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BMP-3 PROMOTES MESENCHYMAL STEM CELL PROLIFERATION VIA THE TGF-ß/ACTIVIN PATHWAY

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ABSTRACT

This study was designed to test the hypothesis that bone morphogenetic protein-3 (BMP-3) promotes adipogenesis using C3H10T1/2 mesenchymal stem cells (MSCs) and 3T3-L1 preadipocytes as *in vitro* model systems. Although having no effects on the commitment of C3H10T1/2 cells to the adipocyte lineage or the differentiation of 3T3-L1 preadipocytes to adipocytes, BMP-3 stimulated DNA synthesis (as determined by [^{3}H]-thymidine incorporation) in both cell types. Furthermore, the mitogenic effects of BMP-3 were mediated by the TGF-β/activin signalling pathway, because BMP-3 activated the TGF-β/activin pathway in C3H10T1/2 cells, and the TGF-β/activin pathway inhibitor SB-431542 blocked BMP-3-stimulated proliferation. Additionally, inhibitors of ERK1/2 and p38 MAPK pathways reduced proliferation under basal conditions. In conclusion, the present study demonstrates that BMP-3 stimulates proliferation of MSCs and preadipocytes *in vitro*, which provides a source for adipogenesis. Consequently, aberrant BMP-3 expression may have consequences not only for normal adipose tissue growth but also for the pathogenesis of obesity.

Keywords: adipogenesis, adipocytes, preadipocytes, self-renewal, p38 MAPK, ERK1/2, JNK, Smad2, C3H10T1/2, 3T3-L1, [^{3}H]-thymidine, SB-431542, SB-202190, U0126

CO-AUTHORSHIP

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α	alpha
β	beta
γ	gamma
δ	delta
A	adenine
ActRIIA	activin receptor type 2A
ActRIIB	activin receptor type 2B
ALK	activin-like kinase
BAT	brown adipose tissue
BMI	body mass index
BMP	bone morphogenetic protein
BMPRII	bone morphogenetic protein receptor type 2
bp	base pair
°C	degrees Celsius
C	cytosine
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CDK	cyclin-dependent kinase
C/EBP	CCAAT-enhancer binding protein
Ci	Curie
cm	centimeter
CO_2	carbon dioxide

Dex	dexamethasone
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DOHaD	developmental origins of health and disease
DPBS	Dulbecco's phosphate-buffered saline
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGR	fetal growth restriction
g	grams
G	guanine
GTP	guanine triphosphate
^{3}H	tritium
h	hour
HCl	hydrochloric acid
HRP	horseradish peroxidase
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	half maximal inhibitory concentration
IGF	insulin-like growth factor
IgG	immunoglobulin G
IL	interleukin
IUGR	intrauterine growth restriction
JNK	c-Jun NH_2-terminal kinase

K_d	dissociation constant
kg	kilogram
l	liter
m	meter
M	molar
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MEK	MAP/ERK kinase
MEKK	MAP/ERK kinase kinase
MEM	minimal essential medium
min	minutes
mJ	milliJoule
MKK	MAP kinase kinase
ml	milliliter
mM	millimolar
mmol	millimole
MMP	matrix metalloproteinase
MPR	maternal protein restriction
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
NaOH	sodium hydroxide
NCS	newborn calf serum
ng	nanogram

nM	nanomolar
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PlGF	placental growth factor
PPAR	peroxisome proliferator-activated receptor
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
R-Smad	receptor-activated Smad
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
s	second
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of mean
siRNA	small interfering ribonucleic acid
T	thymine
TβRII	transforming growth factor beta receptor type 2
TAK	TGF-β-activated kinase
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TGF	transforming growth factor
TNF	tumor necrosis factor
TSP	thrombospondin

TTBS	Tris-Tween buffered saline
UCP	uncoupling protein
UV	ultraviolet
μCi	microCurie
μg	microgram
μl	microliter
μM	micromolar
VEGF	vascular endothelial growth factor
vol	volume
WAT	white adipose tissue
WHO	World Health Organization
wt	weight

Chapter 1

Introduction

1.1 The Obesity Epidemic

1.1.1 Definition of Obesity

Obesity is defined as an increase in body weight, specifically adipose tissue, in sufficient magnitude to produce adverse health issues (Mokdad *et al*, 2003, Spiegelman and Flier, 2001). According to body mass index (BMI), individuals with a BMI $\geq$ 30 kg/m^2 are considered obese (James *et al*, 2006). In a 2004 survey from the World Health Organization (WHO), 23.1% of Canadian adults were reported as obese and 36.1% were overweight (Tjepkema, 2004). Furthermore, WHO reported that worldwide approximately 400 million adults were obese and 1.6 billion were overweight.

Obesity is the result of an imbalance between energy intake and energy expenditure. Ideally, the amount of energy consumed should be equivalent to the amount expended. In the case of obesity however, energy consumption outweighs energy expenditure and the excess energy is stored in the form of adipose tissue (Spiegelman and Flier, 2001). This imbalance can result from an increase in food intake (energy uptake), a decrease in physical activity (energy expenditure), or a combination of both. In today's society, both ends of the spectrum are problematic with the ready access to high-fat foods and the increase in conveniences that promote a sedentary lifestyle.

Aside from an increase in adipose tissue, a number of co-morbidities accompany obesity including hypertension, cancers, musculoskeletal disorders, physical disability, osteoarthritis, depression, anxiety, and psychological disturbances (Dixon, 2009). Furthermore, obesity is a major contributor to the striking rise of metabolic syndrome, which consists of metabolic abnormalities including glucose intolerance, insulin resistance, central obesity, dyslipidemia, and hypertension. When these conditions are concomitant in an individual, they are associated with an increased risk of cardiovascular

disease (Eckel *et al*, 2005). Given the immense impact that obesity has on health and its steady rise in prevalence over the past decades, the understanding of its etiology is essential to creating successful treatment and prevention strategies for the future.

1.1.2 Classification of Obesity

Adipose tissue accumulates in various fat depots of the body including visceral, subcutaneous, and gluteofemoral sites (Bouchard, 1991). Subcutaneous-abdominal locations (android) are those frequently associated with the male body type. Conversely, it is more common for women to carry excess adipose tissue in the gluteofemoral region (gynoid). Nonetheless, visceral or intra-abdominal adiposity is the form of obesity that best predicts an increase in morbidity and mortality (Montague and O'Rahilly, 2000). Individuals carrying excess adipose tissue in the visceral-abdominal region have a greater risk of developing insulin resistance and cardiovascular disease (Rosen and MacDougald, 2006). Although the causal link between visceral adiposity and insulin resistance is highly debated, theories have suggested that the free fatty acid flux from visceral adipose tissue is shunted towards the liver. As a result, triglyceride synthesis is promoted in the liver, which can induce hepatic insulin resistance (Rader, 2007). Conversely, adipose tissue that is located subcutaneously is thought to be less of a risk factor because the free fatty acid flux from these tissues is shunted into the general circulation having a less concentrated effect on the liver. In any case, free fatty acid flux occurs in all adipose tissue and all excess fat mass can pose elevated health risks (Rader, 2007).

1.1.3 Classification of Adipose Tissue

The pool of adipose tissue in mammals consists of two functionally diverse forms of adipose tissue: brown and white (Cypess *et al*, 2009). White adipose tissue (WAT) is the primary energy store in the human body (Rosen and Spiegelman, 2006) and is the

form of adipose tissue that we observe in obesity. Historically, WAT was thought to be an inactive tissue; however, it is now known to serve as an important player in energy metabolism through endocrine, paracrine, and autocrine signalling (Kim and Moustaid-Moussa, 2000). The primary role of WAT is to store energy in the form of triglycerides when energy intake exceeds energy expenditure. Conversely, when energy expenditure exceeds energy intake, WAT can catabolise these triglycerides to produce glycerol and fatty acids that participate in glucose metabolism (Vazquez-Vela *et al*, 2008). Furthermore, WAT also functions as an endocrine organ by secreting adipokines, which include a variety of hormones and peptides such as leptin, adiponectin, TNF-α, and IL-6. (Dyck, 2009; Vazquez-Vela *et al*, 2008).

Brown adipose tissue (BAT) is primarily present in newborns. In adults, the level of BAT is reduced significantly and is only found in dispersed areas throughout the body including interscapular, periaortic, perirenal, and intercostal regions, as well as dispersed in small pockets within WAT (Cannon and Nedergaard, 2004). BAT has similar distribution in both sexes; however, it has been shown that there is a greater mass and activity in women over men (Cypess *et al*, 2009). BAT primarily plays a role in thermogenesis by expending energy through the generation of heat. This mechanism is under the control of uncoupling protein-1 (UCP-1) (Cannon and Nedergaard, 2004). In order to carry out its function, the composition of BAT consists of fewer lipids and contains more mitochondria when compared to WAT (Farmer, 2008). Historically, BAT was not considered important in adults; however, recent studies have revealed evidence that suggests this may be untrue. The prevalence of BAT has been shown to have an inverse correlation to BMI, suggesting that low levels of BAT are related to obesity. Furthermore, this correlation is primarily evident in elderly adults, signifying BAT may

play a role in the prevention of age-related obesity (Cypess *et al*, 2009). Given that BAT can expend high levels of energy; inducing BAT may become a new approach for the treatment and prevention of obesity (Gesta *et al*, 2007).

1.2 Pathogenesis of Obesity

1.2.1 Environmental and Genetic Factors

A number of genes associated with human adiposity have been identified (Froguel and Boutin, 2001) and there are multiple hypotheses that attempt to explain the underlying genetics of obesity. Genetics play a role in the etiology of obesity because they account for an individual's susceptibility to the condition (de Ferranti and Mozaffarian, 2008). The thrifty genotype hypothesis suggests that the ability to store fat in times of excess nutrition may be a positive trait that was selected through evolution (Neel, 1962). Conversely, the fetal programming hypothesis states that the predominant governing force is the fetal environment, which in response to over- or under-nutrition will provoke a postnatal response in a child (Walley *et al*, 2009). Other hypotheses also make an effort to explain genetic factors, however many contradict one another and it remains unclear as to which hypothesis is the most accurate.

