quantified by densitometry. All protein levels were normalized to ß-actin and expressed as a fold change relative to untreated cell culture conditions. **Panels E-G:** MOVAS cells were cultured in the presence of 5 mM glucosamine, 5 mM PBA or both for 18 hrs. mRNA expression for markers of UPR activation was analyzed by qRT-PCR.

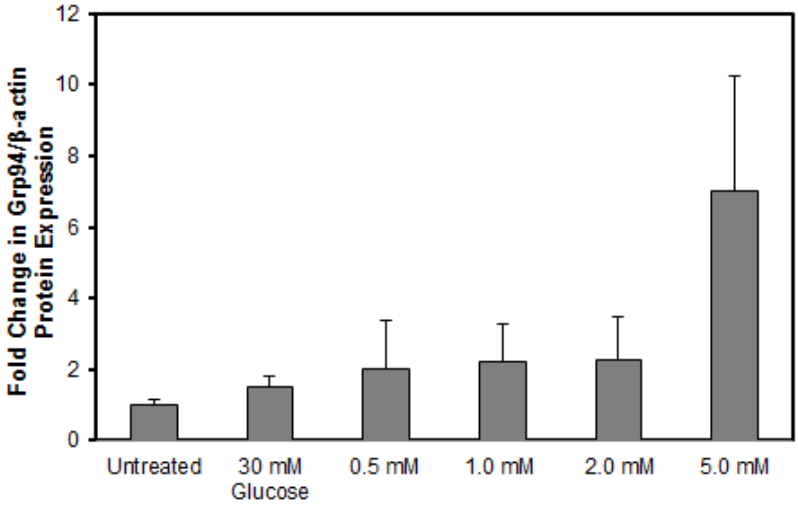
A


Untreated
30 mM Glucose
Glucosamine
0.5 mM
1.0 mM
2.0 mM
5.0 mM
Grp78
Grp94
CHOP
ß-actin

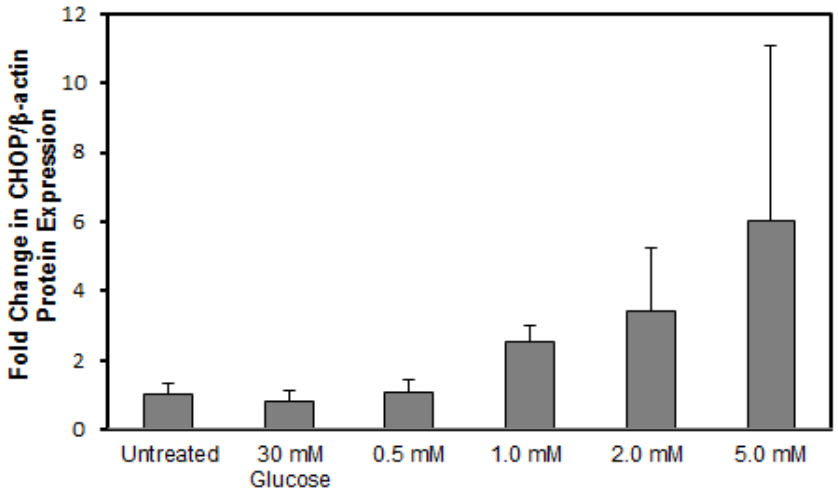
B


Fold Change in Grp78/β-actin Protein Expression
P<0.001
P<0.05
0
5
10
15
20
25
30
35
40
Untreated
30 mM Glucose
0.5 mM
1.0 mM
2.0 mM
5.0 mM

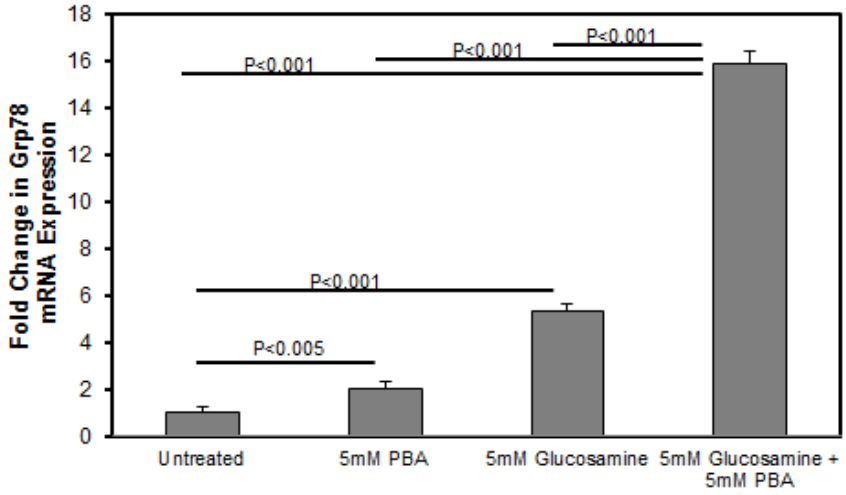
C


Fold Change in Grp94/β-actin Protein Expression
0
2
4
6
8
10
12
Untreated
30 mM Glucose
0.5 mM
1.0 mM
2.0 mM
5.0 mM

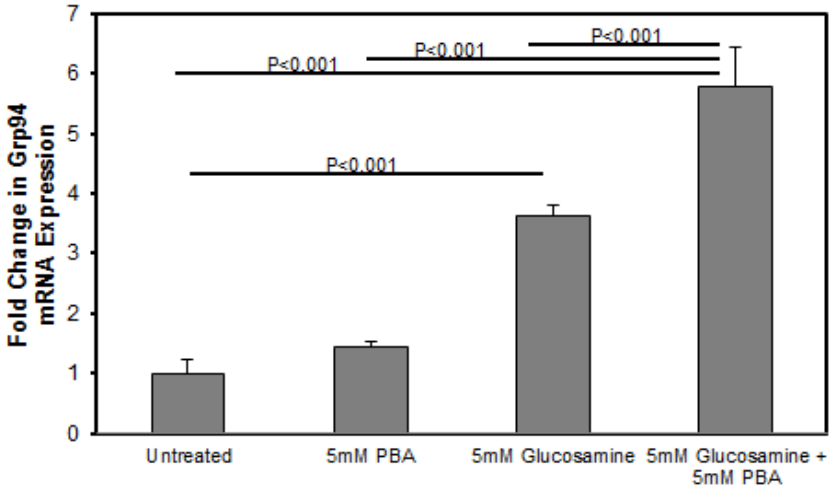
D


Fold Change in CHOP/β-actin Protein Expression
0
2
4
6
8
10
12
Untreated
30 mM Glucose
0.5 mM
1.0 mM
2.0 mM
5.0 mM

E


Fold Change in Grp78 mRNA Expression
0
2
4
6
8
10
12
14
16
18
P<0.001
P<0.001
P<0.001
P<0.001
P<0.005
Untreated
5mM PBA
5mM Glucosamine
5mM Glucosamine + 5mM PBA

F


Fold Change in Grp94 mRNA Expression
0
1
2
3
4
5
6
7
P<0.001
P<0.001
P<0.001
P<0.001
Untreated
5mM PBA
5mM Glucosamine
5mM Glucosamine + 5mM PBA