Nevertheless, the abrupt rise in obesity that has occurred over the past few decades cannot be attributed to genetic factors alone. To emerge so quickly, and on such a large scale, is primarily due to environmental factors; particularly the increase in food availability and the decrease in physical activity. As a result, chronic intake of excess energy is stored in the form of intracellular triglycerides in adipocytes (Spiegelman and Flier, 2001). Nonetheless, the genetic-environmental interactions that occur in an individual are what ultimately account for any increase in adipose tissue.

1.2.2 Adipose Tissue Expansion

With excess nutritional supply, energy is stored in the body as adipose tissue. Adipose tissue expansion occurs by way of two mechanisms; adipocyte hyperplasia and adipocyte hypertrophy (Otto and Lane, 2005), however the regulation of hyperplasia and hypertrophy are poorly understood. In order to supply the expanding tissue with oxygen and nutrients, the vasculature also expands by increasing the number and size of vessels (Lijnen, 2008). Furthermore, the growth of tissue requires continuous remodelling of the extracellular matrix (ECM); a process that involves proteolysis. There is increasing evidence for the involvement of proteolysis in several aspects of adipose tissue growth (Lijnen, 2008). Consequently, adipose tissue expansion is a complex process that includes not only adipocyte hyperplasia and hypertrophy, but also angiogenesis and ECM remodelling.

1.2.2.1 Adipocyte Hypertrophy

Adipocyte hypertrophy is an increase in cell size and is thought to precede adipocyte hyperplasia. Previously, increased lipid storage of already developed fat cells was considered the primary source of obesity because the number of adipocytes was thought to remain constant throughout adulthood. However, an increase in the number of adipocytes in obesity has been established, therefore hypertrophy is now known to work in concert with hyperplasia (Spalding *et al*, 2008). Evidence suggests that as triglyceride storage accumulates in the adipocyte, the cell volume increases until it reaches a critical size, at which point the enlarged cell triggers the beginning of new cell replication and/or preadipocyte differentiation (Bluher, 2009; Faust *et al*, 1978). This was further supported in a model of diet-induced hyperplasia where cells appeared to reach a maximum size prior to the initiation of adipocyte hyperplasia (Jo *et al*, 2009).

1.2.2.2 Adipocyte Hyperplasia

Hyperplasia is defined as an increase in cell number. Proliferative growth of adipocytes has been shown to appear in both the early stages of adipose tissue development, as well as throughout adulthood (Drolet *et al*, 2008; Spalding *et al*, 2008). In addition, approximately 10% of adipocytes are renewed annually providing a continual role for adipocyte hyperplasia (Spalding *et al*, 2008). The regulation of hyperplasia is unclear; however, studies suggest that lipid-laden adipocytes may stimulate its activation (Bluher, 2009). Hyperplastic growth occurs when new adipocytes are recruited from their precursor cells; a direct result of increased adipogenesis. Consequently, the regulation of adipogenesis is a fundamental component of adipose tissue expansion and the pathogenesis of obesity.

1.2.2.2.1 Adipogenesis

As shown in Figure 1.1, adipogenesis is a two-step process which induces the formation of adipocytes. Beginning with a mesenchymal stem cell (MSC), the cell first commits to the adipocyte lineage thus becoming a preadipocyte. The preadipocyte then differentiates into a mature adipocyte (Rosen and MacDougald, 2006). Both MSCs and preadipocytes also undergo proliferation. This transformation process is under the regulation of hormonal activity and transcription factors (Vazquez-Vela *et al*, 2008). A thorough understanding of the factors that control adipogenesis is critical in elucidating the etiology of obesity.

1.2.2.2.1.1 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) were first discovered in the bone marrow of rats more than 40 years ago (Friedenstein *et al*, 1966). They are primarily found in the stromal compartment of the bone marrow, representing approximately 0.001 – 0.01% of

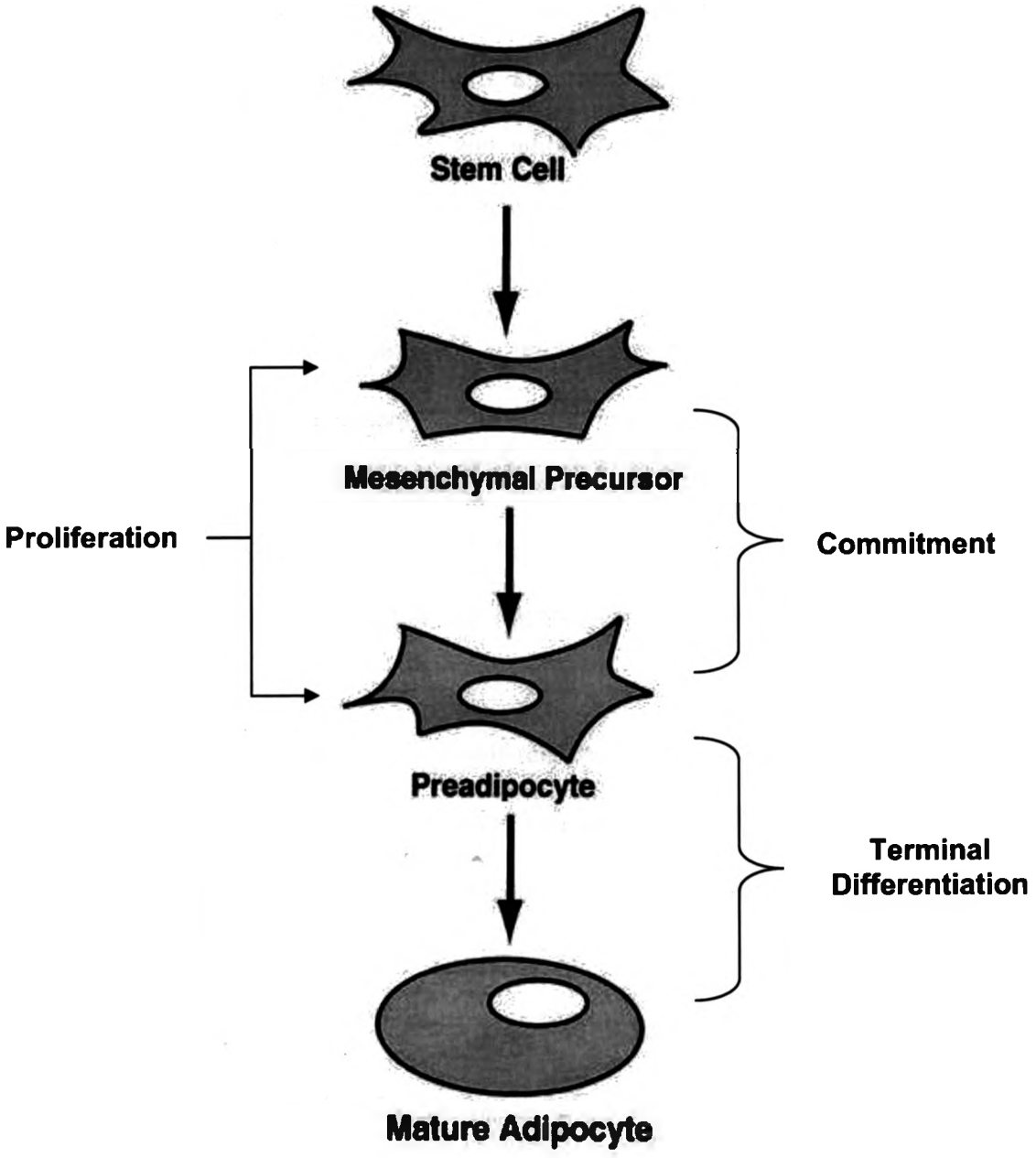


Fig 1.1 Overview of adipogenesis. The process of adipogenesis includes MSC commitment to the adipocyte lineage, terminal differentiation of a preadipocyte to an adipocyte, and proliferation of both MSCs and preadipocytes. (adapted from Gregoire *et al*, 1998)

the cell population (Pittenger *et al*, 1999). However, MSCs are also found in other tissues including periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle, lung, and deciduous teeth (Barry and Murphy, 2004). MSCs are multipotent stem cells that have the capacity to differentiate into a limited set of cell types; these include chondrocytes, myocytes, osteocytes, and adipocytes (Rosen and MacDougald, 2006). Distinct sets of environmental cues are responsible for the transformation of MSCs into each specific cell type. In order to continuously differentiate, a population of MSCs must be maintained to use as a continual source. Therefore, working in concert with differentiation, MSCs also undergo proliferation and self-renewal (Baksh *et al*, 2004). MSCs exist within a dynamic microenvironment, termed a niche, which exerts extrinsic signals that aid in regulating both the self-renewal and differentiation processes. MSCs can also be released from their niche and travel to other body tissues, a process known as mobilization (Liu *et al*, 2009).

A delicate balance between self-renewal and differentiation is essential to maintaining stem cell homeostasis (Zhang and Li, 2005). Growth factors which have regulatory effects on MSCs include TGF-β family, insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), vascular endothelial growth factors (VEGFs), and Wnt family molecules (Liu *et al*, 2009). However, the mechanisms that govern MSC self-renewal and differentiation are not well understood (Baksh *et al*, 2004).

In clinical applications, MSCs have generated an encouraging potential for cell-based therapeutic treatments primarily due to their ability to self-renew. MSCs are an acceptable stem cell source, which can be obtained from individual patients eliminating the complications of the body rejecting foreign tissues (Baksh *et al*, 2004). Therefore,

enhancing our knowledge of the MSC population and its niche is of great interest for regenerative therapeutic strategies.

1.2.2.2.1.2 Commitment to the Adipocyte Lineage

The first step of adipogenesis is the commitment of a MSC to the adipocyte lineage. Signals that promote adipogenic commitment generate preadipocytes from MSC precursors. Although the morphology of preadipocytes is identical to MSCs, this step determines the fate of a MSC because the newly formed preadipocyte has lost the ability to differentiate into non-adipocyte cell types (Rosen and MacDougald, 2006). Evidence suggests that commitment is triggered by factors that induce direct entry into lineage-specific development programs. BMP-4 is a known promoter of the commitment of MSCs to the adipocyte lineage and is one of the few factors which have been identified (Tang *et al*, 2004). Consequently, this step of adipogenesis is poorly understood.

The C3H10T1/2 stem cell line is a well-established cell line derived from C3H mouse embryos (Reznikoff *et al*, 1973). The cells possess a fibroblast morphology and are functionally similar to MSCs. C3H10T1/2 cells have been shown to convert into a stable form of adipocytes, osteocytes, chondrocytes, and myocytes (Jasuja *et al*, 2005; Katagiri *et al*, 1990; Tang *et al*, 2004; Zhang and Stott, 2004). This cell line has been highly effective in the understanding of MSCs and its corresponding lineages.

1.2.2.2.1.3 Adipocyte Differentiation

The second step in adipogenesis is terminal differentiation. Once triggered, preadipocytes transform and acquire the features of a mature adipocyte. Each adipocyte will have acquired characteristics for lipid transport and synthesis, insulin sensitivity, secretion of adipocyte specific proteins, and contain a lipid-filled vesicle (Rosen and MacDougald, 2006). Adipocyte differentiation is controlled by an elaborate network of

transcription factors that coordinate the expression of proteins responsible for producing mature adipocytes. CCAAT-enhancer binding protein-α (C/EBPα) and peroxisome proliferator-activated receptor-γ (PPARγ) are the two principal adipogenic factors. PPARγ is the master regulator and is necessary and sufficient for adipogenesis to occur (Tontonoz *et al*, 1994). Conversely, C/EBPα is a primary player, but adipogenesis has been shown to occur in its absence (Farmer, 2006). It is now known that a cascade of transcription factors lead to the expression of PPARγ and C/EBPα. In this cascade, other C/EBPs have been shown to be involved whereby C/EBPβ and C/EBPδ are expressed in early adipogenesis and are responsible for the subsequent expression of PPARγ and C/EBPα. The precise role of C/EBPβ and C/EBPδ in regulating this cascade is not clear; however, evidence suggests that they may also participate in the terminal differentiation phase of adipogenesis (Farmer, 2006).

3T3-L1 preadipocytes are a well-established cell line system cloned from 3T3 mouse embryo fibroblasts and are used to study adipocyte differentiation (Green and Kehinde, 1975). This cell line undergoes a reputable terminal differentiation program, whereby confluent cells undergo an adipogenic program following exposure to FBS, insulin, inducers of cAMP signaling, and glucocorticoids (Student *et al*, 1980). As a result, the cells first undergo mitotic clonal expansion, whereby the cells re-enter the cell cycle and undergo two rounds of cell division. During this time, the cells express adipogenic transcription factors that facilitate the expression of PPARγ and C/EBPα. Following this event, the cells undergo terminal differentiation and become mature adipocytes (Farmer, 2006).

1.2.2.2.1.4 Proliferation

A vital part of adipogenesis is proliferation, which is the capacity of stem cells to undergo continuous division (Zhang and Li, 2005). MSCs and preadipocytes exhibit self-renewal potential, which allows them to generate identical copies of themselves over extended periods of time (Baksh *et al*, 2004). Mature adipocytes cannot multiply; therefore, it is imperative that both MSCs and preadipocytes continually undergo mitosis. Proliferation is a component of stem cell self-renewal, which requires a balance between stem cell proliferation and differentiation (Zhang and Li, 2005). Consequently, proliferation is an integral part of stem cell regulation and adipogenesis. However, the regulation of MSC and preadipocyte self-renewal remain poorly understood.

1.2.2.2.1.5 Angiogenesis

The growth of adipose tissue requires the continual remodelling of its vascular network. Adipose tissue is highly vascularised with each adipocyte contacting one or more capillary vessels (Lijnen, 2008). Most angiogenic activity in adipose tissue is regulated by VEGFs. However, a number of other factors affecting angiogenesis have been identified in adipose tissue, including placental growth factor (PlGF), FGF-2, osteonectin, angiopoietins, leptin, and thrombospondins (TSPs) (Lijnen, 2008). VEGF-A isoform is a major angiogenic factor and promotes proliferation and migration of endothelial cells. FGF-2 also stimulates endothelial cell proliferation and is known to promote adipocyte differentiation *in vivo*. Conversely, TSP-1 and TSP-2 are inhibitors of angiogenesis through inhibition of endothelial cell proliferation and migration (Voros *et al*, 2005). Meanwhile, PlGF appears to exert it effects only under obese conditions and is absent under normal circumstances. VEGF-A, FGF-2 and PlGF have also been shown to have increased mRNA levels during adipocyte differentiation *in vitro* (Voros *et al*, 2005).

Furthermore, an anti-VEGF antibody resulted in the inhibition of not only angiogenesis, but also adipogenesis. Consequently, the coupling of angiogenesis and adipogenesis are essential for differentiating adipocytes in obesity (Nishimura *et al*, 2007).

1.2.2.2.1.6 ECM Remodelling

The excessive growth of body fat requires extensive modifications of the structure of adipose tissue. There is increasing lines of evidences suggesting a role for proteolysis in several aspects of tissue modulation. For example, protein degradation is required for basement membrane remodelling in hypertrophy; for cell migration to produce new vasculature; and for migration of macrophages into adipose tissue. Most proteolytic activity occurs through the fibrinolytic and matrix metalloproteinase (MMP) systems (Lijnen, 2008). In the fibrinolytic system, the active enzyme plasmin (inactive proenzyme is plasminogen) is responsible for the degradation of fibrin. The MMP system, which is usually found in low levels in the absence of tissue remodelling, is able to cleave ECM components as well as some non-ECM proteins. (Lijnen, 2008).

1.3 Fetal Programming of Obesity

Fetal tissues and organs undergo critical growth periods during their development. During these periods, the system is extremely sensitive to its environment. Once this sensitivity has ended, a fixed functional capacity has been formed (Osmond and Barker, 2000). Programming occurs when a stimulus or insult occurs during the critical period that results in an altered functional capacity that has lasting or lifelong effects (Ozanne, 2001). Stimuli can include reduced nutrition, pre-eclampsia, oxidative stress, and hypoxia (Myatt, 2006).

1.3.1 Developmental Origins of Health and Disease Hypothesis

A strong statistical association has been demonstrated between poor fetal growth and the subsequent development of hypertension, type 2 diabetes, and obesity; visceral obesity in particular (Osmond and Barker, 2000). Initially reported by Barker and colleagues (Barker, 1995), these findings led to the fetal origins hypothesis, which states that an adverse intrauterine environment programs the developmental fetal tissues permanently determining their physiological response, and ultimately producing dysfunction and disease. This hypothesis has now been termed the developmental origins of health and disease (DOHaD) hypothesis (Rinaudo and Lamb, 2008). Although there is a direct link between poor fetal growth and the subsequent development of disease in adulthood, the causal relationship is poorly understood.

1.3.2 Fetal Growth Restriction

Fetal growth restriction (FGR), also termed intrauterine growth restriction (IUGR), is when the fetus fails to achieve its full growth potential (Coupe *et al*, 2009). FGR is defined as a birth weight that is below the 10^{th} percentile for gestational age. (Schwitzgebel *et al*, 2009) FGR is strongly associated with an increased risk of developing metabolic diseases in adulthood (Barker *et al*, 1993).

The thrifty-phenotype hypothesis proposes that fetal development is sensitive to the nutritional environment. In response to poor nutrition, the fetus will optimise the growth of certain organs at the expense of others in order to increase the chance of fetal survival. These adaptations may jeopardise the development of the fetus and ultimately will determine its future (Hales and Barker, 1992). Additionally, postnatal metabolism is altered in order to enhance survival under poor nutritional conditions faced after birth. However, when the environment the fetus encounters does not match the one in which it

has prepared for, detrimental effects can occur. Most FGR babies show rapid postnatal growth, often referred to as catch-up growth (Bol *et al*, 2009). Although this does have some advantages in early life, catch-up growth is a risk factor for developing obesity, type 2 diabetes, and cardiovascular disease later in life (Cianfarani *et al*, 1999; Ong *et al*, 2000).

1.3.3 Maternal Protein Restriction Model

FGR can be induced in the well-studied maternal protein restriction (MPR) model in rats. In this model, pregnant rat dams are fed a diet containing 8% protein, while the control dams are fed a standard diet containing 20% protein. Offspring are nursed by low protein fed dams resulting in a permanent growth restriction. MPR offspring are diabetic, insulin resistant, and hypertensive in adulthood (Ozanne, 2001). Having established our own MPR model, we recently reported the original finding that MPR leads to the development of visceral adiposity in male offspring (Guan *et al*, 2005). In addition, the adiposity characterized in this model is due to adipocyte hyperplasia.