G

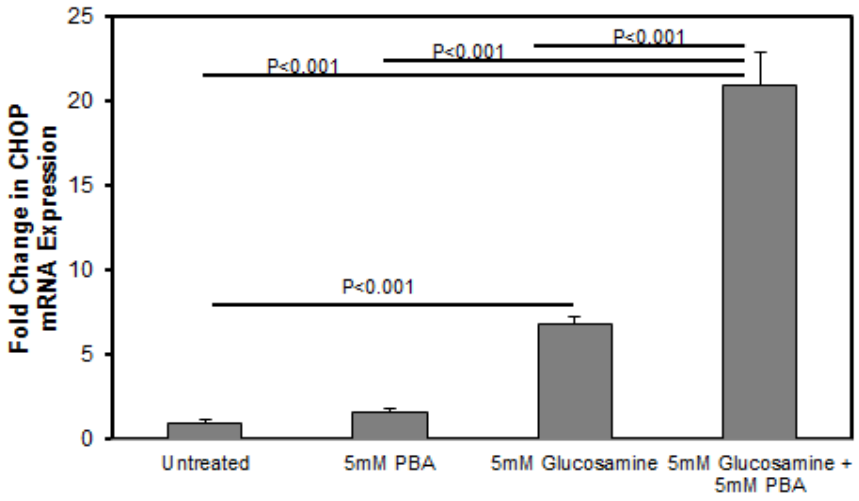

Fold Change in CHOP mRNA Expression
25
20
15
10
5
0
P<0.001
P<0.001
P<0.001
P<0.001
Untreated
5mM PBA
5mM Glucosamine
5mM Glucosamine + 5mM PBA

Figure 5.10 - ThT staining in MEFs and MOVAS cells.

MEFs or MOVAS cells were cultured in the presence of 5 mM PBA, 5 mM glucosamine or both for 18hrs. Live cell fluorescent images were captured. Representative image of three experiments.

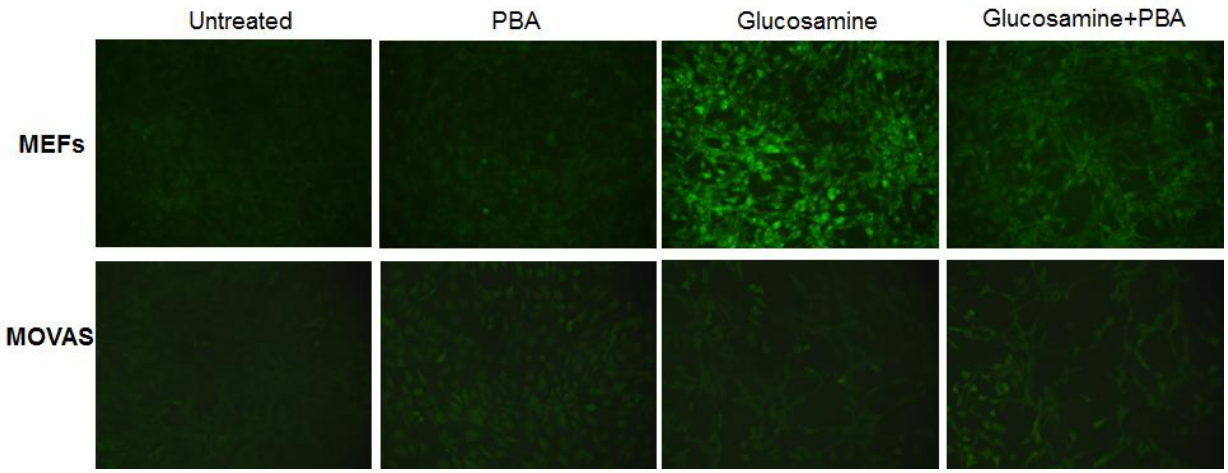
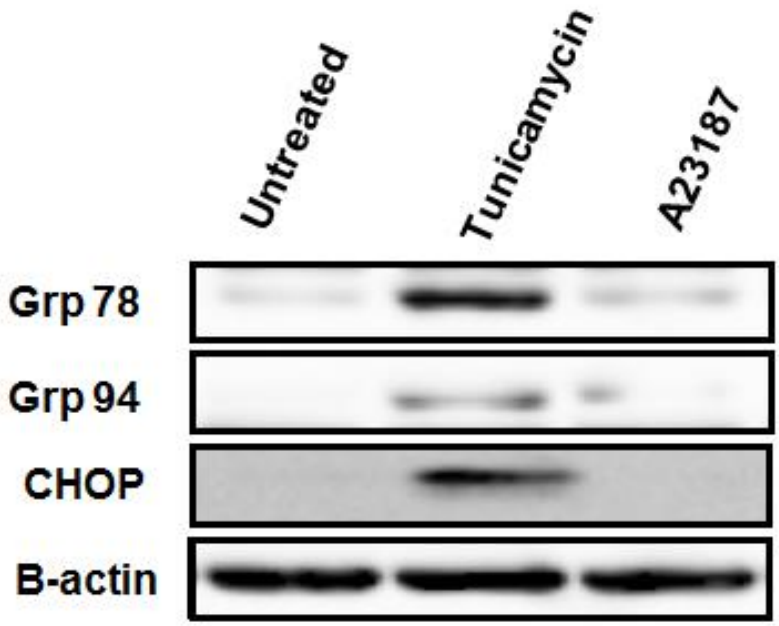

Untreated
PBA
Glucosamine
Glucosamine+PBA
MEFs
MOVAS

Figure 5.11 - Effect of Tunicamycin and A23187 on UPR activation.

MOVAS cells were treated with 0.0625 µg/mL tunicamycin or 0.25 µM A23187 for 18 hrs. Equal amounts of total cell lysates were run on an SDS-PAGE and immunoblots were probed for the expression ER stress markers, GRP78, GRP94 and CHOP. Representative image of three experiments.


Untreated
Tunicamycin
A23187
Grp 78
Grp 94
CHOP
B-actin

5.4 Discussion

In the current study, we proposed to examine the physiological role that GSK-3 plays in modulating the pathogenesis of vascular calcification/ atherosclerosis by using ER stress inducing agents (**Figure 5.1**). Our lab has previously shown that ER stress promotes the accelerated development of atherosclerosis by a mechanism which involves the activation of GSK-3 (McAlpine *et al.* 2012). Furthermore, we have shown that increased leptin levels which are associated with obesity can promote the osteogenic differentiation of VSMC and thus vascular calcification by a mechanism which involves the inhibition of GSK-3β activity (Zeadin *et al.* 2009;Zeadin *et al.* 2012). By using valproate and CT99021, we confirmed that MOVAS cells respond in a similar manner to primary BASMCs and support our previous findings that inhibiting GSK-3 commits VSMCs toward an osteoblast-like phenotype (**Figure 5.3**). Therefore, we hypothesized that increasing GSK-3 activity will cause the phenotypic switch of differentiated 'contractile' VSMCs toward a de-differentiated 'synthetic' phenotype akin to the VSMCs which are found in atherosclerotic lesions.