1.4 TGF-β Family Signalling

The TGF-β superfamily is comprised of growth and differentiation factors including TGF-βs, activins, inhibins, and bone morphogenetic proteins (BMPs). More than 60 members have been identified in multicellular organisms (Feng and Derynck, 2005). As shown in Figure 1.2, TGF-β signalling is conveyed through type I and type II transmembrane serine/threonine kinases to intracellular mediators termed Smad proteins (Kitisin *et al*, 2007). The type II receptors include BMPRII, ActRIIA, ActRIIB, and TβRII, and the type I receptors are activin-like kinases (ALKs) 1 through 7. These

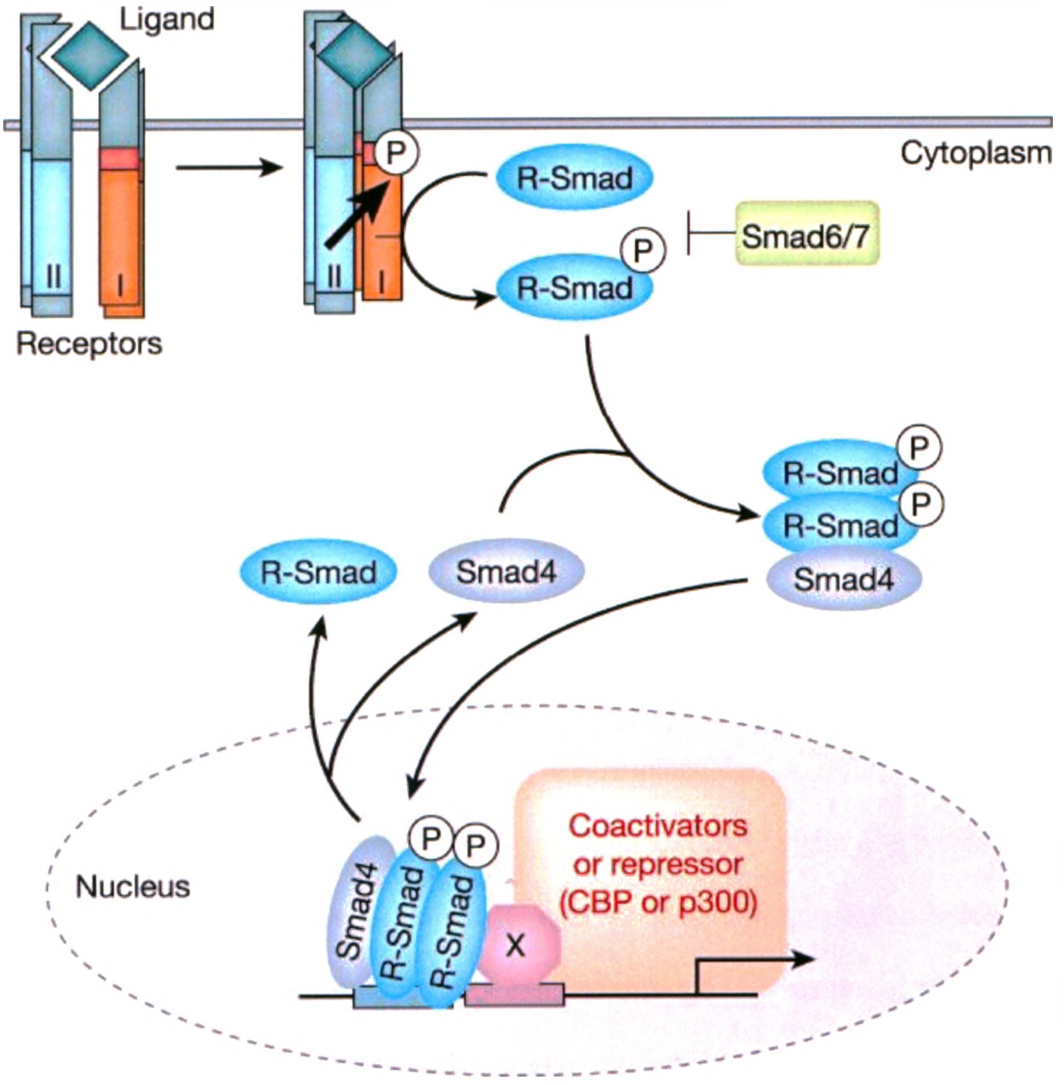
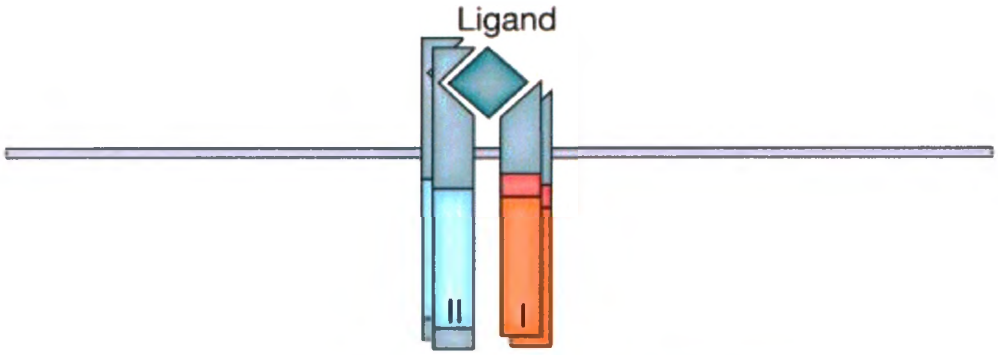


Fig 1.2 Overview of the Smad-dependent TGF-β signalling pathway. Signalling begins by ligand binding to the type II receptor followed by phosphorylation of the type I receptor. The corresponding Smad protein is subsequently phosphorylated, which binds to the common Smad to form a trimeric complex that translocates into the nucleus and regulates target genes. (adapted from Derynck and Zhang, 2003)

receptors exist as homodimers at the cell surface in the absence of ligands (Feng and Derynck, 2005). Ligands are known to bind to receptors in a sequential manner, beginning with its high-affinity receptor. Generally, the type II receptors are of highest affinity, but some ligands will be more strongly attracted to their type I receptor (Allendorph *et al*, 2007). Ligand binding to the type II receptor allows the formation of a heterodimer with the corresponding type I receptor. From here, the ligand induces a conformational change, which results in phosphorylation of the type I receptor. Next, the activated type I receptor phosphorylates the corresponding Smad protein, which initiates the signalling cascade. Smad proteins can be divided into three groups: the receptor-activated Smads (R-Smads), Smad1, Smad2, Smad3, Smad5, and Smad8; the common Smad, Smad4; and the inhibitory Smads, Smad6 and Smad7 (Kitisin *et al*, 2007). Once R-Smads are activated, they bind with the common Smad to form a trimeric complex that translocates into the nucleus and regulates target genes. As shown in Figure 1.3, several combinations of receptors and R-Smads are responsible for defining specific signal transduction cascades including the TGF-β, activin, and BMP signalling pathways.

Given that there are a limited number of receptors and Smad proteins; a vast diversity of transcriptional regulation is attained through various receptor complexes and Smad combinations, as well as transcriptional co-factors (Feng and Derynck, 2005). In addition, downstream of the ligand-receptor complexes, there are Smad-independent pathways that also contribute to the cellular responses of these ligands. As shown in Figure 1.4, TGF-β family molecules can activate other signalling pathways including all of the mitogen-activated protein kinase (MAPK) signalling cascades. Mammalian MAPK pathways can be divided into at least three groups: extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun NH_2-terminal kinase (JNK). The MAPKs



Type II	Type I	R-Smad
BMPRII	ALK-2 (ActRI)	Smad1, Smad5, Smad8
	ALK-3 (BMP-RIA)	
	ALK-6 (BMP-RIB)	
ActRII, ActRIIB	ALK-4 (ActRIB)	Smad2
ActRIIB	ALK-7	Smad2
TβRII	ALK-5 (TβRI)	Smad2, Smad3
	ALK-1	Smad1, Smad5
	ALK-2	

Fig 1.3 Combinations of type I and type II receptors in TGF-β family signalling. The BMP, TGF-β, and activin signalling pathways consist of specific combinations of type I and type II receptors resulting in the phosphorylation of a corresponding set of Smad proteins. (adapted from Derynck and Zhang, 2003)

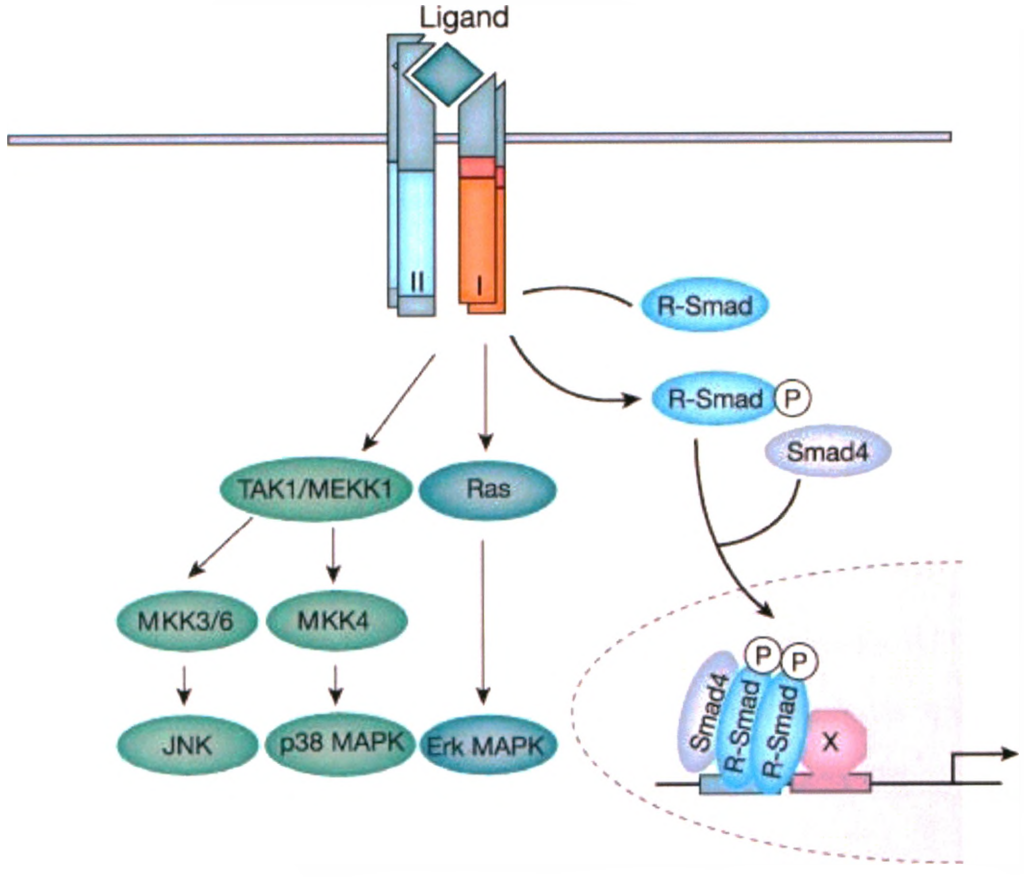


Fig 1.4 Overview of the Smad-independent TGF-β signalling pathways. The type I and type II receptors of the TGF-β signalling pathways can have effects on cascades that are not associated with Smad proteins. These pathways include the JNK, p38 MAPK and ERK pathways. (adapted from Derynck and Zhang, 2003)

are known to coordinate gene transcription, protein synthesis, cell cycle machinery, cell death, and differentiation (Kyriakis and Avruch, 2001). However, the effects of these pathways in TGF-β family signalling are poorly characterized (Derynck and Zhang, 2003).