Glucosamine-supplementation has previously been shown to promote ER stress, hepatic steatosis and accelerated atherosclerosis *in vivo* (Beriault *et al.* 2011). We investigated the effect of glucosamine on GSK-3 activity and found that glucosamine increased total GSK-3 activity only slightly in MOVAS cells. We expected then, that our MOVAS cells would remain in their differentiated

contractile state without any predilection toward osteogenic differentiation or de-differentiation to the synthetic VSMC phenotype. However, glucosamine attenuated the expression of contractile SMC marker, αSM-actin, and synthetic SMC marker, tropomyosin 4. Glucosamine did not significantly alter the expression of synthetic SMC markers, vimentin, or type III collagen (**Figure 5.5B**) as would be predicted based on our proposed model. Unexpectedly, glucosamine did up-regulate the expression of osteoblast-specific markers including Runx2, Osx and osteocalcin (**Figure 5.5A**). In our studies, glucosamine also dose-dependently increased ALP activity by day 21 (**Figure 5.7A**). Several *in vitro* and *in vivo* studies have already demonstrated that ALP is essential for bone mineralization (Levy *et al.* 1991;Maranto and Schoen 1988;Narisawa *et al.* 2007;Shanahan *et al.* 1999;Shioi *et al.* 1995;Shioi *et al.* 2002). Glucosamine has previously been shown to activate the UPR in various cells types such as human aortic smooth muscle cells, monocyte-derived macrophages and HepG2 cells (Hossain *et al.* 2003;Khan *et al.* 2009;Kim *et al.* 2005;Werstuck *et al.* 2006). Therefore, it was not surprising that glucosamine promoted ER stress and activated the UPR in MOVAS cells as determined by an increase in ThT staining and the mRNA and protein expression of Grp78, Grp94 and CHOP (**Figure 5.9 & Figure 5.10**). However, this suggested that the link between ER stress and osteogenic differentiation is more complex than we originally proposed.

Under normo-glycemic conditions of a cell, 1 to 3% of total glucose will be converted to glucosamine-6 phosphate by the enzyme glutamine:fructose-6

phosphate amidotransferase (GFAT) (Marshall *et al.* 1991). Glucosamine is a by-product of increased flux through the hexosamine biosynthetic pathway (HBP). UDP-*N*-acetylglucosamine (UDP-GlcNAc) or activated glucosamine is an essential product because it participates in the proper folding of many proteins by acting as a substrate for both O- and N-linked protein glycosylation. However, elevated concentrations of glucosamine increase levels of O-linked protein glycosylation (Werstuck *et al.* 2006). Recently, it has been shown that elevated levels of O-linked glycosylation accelerate vascular calcification in arteries of a diabetic mouse model through a mechanism which involves AKT (Heath *et al.* 2014). O-linked glycosylation of AKT at T430 and T479 promotes AKT phosphorylation at S473 and subsequent kinase activation. Importantly this same study demonstrated that the knockdown of β-N-acetylglucosaminidase (OGA), which is the enzyme responsible for the removal of O-linked protein glycosylation, induces the expression of Runx2 and osteocalcin *in vitro*. Therefore, it is possible that chronic exposure to glucosamine is having a similar effect in our *in vitro* model for vascular calcification and that his mechanism is independent of any effect on GSK-3 activity.

To further investigate the link between ER stress and the osteogenic differentiation of VSMCs, we used the chemical chaperone, PBA (Engin and Hotamisligil 2010). We investigated the effect of PBA on GSK-3 activity and found that PBA significantly attenuated total GSK-3 activity in MOVAS cells (Figure 5.4). In line with our proposed hypothesis, these findings were associated

with a dramatic increase in the expression of osteoblast-specific markers in both the absence and presence of glucosamine (**Figure 5.6 A-C**). PBA dramatically promoted ALP activity as early as day 7 with a concomitant increase in Alizarin Red S staining by day 14 (**Figure 5.7 B & C**). As an extension of these findings, PBA also attenuated the expression of contractile SMC marker, α-SM-actin, and synthetic SMC marker, type III collagen. Also, as would be expected, PBA did not affect expression of synthetic SMC markers, vimentin, or tropomyosin 4. It is, therefore, possible that PBA promotes the osteogenic differentiation of VSMCs by inhibiting GSK-3 activity. Recent studies, however, have also suggested that PBA can act as a histone deacetylase (HDAC) inhibitor (Shi *et al.* 2007;Svechnikova *et al.* 2008;Takai and Narahara 2010). HDAC inhibitors have previously been shown to promote cell differentiation including osteoblast differentiation (Azechi *et al.* 2013;Eom and Kook 2014;Svechnikova *et al.* 2008). For example, Azechi *et al*. (2013) demonstrated that the HDAC inhibitor, trichostatin A (TSA), promotes Pi-induced mineralization of HASMCs by up-regulating ALP expression thus increasing the risk of vascular calcification likely by allowing for the hyperacetylation of Runx2. We also showed that 100 nM TSA significantly up-regulates ALP activity in MOVAS cells (supplementary data, **Figure 5.12**). Therefore, it is possible that PBA is promoting the osteogenic differentiation of VSMCs by (1) attenuating GSK-3 activity and/or (2) inhibiting histone deacetylase activity. Future studies are necessary to delineate the role of GSK-3 activity in PBA-mediated osteogenic differentiation of VSMCs. This is an important finding

because PBA is currently being used to treat urea-cycle disorders in children. Although PBA has been reported to attenuate atherosclerosis *in vivo*, its effects on vascular calcification have not been investigated (Erbay *et al.* 2009).

Interestingly, when we examined the effect of PBA on ER stress and activation of the UPR, we observed that PBA, like glucosamine increases ThT staining (**Figure 5.10**). Additionally, PBA increases the mRNA expression of Grp78 in the absence and presence of glucosamine while further enhancing Grp94 and CHOP expression in the presence of glucosamine (Figure 5.9E-G). We speculate that this observed increase in ER stress is secondary to the effect of PBA on the osteogenic differentiation of VSMCs. It is likely that for VSMCs to differentiate, the cells need to elaborate their protein synthesis ability thus increasing overall ER activity and their requirement for chaperone proteins. In support of this, we demonstrate that ER stress inducers, tunicamycin and A23187, attenuate ALP activity (**Figure 5.8**) despite that tunicamycin promotes UPR activation and A23187 does not (**Figure 5.11**). Tunicamycin is a glucosamine-containing nucleoside that inhibits N-linked glycosylation and has previously been demonstrated to suppress the osteogenic differentiation of a preosteoblast cell line (MC3T3E1 cells) (Jang *et al.* 2011).

Glucosamine and PBA independently increased the mRNA expression of two markers for mature differentiated contractile VSMCs: SM22α (glucosamine: $P<0.005$ and PBA: $P>0.05$) (**Figure 5.5B** and **Figure 5.6E**) and SM-MHC (glucosamine: $P>0.05$ and PBA: $P<0.001$) (**Figure 5.5B** and **Figure 5.6F**). At first

glance, this appears to contradict our suggestion that glucosamine and PBA cause the osteogenic differentiation of contractile VSMCs. However, it has previously been reported that VSMCs can co-express osteoblastic and VMSC – specific genes (Proudfoot *et al.* 1998) which may identify a unique phenotype that exists along a phenotype continuum (Shioi *et al.* 2002).

It has been exceptionally difficult to utilize current ER stress inducing and modulating agents to study the effect of ER stress on GSK-3 activity and osteogenic differentiation. For example, similar to our findings with PBA, although TUDCA increases ALP activity in the absence and presence of glucosamine, we also observe an increase in ThT staining above the effect with glucosamine alone (supplementary data**, Figure 5.13**). The current ER stress tools display multiple and sometimes conflicting effects that confound the interpretation of the experiments. Although there are published studies from other investigators that have implicated ER stress as an inducer of vascular calcification, our findings, presented here, may raise some questions with respect to the conclusions of these reports. Previously, the IREα-XBP1 branch of the UPR had been implicated in the process of osteoblast differentiation through the transcriptional up-regulation of Osx (Tohmonda *et al.* 2011). This study demonstrated that the UPR has two functions during osteoblast differentiation: (i) to expand the capacity of the ER to cope with increased demand and (ii) to promote the maturation of pre-osteoblasts to osteoblasts through the IREα-XBP1. MEFs were used for these studies and effects with ER stress-inducing agent, thapsigargin, were only

evident upon stimulation of cells with the pro-osteogenic signal, bone morphogenic protein 2 (BMP-2).

The PERK-eIF2α-ATF4 UPR pathway was implicated in stearate - BSA - mediated osteogenic differentiation of MOVAS cells (Masuda *et al.* 2012). However, our control experiments with cell culture compatible BSA from two different sources (Sigma-Aldrich and Calbiochem) significantly attenuated ALP activity (supplementary data, **Figure 5.14**), therefore, we could not investigate the effect of palmitate (or stearate), for example, on the osteogenic differentiation of MOVAS cells. Furthermore, the study by Masuda *et al.* (2012) also utilized either 3.0 or 5.0 mM glycerophosphate which could be priming vascular cells towards osteogenic differentiation. It appears that glucosamine and 4-PBA can promote the osteogenic differentiation of MOVAS cells even in the absence of any additional stimulating agents. Therefore, our studies with ER stressors were conducted in the absence of any additional well-described pro-osteogenic signals such as BMP-2 or βGP. In fact, our lab has previously been shown that βGP promotes osteoblast differentiation by up-regulating the activation of SMAD 1/5/8 (Bear *et al.* 2008). Therefore, in these experiments we opted not to add βGP in order to avoid effects that could complicate our interpretation of the independent contribution of glucosamine or 4-PBA in MOVAS cells. Finally, it is worth mentioning that much of the current literature examines the role of ER stress – mediated osteogenic differentiation in pre-osteoblast as opposed to vascular cells where the mechanism(s) will certainly be different. Given their multiple effects,

future studies on vascular calcification should aim to investigate the role of each ER stressor independently as well as in the presence of various physiological stimuli.

In conclusion, we have shown that ER stressor, glucosamine, and ER stress modulator, PBA, promote the osteogenic differentiation of MOVAS cells by different mechanisms which appear to be independent of their effect on ER stress. This is supported by the results of our ALP assays with ER stress-inducing agents, tunicamycin and A23187. While both tunicamycin and A23187 attenuate ALP activity, only tunicamycin increases the expression of markers of the UPR. The increase in ER stress which we observe in our cell culture model is likely a consequence of the osteogenic differentiation that VSMCs are undergoing. During osteoblast differentiation, the UPR acts to expand the capacity of the ER to manage the increase in the production of extracellular matrix proteins and to avoid apoptosis induced by ER overload. Furthermore, the current ER stress tools display multiple and sometimes conflicting effects. This makes it a challenge to investigate the effect of the ER stress - GSK-3 axis on osteogenic differentiation in VSMCs. We propose that future studies will need to focus on (1) delineating the mechanisms of various ER stressors/attenuators independently of one another; (2) using consistent cell culture models; and (3) repeating these experiments in various mouse models to determine if they in fact promote vascular calcification under physiological conditions.

SUPPLEMENTARY DATA

Figure 5.12 - HDAC Inhibitor, TSA, Promotes MOVAS Cell ALP Activity.

MOVAS cells were treated with either 100 nM TSA or an equal volume of ethanol (vehicle) control for 14 days. Cell lysates were subject to an ALP assay by measuring the cleavage of pNPP at 405 nM. ALP values were normalized to total protein.

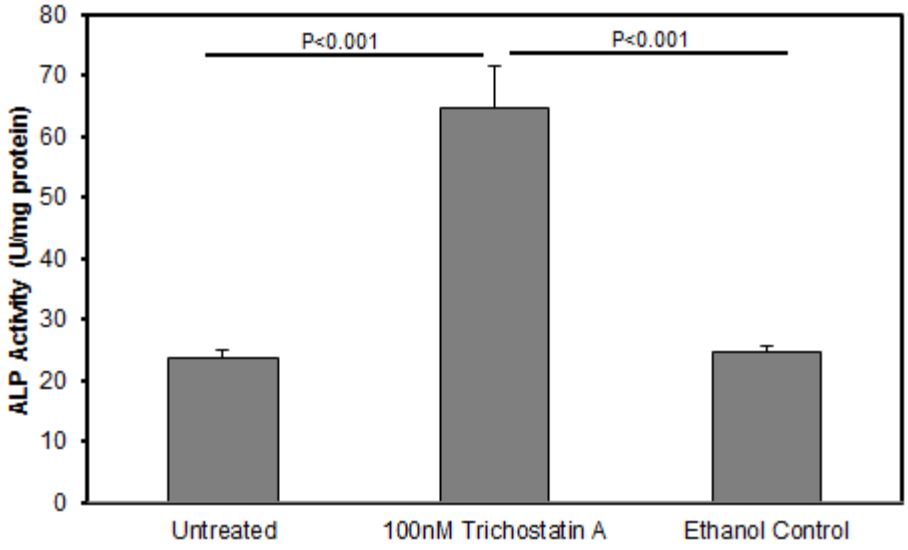
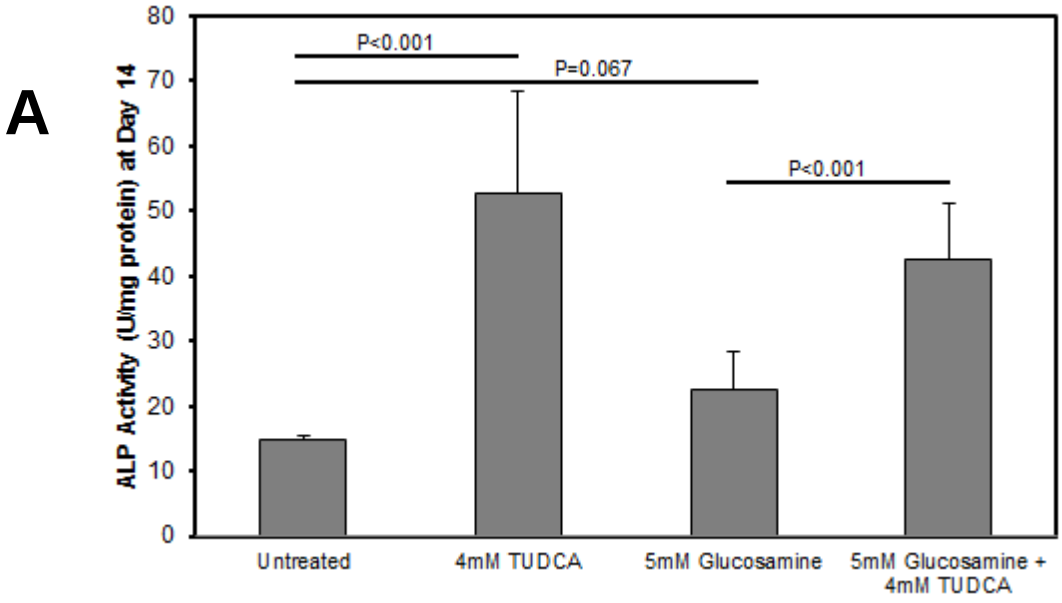