1.4.1 Smad-Dependent Pathways

TGF-β family signalling is conveyed through an arrangement of type I and type II receptors forming the BMP, TGF-β, and activin signal transduction pathways. Traditionally, BMP ligands signal through the BMP pathway. BMP signalling has been shown to occur through type II receptors BMPRII, ActRIIA, and ActRIIB, in concert with type I receptors ALK-2, ALK-3, and ALK-6. These receptor combinations activate Smad1, Smad5, and/or Smad8 (Kitisin *et al*, 2007). Alternatively, TGF-β molecules typically signal through the type II receptor TβRII and the type I receptor ALK-5. This receptor complex activates Smad2 and/or Smad3 (Kitisin *et al*, 2007). Lastly, activin and nodal ligands convey signals through the ActRIIA and ActRIIB type II receptors in conjunction with the type I receptor ALK-4. This pathway also activates Smad2 and/or Smad3 (Kitisin *et al*, 2007).

1.4.2 Smad-Independent Pathways

TGF-β family signalling can also be exerted through Smad-independent pathways such as the MAPKs; a family of mitogen-activated protein kinases. These include the p38 MAPK, ERK, and JNK signal transduction pathways. MAPK signalling occurs in a cascade of kinases upstream of the MAPK proteins. A MAPKKK is activated first, which activates a MAPKK through phosphorylation, and then in turn the MAPKKs phosphorylate the MAPKs (Wagner and Nebreda, 2009).

The p38 MAPKs are a mammalian stress-activated MAPK family. There are four known isoforms including p38α, p38β, p38γ, and p38δ, where p38α is the most abundant isoform and is found in the majority of cell types (Kyriakis and Avruch, 2001). The JNK proteins are also a stress-activated family and include JNK1, JNK2, and JNK3. JNK1 and JNK2 are found in most cell types, however JNK3 is found primarily in the brain. JNK and p38 MAPKs are activated in response to environmental and genotoxic stresses. They play roles in inflammation and tissue homeostasis, and they are known to control proliferation, differentiation, survival, and migration of several cell types (Wagner and Nebreda, 2009). TAK1 and MEKK1 are the MAPKKKs that function upstream of p38 and JNK. As a result of their activation, they carry on to activate JNK through MKK4 and MKK7, and p38 through MKK6 and MKK3 (Derynck and Zhang, 2003; Wagner and Nebreda, 2009).

Conversely, ERK is not considered a stress-activated pathway. ERK signalling is primarily regulated by Ras; a GTPase which recruits MAPKKKs such as Raf to activate MEK1/MEK2, which in turn activates ERK (Kyriakis and Avruch, 2001). Extracellular stimuli such as growth factors, cytokines, or hormones initiate a cascade of factors that are responsible for activating the ERK pathway. Once activated, ERK is known to regulate a multitude of cellular activates including gene expression, mitosis, programmed cell death, differentiation, and embryogenesis (Mebratu and Tesfaigzi, 2009).

1.4.3 Pharmacological Pathway Inhibitors

Small molecule inhibitors are invaluable tools to understand the composition and function of signalling pathways in various biological processes. TGF-β family signalling has multiple kinase inhibitors that can successfully block each pathway. The pharmacological TGF-β/activin inhibitor SB-431542 is an inhibitor of the type I receptors

ALK-4, ALK-5, and ALK-7 (Inman *et al*, 2002). This inhibitor is effective in blocking phosphorylated Smad2 in response to TGF-β and activin induced activation. Conversely, it has no effect on the activation of Smad1, Smad5, or Smad8 of the BMP pathway. A concentration of 10 μM is often used in the literature; however, it has been shown that the transcriptional activity of TGF-β/activin reporter genes has an IC_{50} of 1 μM for ALK-4, 0.75 μM for ALK-5, and 2 μM for ALK-7 (Inman *et al*, 2002).

The pyridinyl imidazole compound SB-202190 is a potent inhibitor of the p38 MAPK family *in vitro* and *in vivo*. SB-202190 has been shown to inhibit both p38α and p38β isoforms by binding to the ATP site, and has no effect on ERK or JNK proteins (Young *et al*, 1997). SB-202190 is often used in 10 – 20 μM concentrations throughout the literature (Shanware *et al*, 2009).

The ERK inhibitor U0126 is a selective inhibitor of MEK1 and MEK2 (Chen *et al*, 2009). U0126 is often used at approximately 10 μM ranges, however at a range of 5 μM it was shown to be sufficient and did not have any effect on the p38 MAPK pathway (Geest *et al*, 2009).

Although pharmacological inhibitors can be an excellent tool in delineating the effects of signalling pathways, their use can be limited by their lack of specificity. Although studies have been performed to show the specificity of a SB-431542, SB-202190, and U0126 inhibitors, conflicting reports still remain in the literature.

1.5 BMP Family

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily; members of which are known to be involved in the regulation of proliferation,

differentiation, apoptosis, and stem cell commitment (Bahamonde and Lyons, 2001; Daluiski *et al*, 2001; Massague, 1998; Tang *et al*, 2004; Zhang *et al*, 2007). Fourteen human BMPs have been identified (Tseng and He, 2007). BMP expression can be widespread and dynamic throughout development and they are often found in areas of epithelial-mesenchymal interaction (Tseng and He, 2007). The BMP family generally can bind to a wide variety of receptors including ALK-3, ALK-6, BMPRII, ActRIIA, and ActRIIB. The differences between binding affinities and capacities lie in the structural differences between BMP ligands (Allendorph *et al*, 2007).

1.5.1 BMP-3

BMP-3 was originally isolated as a constituent of osteogenin (Sampath *et al*, 1987), a protein which initiates bone differentiation. Consequently, BMP-3 was initially thought to be osteogenic; however, BMP-3 has proved to be a divergent member of the TGF-β superfamily and cannot be grouped with the osteoinductive members. Additionally, BMP-3 only shares approximately 50% identity in amino acid sequence with other BMPs (Bahamonde and Lyons, 2001). Since BMP-3 is an unusual member within the BMP family, many of its characteristics and functions are inconsistent with conventional BMPs. However, very little has been reported regarding its expression, function, and regulation; therefore, BMP-3 remains very poorly understood.

1.5.1.1 Molecular Structure

BMP-3 exhibits the classic TGF-β family architecture with each monomer containing a cystine knot motif, four beta strands, and the conserved α-helix H3. BMP-3 exists as a heterodimer consisting of two monomers that connect by an inter-subunit

disulfide bond. The covalently linked dimer gives the overall shape of a butterfly with two fingers extending outwards from the cystine knot (Allendorph *et al*, 2007).

1.5.1.2 Sites of Expression

BMP-3 has been shown to be expressed in bone and it accounts for 65% of the total BMP found in demineralized bone (Daluiski *et al*, 2001; Luyten *et al*, 1989). In a study examining chick limb development, BMP-3 transcripts were first detected in the mesenchyme during early development and were continually expressed in osteogenic tissues throughout the development of bone (Gamer *et al*, 2008).

1.5.1.3 Functions

Few studies have been performed to investigate the function of BMP-3. From the few that have been completed, BMP-3 has been shown to antagonize BMP-2-induced osteogenic commitment and differentiation *in vitro* and act as a negative regulator of bone density *in vivo* (Daluiski *et al*, 2001). In addition, *Bmp3*$^{-/-}$ mice showed a novel skeletal phenotype displaying an increase in bone density, and total trabecular bone volume in mutants was double that of the wild type (Daluiski *et al*, 2001). Therefore, BMP-3 is known to play a role in the regulation of bone.

1.5.1.4 Mechanisms of Action

There is increasing evidence that suggests BMP-3 signals through the TGF-β/activin pathway. First, BMP-3 has been shown to induce the expression of a TGF-β/activin-responsive reporter. Conversely, BMP-3 does not induce the expression of a BMP-responsive reporter (Daluiski *et al*, 2001). Second, unlike other BMPs, BMP-3 has been shown to primarily bind ActRIIB. In addition, BMP-3 has a 30-fold higher affinity to ActRIIB (K_d = 52.6 nM) than to ActRIIA (K_d = 1.8 μM), an ability that most activin receptor ligands do not possess (Allendorph *et al*, 2007). Therefore, BMP-3 may exert its

effects through binding the ActRIIB receptor and through activation of the TGF-β/activin signal transduction pathway.

1.5.1.5 Selected as Candidate Gene

A candidate gene approach was used to capitalize on our previously published visceral adipose tissue gene expression profiling database generated with our early-life programmed rat model of increased visceral adiposity (Guan *et al*, 2005). Our primary objective was to identify novel factors that promote adipogenesis. Consequently, candidate genes were selected based on the following criteria. ***(a)*** They are known to be involved in the commitment of MSCs to specific cell lineages, because adipogenesis begins with the commitment of MSCs to the adipocyte lineage and the regulation of which is largely unknown. ***(b)*** They stimulate non-adipose cell proliferation and/or differentiation, since the second step in adipogenesis is terminal differentiation of preadipocytes to mature adipocytes. Importantly, a critical but poorly understood component of adipogenesis involves self-renewal/proliferation of MSCs and preadipocytes. ***(c)*** Their expression is up-regulated in our rat model. One such candidate is the gene encoding BMP-3, the expression of which is increased approximately 2-fold (Guan *et al*, 2005).