Figure 5.13 - TUDCA promotes ALP activity and ThT staining in MOVAS cells.

MOVAS cells were cultured in the presence of 4mM TUDCA, 5mM glucosamine or both. **Panel A:** Cell lysates were subject to an ALP assay after 14 days and values normalized to total protein. **Panel B:** Live cell fluorescent images were captured using ThT staining after 18 hrs.



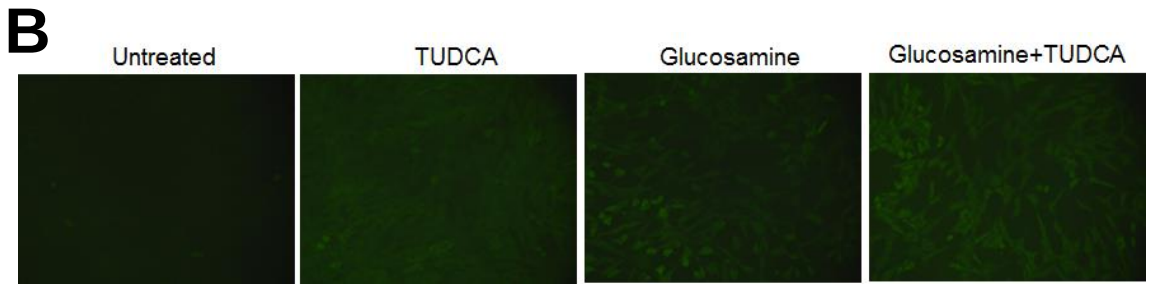
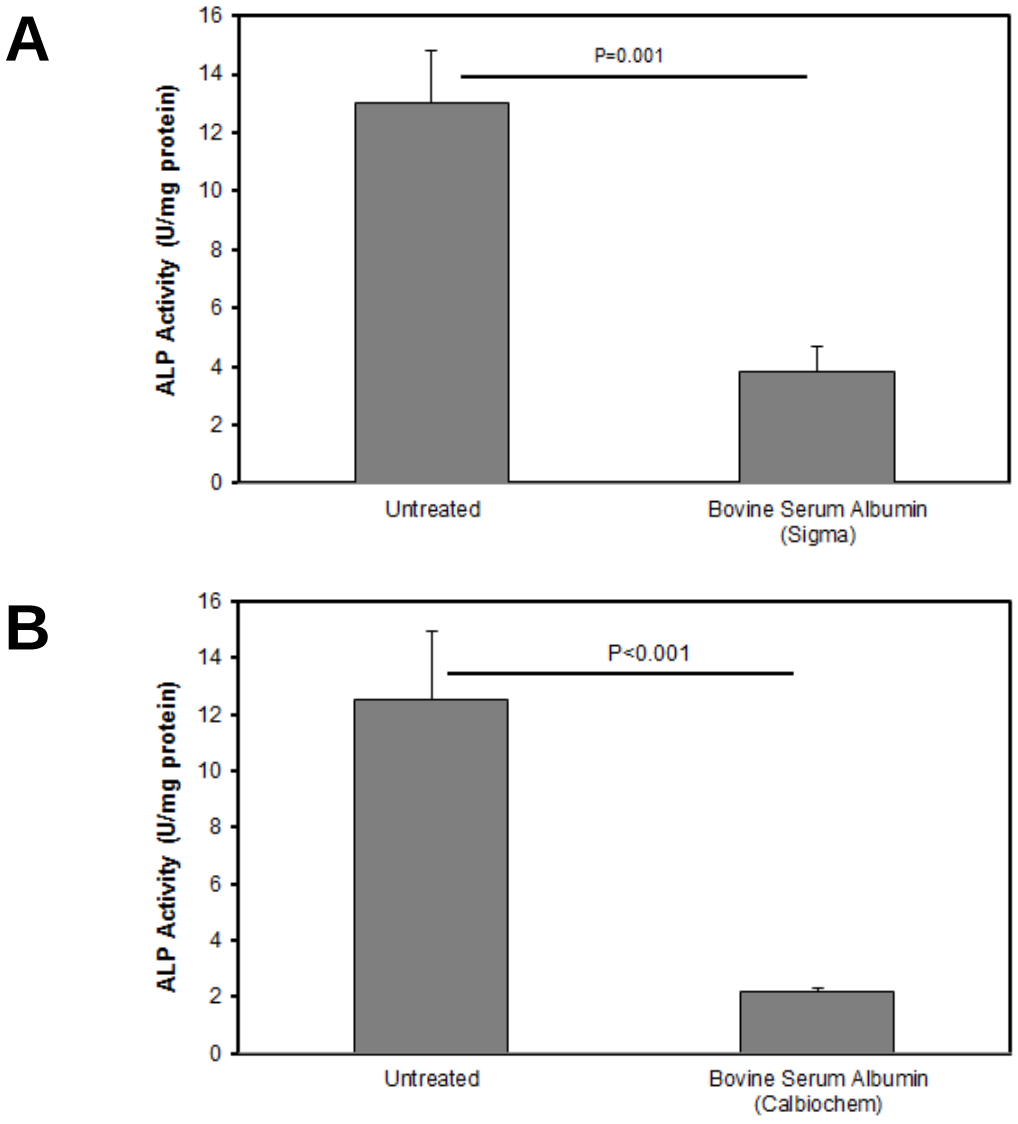


Figure 5.14 - BSA Attenuates ALP Activity in MOVAS Cells.

Cells were treated with 4% BSA obtained from either Sigma **(Panel A)** or Calbiochem **(Panel B)** for 14 days. Cell lysates were subject to an ALP assay by measuring the cleavage of pNPP at 405 nM. ALP values were normalized to total protein.



CHAPTER 6: General Discussion

The prevalence of obesity, defined as a BMI of 30 or higher, has increased significantly from 6.1% in 1985 to 18% in 2010, according to the Canadian Community Health Survey (CCHS). Alarmingly, this number is predicted to reach 21% by 2019 (Twells *et al.* 2014). This is of great concern, as obesity is a major risk factor for insulin resistance, type 2 diabetes, CVD, and vascular calcification. We are just beginning to understand the mechanisms linking obesity to these pathologies.