1.6 Rationale and Hypothesis

Recently, we and others reported an increase in the expression of BMP-3 in the adipose tissue of two distinct rodent models of obesity. In our MPR rat model, we illustrated a 1.9-fold increase of BMP-3 expression (Guan *et al*, 2005). Similar findings were shown in a mouse model of diet-induced obesity reporting a 1.81-fold increase in BMP-3 expression. Additionally, the increase of expression was seen prior to the

increase in fat mass (Koza *et al*, 2006). BMP-3 is a divergent member of the TGF-β superfamily and is known to be an inhibitor of osteogenesis (Bahamonde and Lyons, 2001). Given that there is a reciprocal relationship between osteogenesis and adipogenesis whereby factors that promote osteogenesis inhibit adipocyte formation (Jeon *et al*, 2003; Lecka-Czernik *et al*, 2002), coupled with the recent findings that BMP-3 expression was increased in the adipose tissue of two distinct rodent models of obesity and that the enhanced BMP-3 expression proceeded increases in fat mass, the present study was undertaken to examine the hypothesis that BMP-3 stimulates adipogenesis. In addition, we explored the possibility that BMP-3 may stimulate proliferation of MSCs and/or preadipocytes, because other members of the TGF-β superfamily are known to promote proliferation of stem cells and other progenitor cells. The C3H10T1/2 stem cells and 3T3-L1 preadipocytes were used as *in vitro* model systems.

Chapter 2

Materials and Methods

2.1 Cell Culture

The 3T3-L1 preadipocyte line and the C3H10T1/2 MSC line were obtained from the American Type Culture Collection (Manassas, VA, USA). 3T3-L1 preadipocytes were cultured in standard growth medium consisting of Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and 10% Newborn Calf Serum (NCS; Sigma). C3H10T1/2 stem cells were cultured in standard growth medium consisting of Minimal Essential Medium (MEM; Sigma) and 10% Fetal Bovine Serum (FBS; Sigma). All cultures were maintained in a humidified incubator at 37°C and 5% CO_2. Growth medium was replaced every other day.

2.2 Commitment to the Adipocyte Lineage

C3H10T1/2 stem cells were plated at low density (20 – 30% confluence) on 12-well plates and cultured in standard growth medium for two days. Cells were then treated with increasing concentrations of recombinant human BMP-3 and/or recombinant human BMP-4 (R&D Systems, Minneapolis, MN, USA) until two days post-confluence (day 0), at which time they were induced to differentiate by the addition of a standard differentiation cocktail containing 0.5 mM of 3-isobutyl-1-methylxanthine (IBMX; Sigma), 0.25 μM of dexamethasone (SABEX, Boucherville, QC, Canada), and 1 μg/ml of insulin (Eli Lilly Canada Inc., Toronto, ON, Canada). After 48 h (day 2), the medium was replaced with growth medium supplemented with 1 μg/ml insulin. Subsequently, the medium was changed at days 4 and 6 with fresh growth medium. Both positive (100 ng/ml BMP-4) and negative (cocktail only) controls were also included. At day 8, differentiation was assessed by Oil Red O staining of lipid droplets, as described below.

2.3 Adipocyte Differentiation

3T3-L1 preadipocytes were plated at low density (20 – 30% confluence) on 12-well plates, grown in standard growth medium, and allowed to reach confluence. At two days post-confluence (day 0), cells were induced to differentiate by the addition of a differentiation cocktail containing 0.5 mM IBMX, 0.25 μM dexamethasone and 1 μg/ml insulin, as described above. After 48 h (day 2), the medium was replaced with growth medium supplemented with 1 μg/ml insulin. Subsequently, the medium was changed at days 4 and 6 with fresh growth medium. On day 8, >90% of cells had acquired adipocyte phenotype. In order to study the effects of BMP-3 on adipocyte differentiation, 3T3-L1 preadipocytes were subjected to the same differentiation protocol except that insulin was replaced with BMP-3 (50, 100, and 200 ng/ml). Positive (standard cocktail) and negative (IBMX and dexamethasone only) controls were also included. At day 8, differentiation was assessed by Oil Red O staining of lipid droplets, as described below.

2.4 Oil Red O Staining

On day 8 of adipocyte differentiation, adipocyte monolayers were washed with DPBS, fixed for 1 h with 10% formalin at room temperature, and incubated in 60% isopropanol for 5 min. Oil Red O (3 g/l; Sigma) in 99% isopropanol was diluted with water, filtered, and added to the fixed cell monolayers for 5 min. Nuclei were then stained with hematoxylin for 10 s. Cell monolayers were then washed with water, and the stained triglyceride droplets were visualized and photographed.

2.5 Proliferation Assay - [^{3}H]-Thymidine Incorporation

C3H10T1/2 and 3T3-L1 cells were plated on 24-well plates at low density (20 – 30% confluence) and cultured in growth medium for three days until 50 – 60% confluence. Cells were growth arrested in serum-free medium for 20 – 24 h and were then treated in serum-free medium with increasing concentrations of BMP-3 (1 – 100 ng/ml) for 24 h, or as indicated otherwise. During the last 4 h of treatment, cells were pulse labelled with [^{3}H]-thymidine (0.5 μCi/well; 75.2 Ci/mmol, PerkinElmer Life and Analytical Sciences, Woodbridge, ON, Canada). Cells were washed twice with ice-cold phosphate-buffered saline (PBS), once with 5% trichloroacetic acid (TCA), and twice with 95% ethanol. Cells were then solubilised by the addition of 400 μl of 0.5 M NaOH. The solubilised cell lysate (300 μl) was added to 4 ml of scintillation fluid, and the incorporation of [^{3}H]-thymidine into DNA was determined by scintillation counting. Results were expressed as a percentage of control.

For inhibitor studies, C3H10T1/2 stem cells were plated at low density (20 – 30% confluence) on a 24-well plate and cultured in growth medium for 3 days until 50 – 60% confluence. Cells were growth arrested in serum-free medium for 20 – 24 h and then pre-treated for 1 h with an inhibitor of the TGF-β/activin pathway, SB-431542 (Sigma); an inhibiter of the p38 pathway, SB-202190 (Alexis Biochemicals, Plymouth Meeting, PA, USA); or an inhibitor of the ERK pathway, U0126 (Calbiochem, EMD Chemicals Inc., San Diego, CA, USA). Subsequently, cells were treated with or without 100 ng/ml BMP-3 for 24 h. During the last 4 h of treatment, cells were pulse labelled with [^{3}H]-thymidine (0.5 μCi/well). Cells were washed and solubilised, as described above, and the incorporation of [^{3}H]-thymidine into DNA was determined by scintillation counting. Results were expressed as a percentage of control.

2.6 Analysis of TGF-β/Activin Pathway Expression - RT-PCR

Expression of ActRIIB, ALK-4, Smad2, Smad3, and Smad4 was analyzed by standard RT-PCR. Briefly, total RNA was isolated from C3H10T1/2 stem cells using RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada) coupled with on-column DNase digestion with the RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed in a volume of 20 µl with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. For every RT reaction, one RNA sample was set-up without reverse transcriptase enzyme to provide a negative control against possible genomic DNA contamination. PCR reactions were carried out in a total volume of 50 µl containing 1 µl of RT (or as indicated otherwise) and 1.5 units of Platinum Taq DNA polymerase (Invitrogen Life Technologies, Burlington, ON, Canada). The primers specific for mouse ActRIIB, ALK-4, Smad2, Smad3, and Smad4 as well as their expected product sizes are shown in Table 2.1. PCR reactions were performed for 32 cycles with denaturing at 95°C, annealing at 58°C, and extension at 72°C. PCR products were confirmed with standard restriction enzyme digestions and sequencing analysis.

2.7 Western Blot Analysis

C3H10T1/2 cells were plated at medium density (30 – 40% confluence) on 10 cm^2 cell culture dishes. Three days after plating, cells were growth arrested in serum-free medium for 20 – 24 h and then treated with 100 ng/ml BMP-3 in serum-free medium. At discrete times thereafter (0 – 120 min), cells were lysed in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% wt/vol SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% wt/vol bromphenol blue) and stored at -80°C. For the inhibitor experiments, cells were

Table 2.1. Primers for TGF-β/activin pathway components RT-PCR

Gene	Primer Sequence	Product Size
ActRIIB	Forward: 5'-ATCGTCATCGGAAACCTCCC Reverse: 5'-CAGCCAGTGATCCTTAATC	929-bp
ALK-4	Forward: 5'-ACCGCTACACAGTGACCATT Reverse: 5'-TCTTCACATCTTCCTGCACG	630-bp
Smad2	Forward: 5'-CGGAGATTCTAACAGAACTG Reverse: 5'-TGCTTGAGCATCGCACTGAA	846-bp
Smad3	Forward: 5'-AGCACACAATAACTTGGACC Reverse: 5'-TAAGACACACTGGAACAGCGGATG	636-bp
Smad4	Forward: 5'-GATCTATGCCCGTCTGTGGAGGTG Reverse: 5'-AATACTGGCCGGCTGACTTGTGGA	403-bp

pre-treated with various concentrations of SB-431542, SB-202190, or U0126 for 1 h in serum-free medium prior to treatment with 100 ng/ml of BMP-3 for 5 min or 1 h. Cells were lysed in SDS sample buffer and stored at -80°C.

To test activation of the JNK pathway, C3H10T1/2 cells were growth arrested in serum-free medium for a period of 20 – 24 hours. Cells were treated with or without 100 ng/ml of BMP-3 in serum-free medium for 24 h. As a positive control, growth medium was removed and cells were exposed to 40 mJ of ultraviolet light. Subsequently, growth medium was replaced and cells were allowed to recover for 30 min in a 37°C incubator. All cells were lysed in SDS sample buffer and stored at -80°C.