Adipose tissue produces a large number of hormones and cytokines, including TNF-α, IL-6, leptin, adiponectin, resistin, and obesity is associated with significant changes in the secretory activities of adipocytes (Hajer *et al.* 2008). Evidence suggests that the shift in adipocyte metabolic activity is an important link between obesity and associated morbidity and mortality.

Until recently, vascular calcification was considered a benign finding associated with aging, uremia, diabetes, and atherosclerosis. Vascular calcification has since been correlated with advanced CVD and is now understood to be a significant predictor of cardiovascular events (Bobryshev *et al.* 2008;Detrano *et al.* 2008;Rennenberg *et al.* 2009;Vliegenthart *et al.* 2002). Advances in imaging modalities, including fluoroscopy, intravascular ultrasound (IVUS) and electron beam computed tomography (EBCT), have permitted researchers to quantify and correlate vascular calcification to cardiovascular risk

and clinical events (Speer and Giachelli 2004). The molecular mechanisms that underly these observations are still being worked out. The adipose tissue-specific cytokine, TNF-α, for example, has been shown to be able tp stimulate the mineralization of aortic calcifying vascular cells (CVCs) *in vitro* (Tintut *et al.* 2000). *In vivo*, TNF-α has been shown to promote the expression of the Msx2-Wnt osteogenic pathway within the aorta and contribute to aortic calcium accumulation in an $LDLR^{-/-}$ mouse model of type 2 diabetes (Al Aly *et al.* 2007).

One of the aims of this thesis was to investigate the potential link between obesity and CVD by examining the effect of the adipocytokine leptin in the context of vascular calcification (**Chapter 3**). A second aim was to delineate the molecular mechanism underlying this effect (**Chapter 4**).

In these studies, we demonstrate that leptin treatment augments vascular calcification in an $ApoE^{-/-}$ mouse model of atherosclerosis. This increase in calcification was associated with an increase in the expression of osteoblast-specific markers including osteocalcin, OPN and ALP. Evidence from our *in vivo* study together with several other *in vitro* studies in the field (Handschin *et al.* 2007;Parhami *et al.* 2001;Reseland *et al.* 2001;Thomas *et al.* 1999) have firmly established the ability of leptin to mediate osteogenic differentiation. The signaling mechanism by which this occurs in VSMCs is not clear. Some reports have implicated a role for Wnt signaling in the process of osteoblast differentiation. Therefore, we examined whether or not leptin can signal through the GSK-3β/β-catenin axis to promote the osteogenic differentiation of BASMCs.

The results of these experiments demonstrate that leptin induces GSK-3β phosphorylation/inactivation and regulates the expression of genes involved in osteoblast differentiation. Furthermore, we have shown, for the first time, that constitutively active GSK-3β attenuates leptin-induced osteoblast differentiation suggesting that leptin signals through GSK-3β to regulate osteoblast differentiation.

There are some important questions that can be addressed about the *in vivo* study reported in Chapter 3 including: (1) Why was 125 μg of leptin/mouse/day chosen as the dose to use?; (2) What was the circulating leptin level in the experimental mice?; and (3) Why was there no difference in atherosclerotic lesion size?

The dose of leptin used in our *in vivo* study was based on two previous reports. The first showed that 125 μg was the necessary dose to promote weight loss and restore fertility in leptin-deficient mice (Malik *et al.* 2001). Another study demonstrated that daily injections of 125 μg leptin for 4 weeks promoted atherosclerosis and thrombosis in ApoE$^{-/-}$ mice (Bodary *et al.* 2005). Therefore, we also injected mice with 125 μg of leptin daily but for 8 weeks assuming that vascular calcification requires more time to develop compared to atherosclerosis.

Serum leptin levels in lean subjects are typically between 5 - 15 ng/mL but levels have been reported to be ≥ 25 - 30 ng/mL in obese individuals (Sinha *et al.* 1996). We did not measure leptin levels in our mice during the course of the experiment. However, to put leptin levels into context, another seminal study from

this field reported leptin levels in wild-type C57BL/6J mice maintained on either a normal chow or high fat diet for 8 weeks: normal chow diet - 2.2±0.4 ng/mL versus high fat diet - 20±4.0 ng/mL. The corresponding body weight of the mice in this experiment was also reported: normal chow diet – 34 g versus high fat diet - 41 g (Schafer *et al.* 2004). We speculate that the leptin levels in our control mice (also maintained on a high fat diet) would likely have been lower than those reported by Schafer *et al.* (2004) because our reported body weights were much lower (approximately ~20 g; **Table 3**). Chiba *et al.* (2008) reported that leptin levels in ApoE$^{-/-}$ maintained on a Paigen diet were approximately 1 ng/ml. It is not clear what the average body weight of these male mice was relative to the mice in the report from Schafer *at al*. (2004). However, when taken together, these studies show that mice display leptin levels that are similar to those found in humans.

Lastly, prior studies had shown that leptin increases total lesion area (Bodary *et al.* 2005;Chiba *et al.* 2008). Bodary *et al.* (2005) specifically examined the pro-atherogenic effects of leptin within the carotid and brachiocephalic arteries and the thoracic aorta. They did not find a significant increase in total lesion area when examining lesions within the thoracic aorta of leptin-treated mice, even though the same animals showed a significant increase in total lesion area within the brachiocephalic and carotic artery. Chiba *et al.* (2008) also reported that leptin increased the percentage of atherosclerotic area when the whole aortic surface was examined. Our studies failed to show an increase in

atherosclerosis at the aortic root or on the surface of the thoracic aorta, after the administration of leptin to ApoE$^{-/-}$ mice (**Figure 3.1**). Neither Bodary *et al.* nor Chiba *et al.* measured the effect of leptin on atherosclerotic lesion area at the aortic root. It is possible that the pro-atherogenic effects of leptin are site-specific. Whether these differences account for the observed discrepancy is not known. However, our findings suggest that the effect(s) of leptin on vascular calcification are independent of any pro-atherogenic effects.

There exist some key differences in methodology between our study and that by Bodary *et al.* (2005), including: (1) the length of leptin treatment (8 weeks vs. 4 weeks) and (2) the gender of the mice (female vs. male). There are also significant differences between our study and that by Chiba *et al.* (2008), including: (1) the dose of leptin used (125 µg/mouse/day vs. 5 µg/mouse/day); (2) method of leptin delivery (intraperitoneal injections vs. mini-osmotic pump that delivered leptin at a rate of 0.25 µl/h); (3) length of leptin treatment (8 weeks vs. 4 weeks); and (4) type of diet. These differences might explain the discrepancy in certain findings. However, neither Bodary *et al.* nor Chiba *et al.* examined vascular calcification in leptin-treated mice.

Vascular calcification is a heterogeneous disorder with overlapping yet distinct mechanisms of initiation and progression. The predominant school of thought is that a subpopulation of VSMCs can undergo a phenotypic change and behave much like the bone-forming cells known as osteoblasts. Normally functioning, contractile VSMCs express SM-actin, SM22α, and SM-MHC that

directly contribute to the maintenance of vascular tone in the medial layer of the arterial wall. Under pathological conditions, however, VSMCs can be induced to undergo osteogenic differentiation. For example, we and others have shown that primary cultures of BASMCs can differentiate into osteoblast-like cells when cultured *in vitro* (Figure 1.2) (Bear *et al.* 2008;Steitz *et al.* 2001;Yang *et al.* 2005). It is noteworthy, however, that adventitial myofibroblasts have also been implicated in this process (Cheng *et al.* 2003;Shao *et al.* 2003;Towler *et al.* 1998). For example, Msx2 (an osteoblast-specific transcription factor) expression and von kossa staining was detected in a subset of peri-aortic adventitial cells in $LDLR^{-/-}$ mice fed a high fat diet (Cheng *et al.* 2003). These results suggest a role for aortic fibroblasts (pericytes) in vascular calcification.

In Chapter 4 and 5, we show that leptin promotes vascular calcification through a GSK-3β-dependent mechanism. However, we did not conclusively identify the events upstream of GSK-3β inactivation. Based on the literature, in our working model (**Figure 4.5**) we propose that leptin may be activating either ERK 1/2 and/or Akt to promote the inactivation of GSK-3β and subsequent osteoblast differentiation. Studies have already shown that the activation of ERK and Akt mediate leptin's actions (Liu *et al.* 2014;Wang *et al.* 2012a;Wang *et al.* 2012b). Upon leptin binding to its receptor, receptor dimerization activates JAK2 which is noncovalently bound to Ob-Rb. JAK2 autophosphorylates on Tyr813 which can stimulate activation of the IRS/PI3K pathway via SH2B1. In addition, JAK2 phosphorylates Ob-Rb on Tyr 985/1077/1138. SHP2 can bind to

phosphorylated Tyr 985 residue and mediate the activation of the MAPK pathway via Grb2. In support of our hypothesis, both MAPK (Ding *et al.* 2005;Stambolic and Woodgett 1994) and Akt (Cross *et al.* 1995;van Weeren *et al.* 1998) have been shown to independently promote the inactivation of GSK-3 in various cell lines. Indeed, previous studies have also demonstrated that ERK and Akt are involved in the osteoblastic differentiation and mineralization of VSMCs (Bear *et al.* 2008;Liu *et al.* 2011;Taylor *et al.* 2011). Skeletal development requires a delicate balance of osteoblast proliferation, survival and differentiation which is mediated by the relative strengths of ERK 1/2 versus Akt activation (Raucci *et al.* 2008). As an extension of these findings, a recent study demonstrated that the leptin mediated - osteoblast differentiation of calcifying VSMCs can occur via BMP4-RANKL in an ERK 1/2 and Akt – mediated manner (Liu *et al.* 2014). Therefore, in light of these studies, further research should be focused upon understanding the relative contributions of ERK 1/2 and Akt in leptin-mediated osteogenic differentiation of VSMCs.

One of the limitations of our current investigations is that the experiments of the latter chapters were performed exclusively *in vitro. In vivo* this is a complex process, and we cannot eliminate the possibility that other cell types likely contribute to the overall process of vascular calcification. For example, macrophages are known to secrete TNF-α in response to factors such as oxidized or acetylated LDL, physically damaged extracellular matrix and bacterial infection (Trion and van der 2004). In addition to its effect on aspects of

atherosclerosis such as endothelial permeability, monocyte adhesion and macrophage differentiation, TNF-α has been shown to promote the mineralization of CVCs (Tintut *et al.* 2000) and regulate the expression and secretion of leptin (Kirchgessner *et al.* 1997). As an extension of these findings, a separate study demonstrated that monocytes/macrophages enhance vascular calcification of SMCs *in vitro* via two mechanisms: cell-cell interaction and production of soluble factors such as TNF-α (Tintut *et al.* 2002). Therefore, it is likely that the proximity of monocytes/macrophages to VSMCs may contribute to obesity-related hyperleptinemia as well as exacerbate vascular calcification *in vivo*.

Several of the outstanding questions which arose from our studies would benefit from future work using the novel, Cre/*loxP* (cyclization recombination/locus of X-over P_1) tissue-specific GSK-3α and β knockout mouse models. Atherosclerosis-prone $LDLR^{-/-}$ mice carrying floxed GSK-3α or β have been successfully created in the lab of Dr. Geoff Werstuck by Cameron McAlpine. This will be an invaluable tool in studying the role of GSK-3 *in vivo* because, while GSK-3α whole-body knockout mice exist with little or no overt phenotype (MacAulay *et al.* 2007), GSK-3β whole-body knockout mice are not viable (Hoeflich *et al.* 2000). Our lab has shown that $LDLR^{-/-}$/$GSK\text{-}3\alpha^{-/-}$ and $LDLR^{-/-}$/$GSK\text{-}3\alpha^{+/-}$ mice fed a high fat diet develop significantly smaller atherosclerotic lesions when compared to $LDLR^{-/-}$/$GSK\text{-}3\alpha^{+/+}$ control mice (Banko *et al.* 2014, in press). Atherosclerotic lesions are 60% and 80% smaller in $LDLR^{-/-}$/$GSK\text{-}3\alpha^{+/-}$ and $LDLR^{-/-}$/$GSK\text{-}3\alpha^{-/-}$ mice, respectively. These mouse models would be useful

(1) to directly distinguish the contribution of GSK-3 activity in various vascular cells (e.g. SMCs vs. macrophages), and (2) to determine the contributions of GSK-3α versus β in vascular calcification.

It would be relevant to generate macrophage-specific and SMC-specific GSK-3α and β knockout mice on an $ApoE^{-/-}$ background at first to eliminate any effects of diet-induced insulin-resistant diabetes mellitus and obesity with hypertriglyceridemia. These experiments can be complemented in the absence or presence of leptin-treatment using a similar methodology to that described in Chapter 3. Certainly, repeating these experiments in an $LDLR^{-/-}$ mouse which develops characteristics relevant to the human population (obesity and diabetes) would also be of great interest. Cameron McAlpine has already provided some preliminary data from the macrophage-specific GSK-3α and β knockout mice on an $LDLR^{-/-}$ background ($LDLR^{-/-}GSK3\alpha^{fl/fl}LyzCre^{+/-}$ and $LDLR^{-/-}GSK3\beta^{fl/fl}LyzCre^{+/-}$, respectively). After 10 weeks on a high fat diet, knocking out GSK-3α and β in macrophages has no effect on plasma or liver lipid levels. However, $LDLR^{-/-}GSK3\alpha^{fl/fl}LyzCre^{+/-}$ mice display smaller lesions at the aortic root relative to floxed controls while the $LDLR^{-/-}GSK3\beta^{fl/fl}LyzCre^{+/-}$ mice have the same lesion size as controls but their atherosclerotic lesions appear to be less complex (unpublished data).The myeloid-specific promoter that has been used in our lab is LysozymeM-Cre and according to the literature, SM22α-Cre could be used as the SMC-specific promoter in future SMC tissue-specific knockout experiments (Lepore *et al.* 2005;Naik *et al.* 2012;Nguyen *et al.* 2013). For a more elaborate and slightly

more complex model, LDLR$^{-/-}$GSK3α$^{fl/fl}$SM22αCre$^{+/-}$ and LDLR$^{-/-}$ GSK3β$^{fl/fl}$SM22αCre$^{+/-}$ mouse strains can be crossed with a Rosa26-Cre reporter (R26R) strain which expresses β-galactosidase activity following Cre-mediated excision. This would allow for genetically labelling cells of SM origin even after their osteogenic differentiation in vascular calcification.