Equal volumes of cell lysate were subjected to a standard 10% SDS-PAGE. After electrophoresis, proteins were transferred to PVDF transfer membrane (Amersham Hybond^{TM}-P, GE Healthcare Canada, Baie d'Urfe, QC, Canada) using a Bio-Rad Mini Transfer Apparatus. The PVDF membrane was blocked for 1 h at room temperature with 5% milk in TTBS (0.05% Tween-20 in TBS), and incubated with primary antibodies in TTBS overnight at 4°C. The phosphorylated Smad2, p38, Erk1/2, and JNK proteins were detected using the Phospho-Smad2 Antibody (Ser465/467; Cell Signaling, Danvers, MA, USA), Phospho-p38 MAP Kinase Antibody (Thr180/Tyr182; Cell Signaling), Phospho-p44/42 MAP Kinase Antibody (Thr202/Tyr204; Cell Signaling), and Phospho-SAPK/JNK Antibody (Cell Signaling), respectively. All primary antibodies were used at 1:1000 dilutions. After three 10 min washes with TTBS, the membrane was incubated with Anti-Rabbit IgG-HRP (R&D Systems) secondary antibody (1:500 dilution) and developed using chemiluminescence (Western Lightning^{TM} Plus-ECL, PerkinElmer Life and Analytical Sciences). The membrane was then exposed to X-ray film (Eastman Kodak, Rochester, NY, USA) for 10 s to 1 h.

Each membrane was stripped and re-probed to detect total Smad2, p38, ERK1/2, or JNK proteins. Briefly, membranes were incubated in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl pH 6.7) for 30 min at 55°C. After three 10 min washes in TTBS, the membrane was blocked, as described previously. Next, the membrane was probed using Smad2 Mouse Antibody (L16D3; Cell Signaling), p38 MAP Kinase Antibody (Cell Signaling), p44/42 MAP Kinase Antibody (Cell Signaling), or SAPK/JNK Antibody (Cell Signaling). All primary antibodies were used at 1:1000 dilutions. Subsequently, the membranes were probed with Anti-Rabbit IgG-HRP secondary antibody (1:500) or Anti-Mouse IgG-HRP secondary antibody (R&D Systems) (1:10,000) and developed, as described above.

Chapter 3

Results

3.1 BMP-3 does not promote the commitment of C3H10T1/2 stem cells to the adipocyte lineage

Given that BMP-3 inhibits osteogenesis (Daluiski *et al*, 2001) and there is evidence for a reciprocal relationship between osteogenesis and adipogenesis wherein factors that induce osteogenesis inhibit the formation of adipocytes and vice versa (Jeon *et al*, 2003; Lecka-Czernik *et al*, 2002), we hypothesized that BMP-3 promotes adipogenesis. As a first step in examining this hypothesis, we studied the effects of BMP-3 on the commitment of MSCs to the adipocyte lineage using C3H10T1/2 stem cells as an *in vitro* model system. As shown in Fig. 3.1, treatment of C3H10T1/2 stem cells with BMP-4, which is known to promote the commitment of MSCs to the adipocyte lineage, led to adipocyte formation as evidenced by lipid accumulation following the induction of adipogenesis with a standard differentiation cocktail. In contrast, treatment of these cells with various concentrations of BMP-3 was ineffective (Fig. 3.1). We also examined the possibility that BMP-3 might function in concert with BMP-4 to promote the commitment to the adipocyte lineage. To do so, we treated C3H10T1/2 stem cells with 200 ng/ml BMP-3 and 10 ng/ml BMP-4; a concentration that was shown to be insufficient to drive these cells towards the adipocyte lineage when administered alone. There was no evidence of lipid accumulation when these cells were subjected to a standard adipogenic protocol following the combined treatment (Fig. 3.1).

3.2 BMP-3 does not promote adipocyte differentiation

The second step in adipogenesis is the terminal differentiation of preadipocytes to mature adipocytes. To determine if BMP-3 promotes adipocyte differentiation, we

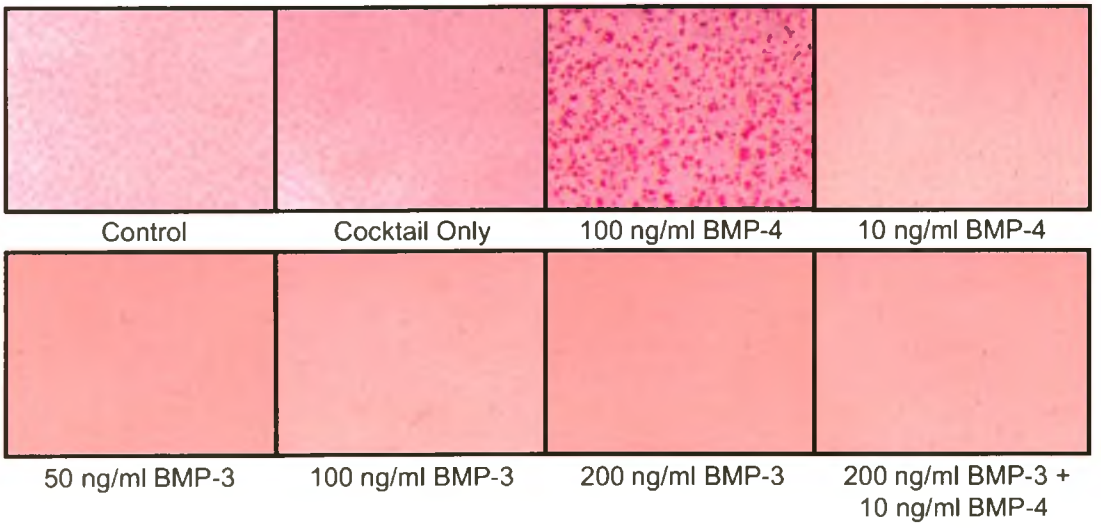


Figure 3.1 Effects of BMP-3 on the commitment of C3H10T1/2 stem cells to the adipocyte lineage. C3H10T1/2 stem cells were cultured in standard growth medium (10% FBS) with or without various concentrations of recombinant BMP-3 and/or BMP-4 for 5 – 6 days. At two days post-confluence (day 0), cells were induced to differentiate by the addition of a standard differentiation cocktail containing 500 µM IBMX, 0.25 µM dexamethasone, and 1 µg/ml insulin. After 48 h (day 2), the medium was replaced with growth medium supplemented with 1 µg/ml insulin. At days 4 and 6, medium was replaced with standard growth medium. At day 8, differentiation was assessed by Oil Red O staining. Microphotographs (10 X magnification) from one representative experiment are shown. Similar results were obtained from three independent experiments.

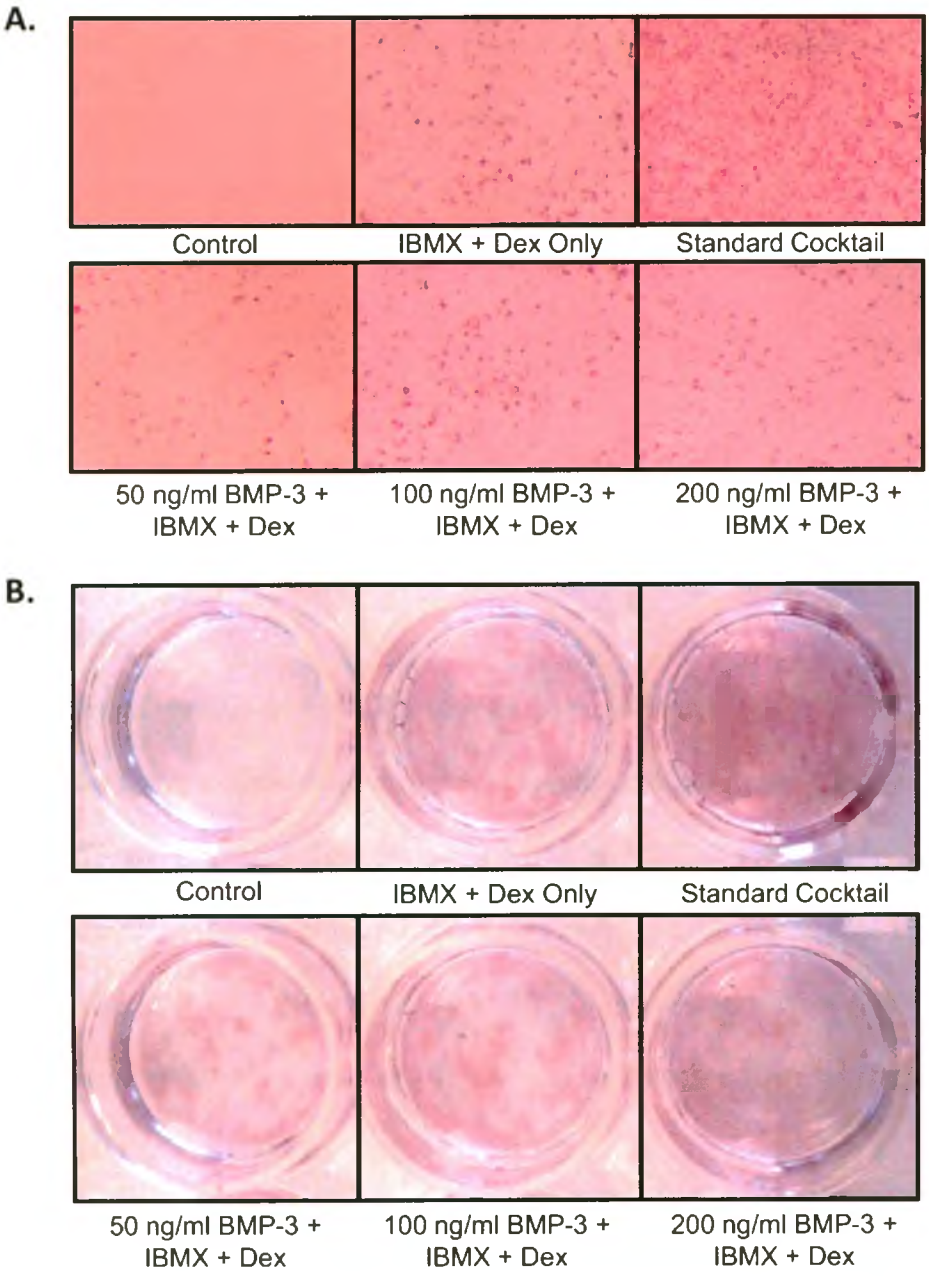


Figure 3.2 Effects of BMP-3 on preadipocyte differentiation. 3T3-L1 preadipocytes were cultured in standard growth medium (10% FBS). At two days post-confluence (day 0), cells were induced to differentiate by the addition of a standard differentiation cocktail containing 500 µM IBMX, 0.25 µM dexamethasone, and 1 µg/ml insulin. After 48 h (day 2), the medium was replaced with growth medium supplemented with 1 µg/ml insulin. At days 4 and 6, medium was replaced with standard growth medium. For BMP-3 conditions, insulin was replaced with 50, 100, or 200 ng/ml BMP-3. At day 8, differentiation was assessed by Oil Red O staining. Microphotographs (**A**, 10 X magnification; **B**, without magnification) from one representative experiment are shown. Similar results were obtained from three independent experiments.

treated 3T3-L1 preadipocytes with an established protocol in which insulin, one of the three components of a standard differentiation cocktail, was substituted with the test compound BMP-3 at various concentrations. Using this protocol, greater than 95% of preadipocytes were converted to lipid-filled mature adipocytes in the presence of insulin (Fig. 3.2; served as a positive control). In the absence of insulin, less than 40% of adipocytes were formed and this rate of adipocyte differentiation was not altered by the addition of BMP-3 (Fig. 3.2).