In the final chapter of this thesis, we extrapolated from previous studies in our lab which demonstrated that the pro-atherogenic effects of ER stress may be mediated through the activation of GSK-3 and that inhibiting GSK-3 attenuates atherosclerosis in hyperglycemic, hyperhomocysteinemic, and dysplipidemic ApoE$^{-/-}$ mouse models (Bowes *et al.* 2009;McAlpine *et al.* 2012). More recent work with LDLR$^{-/-}$/GSK-3α$^{-/-}$ mice support these previous findings by showing that whole-body GSK-3α knockout mice develop significantly smaller atherosclerotic lesions compared to LDLR$^{-/-}$/GSK-3α$^{+/-}$ and LDLR$^{-/-}$/GSK-3α$^{+/+}$ control mice. Several independent risk factors for cardiovascular disease including, hyperglycemia (Werstuck *et al.* 2006), hyperhomocysteinemia (Werstuck *et al.* 2001;Zhou *et al.* 2004), elevated levels of unesterified cholesterol (Li *et al.* 2005b) or palmitate (Cunha *et al.* 2008) and obesity (Ozcan *et al.* 2004), can also promote the activation of the UPR. Therefore, the objective of Chapter 5 was to examine the physiological role that GSK-3 plays in modulating the pathogenesis of vascular calcification/atherosclerosis in VSMCs using agents that are known to modulate ER stress (e.g. glucosamine, PBA and TUDCA) (**see Figure 5.1 – Proposed model**).

Surprisingly, our studies showed an increase in the osteogenic differentiation of VSMCs in the presence of either glucosamine or PBA. Glucosamine-treatment showed the following effects: (1) increases in ER stress and UPR activation which was associated with only a slight increase in GSK-3 activity, (2) attenuation in the expression of contractile SMC markers, αSM-actin, and synthetic SMC marker, tropomyosin; (3) no significant change in the expression of synthetic SMC markers (vimentin, or type III collagen); (4) up-regulation in the expression of osteoblast-specific markers (Runx2, Osx and osteocalcin); and (5) dose-dependent increase in ALP activity. PBA-treatment showed a similar pattern of activity as glucosamine in all experiments, albeit the effects of PBA were much more pronounced. PBA significantly attenuated total GSK-3 activity. The ability of PBA to induce ER stress in MOVAS cells was confirmed using the ThT-fluoresence method to identify misfolded proteins. To the best of our knowledge, this is the first report in which PBA increases, rather than decreases, ER stress. Interestingly, tunicamycin and A23197, which promote ER stress by blocking protein N-glycosylation (Feige and Scheffler 1987) and disrupting ER Ca^{2+} balance (Li *et al.* 1993), respectively, attenuated ALP activity at very low concentrations (Figure 5.8). However, tunicamycin showed signs of UPR activation while A23187 did not (**Figure 5.11**). Unfortunately, we were unable to investigate the role of palmitate in this *in vitro* model because of the effects of BSA alone on ALP activity (supplementary data, **Figure 5.14**). These findings are certainly at odds with our proposed hypothesis. They

demonstrate a disconnect between effects on ER stress and effects on osteogenic differentiation in VSMCs making it difficult to investigate any role that GSK-3 may be playing in this process using “ER stressors/ modulators.”

It is plausible that the increase in ER stress which we observe in our cell culture model is a consequence of the osteogenic differentiation that VSMCs are undergoing. During osteoblast differentiation, the UPR acts to expand the capacity of the ER to manage the increase in the production of extracellular matrix proteins and to avoid apoptosis induced by ER overload. Furthermore, some ER stress agents are likely able to signal through additional pathways that are independent of the UPR making it more difficult to delineate whether ER stress is causative or associative.

Tunicamycin, a potent inducer of ER stress has previously been shown to suppress the osteogenic differentiation of a preosteoblast cell line (MC3T3E1 cells) by inducing CREBH (cyclic AMP response element-binding protein H) expression and cleavage (Jang *et al.* 2011). In contrast, elevated levels of O-linked glycosylation accelerate vascular calcification in arteries of a diabetic mouse model through a mechanism which involves Akt (Heath *et al.* 2014). This is relevant to our studies since under normo-glycemic conditions of a cell, 1 to 3% of total glucose will enter the HBP and be converted to glucosamine-6-phosphate by the enzyme GFAT (Marshall *et al.* 1991) eventually yielding UDP-GlcNAc. UDP-GlcNAc participates in the proper folding of many proteins by acting as a substrate for both O- and N-linked protein glycosylation. However, elevated

concentrations of glucosamine increase levels of O-linked protein glycosylation (Werstuck *et al.* 2006). Additionally, ER stress modulator, PBA, can act as an HDAC inhibitor (Shi *et al.* 2007;Svechnikova *et al.* 2008;Takai and Narahara 2010). HDAC inhibitors, such as TSA, have previously been shown to promote cell differentiation including osteoblast differentiation (Azechi *et al.* 2013;Eom and Kook 2014;Svechnikova *et al.* 2008). Therefore, it is possible that PBA is promoting the osteogenic differentiation of VSMCs by (1) attenuating GSK-3 activity and/or (2) inhibiting histone deacetylase activity.

In contrast to previously reported studies which examine the role of ER stress in osteogenic differentiation, our studies were conducted in the absence of any additional well-described pro-osteogenic signals such as BMP-2 (Saito *et al.* 2011;Tohmonda *et al.* 2011) or βGP (Masuda *et al.* 2012;Nakamura *et al.* 2013). Finally, it is worth mentioning that much of the current literature examines the role of ER stress – mediated osteogenic differentiation in pre-osteoblast cell lines (Hamamura and Yokota 2007;Nakamura *et al.* 2013;Saito *et al.* 2011;Tohmonda *et al.* 2011) as opposed to vascular cells where the mechanism(s) may differ still from true osteogenesis. We propose that future studies will need to focus on (1) delineating the mechanisms of various ER stressors/attenuators independently of one another; (2) using consistent cell culture models; and (3) repeating these experiments in various mouse models to determine if they in fact promote vascular calcification under physiological conditions.

In conclusion, the results of this thesis suggest that (1) leptin and GSK-3 play a critical role in the development of vascular calcification; and (2) our studies also shed light on the complexity of the specific role that ER stress inducing – and modulating – agents play in osteogenic differentiation. There exists a significant association between obesity, diabetes and vascular calcification, as well as a significant increase in the prevalence of these disease states. Therefore, an understanding of specific mechanisms will provide therapeutic options for the prevention and/or reversal of vascular calcification.

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