3.3 BMP-3 stimulates proliferation of C3H10T1/2 stem cells and 3T3-L1 preadipocytes

A critical provision for adipogenesis is proliferation/self-renewal of MSCs and preadipocytes because terminally differentiated adipocytes are unable to undergo mitosis. Consequently, we examined the effects of BMP-3 on MSC and preadipocyte proliferation using a standard [^{3}H]-thymidine incorporation assay. As shown in Fig. 3.3A and 3.3B, treatment of C3H10T1/2 stem cells and 3T3-L1 preadipocytes with BMP-3 led to a concentration-dependent increase in [^{3}H]-thymidine incorporation reaching 300% of the control at 100 ng/ml BMP-3 ($p < 0.001$).

3.4 Expression of the key TGF-β/activin signalling components in C3H10T1/2 stem cells

Owing to a lack of information on the regulation of MSC self-renewal and considering that BMP-3 has similar effects on C3H10T1/2 stem cells and 3T3-L1 preadipocytes; we chose to study the molecular mechanisms that underlie BMP-3 stimulation of C3H10T1/2 stem cell proliferation. Based on our critical analysis of the

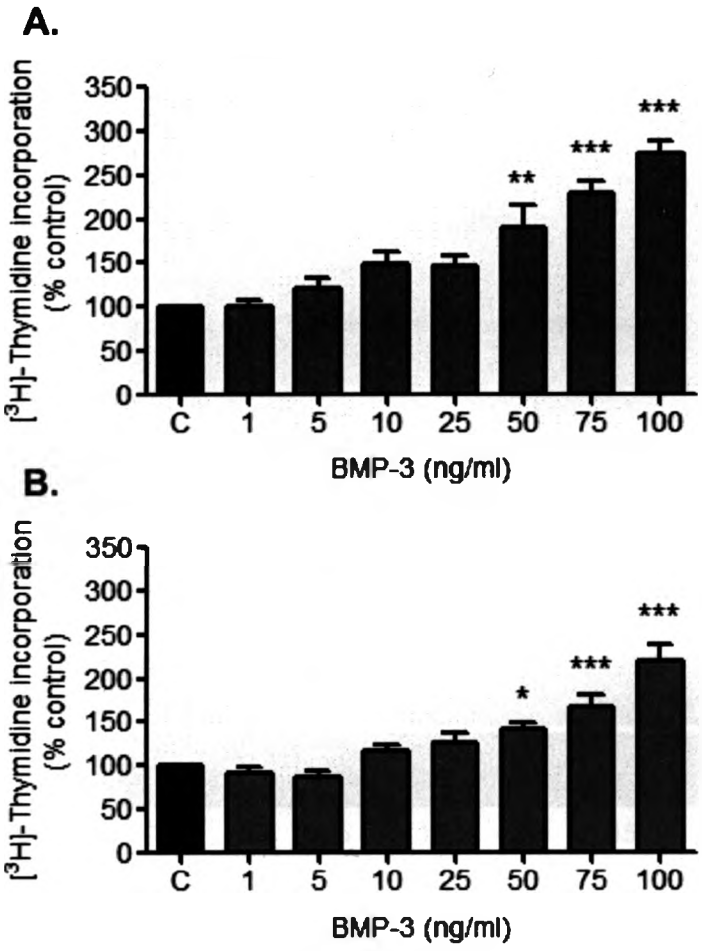


Figure 3.3 Effects of BMP-3 on proliferation of C3H10T1/2 stem cells and 3T3-L1 preadipocytes. C3H10T1/2 stem cells **(A)** and 3T3-L1 preadipocytes **(B)** were cultured in standard growth medium (10% FBS) until 50 – 60% confluence, at which time cells were starved for 24 h in serum-free medium. Cells were then treated with increasing concentration of BMP-3 (1 – 100 ng/ml) in serum-free medium for 24 h. During the last 4 h of treatment, cells were pulse labeled with [^{3}H]-thymidine (0.5 μCi/well) and the rate of [^{3}H]-thymidine incorporation was determined. Data are means ± SEM, n = 4 – 5 independent experiments, each performed in triplicate (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* control).

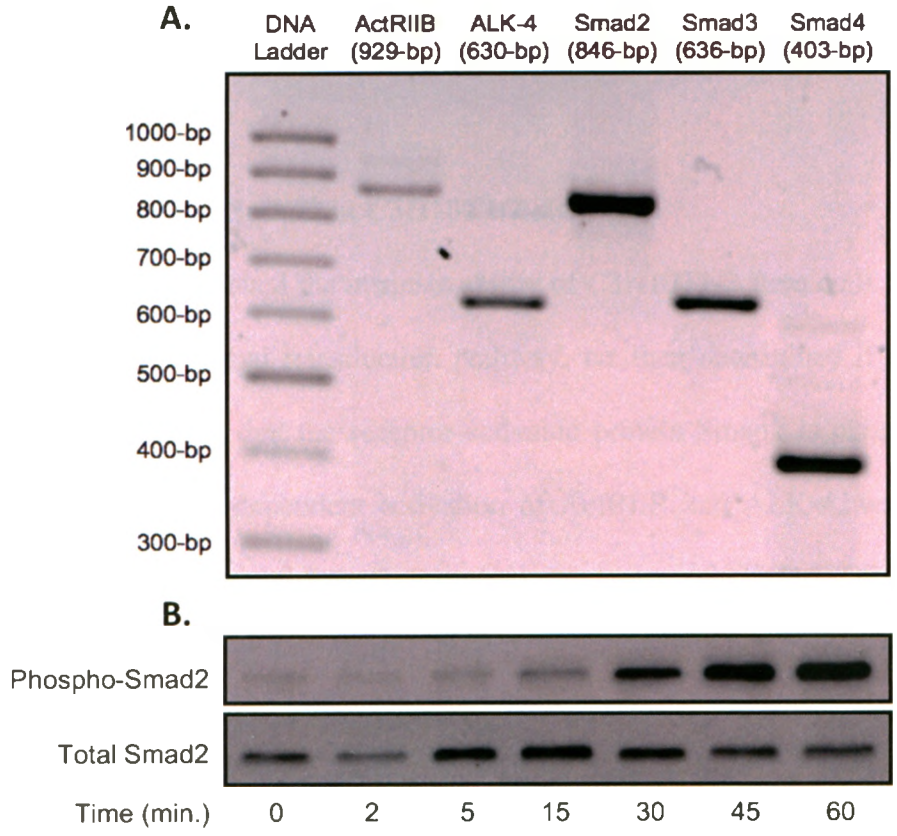


Figure 3.4 (A) Expression of TGF-β/activin pathway components by C3H10T1/2 stem cells. Total cellular RNA was prepared from cultured C3H10T1/2 stem cells and the mRNAs encoding mouse activin receptor type I (ALK-4), type II (ActRIIB), Smad2, Smad3, and Smad4 were assessed with standard RT-PCR. **(B) BMP-3 activates Smad2 in C3H10T1/2 stem cells.** C3H10T1/2 stem cells were cultured in standard growth medium (10% FBS) until 50 – 60% confluence, at which time cells were starved for 24 h in serum-free medium. Cells were then treated with 100 ng/ml BMP-3 in serum-free medium, and at indicated times thereafter, cell lysates were prepared and subjected to standard western blot analysis with antibodies specific for phosphorylated Smad2 and total Smad2 proteins. Results from one representative western blot are shown. Similar results were obtained from two independent experiments.

pertaining literature, we investigated the involvement of the TGF-β/activin signalling pathway. As a first step, we examined the expression of the key TGF-β/activin signalling components with standard RT-PCR. Our results revealed that C3H10T1/2 stem cells express mRNAs encoding the type I receptor (ALK-4), type II receptor (ActRIIB), and Smad proteins (Smad2, Smad3, Smad4) (Fig. 3.4A).

3.5 BMP-3 activates Smad2 in C3H10T1/2 stem cells

Having established the intrinsic ability of C3H10T1/2 stem cells to signal through the TGF-β/activin signal transduction pathway, we then determined if BMP-3 activates this pathway. Given that the receptor-activated protein Smad2 is phosphorylated upon the sequential ligand-dependent activation of ActRIIB and ALK-4, we determined the phosphorylation status of Smad2 following treatment of C3H10T1/2 stem cells with BMP-3. As shown in Fig. 3.4B, BMP-3 increased levels of phosphorylated Smad2 without altering total Smad2 protein levels.

3.6 The TGF-β/activin signalling pathway mediates BMP-3 stimulation of C3H10T1/2 stem cell proliferation

Although BMP-3 activated the TGF-β/activin signalling pathway as reflected in the increased level of phosphorylated Smad2 protein in C3H10T1/2 stem cells, this information is insufficient to conclude whether these pathways are involved in mediating BMP-3 stimulation of cell proliferation. As a first step, we determined effective concentrations of the TGF-β/activin pathway inhibitor SB-431542 at blocking the BMP-3 induced phosphorylation of Smad2. As shown in Fig. 3.5A, the pharmacological

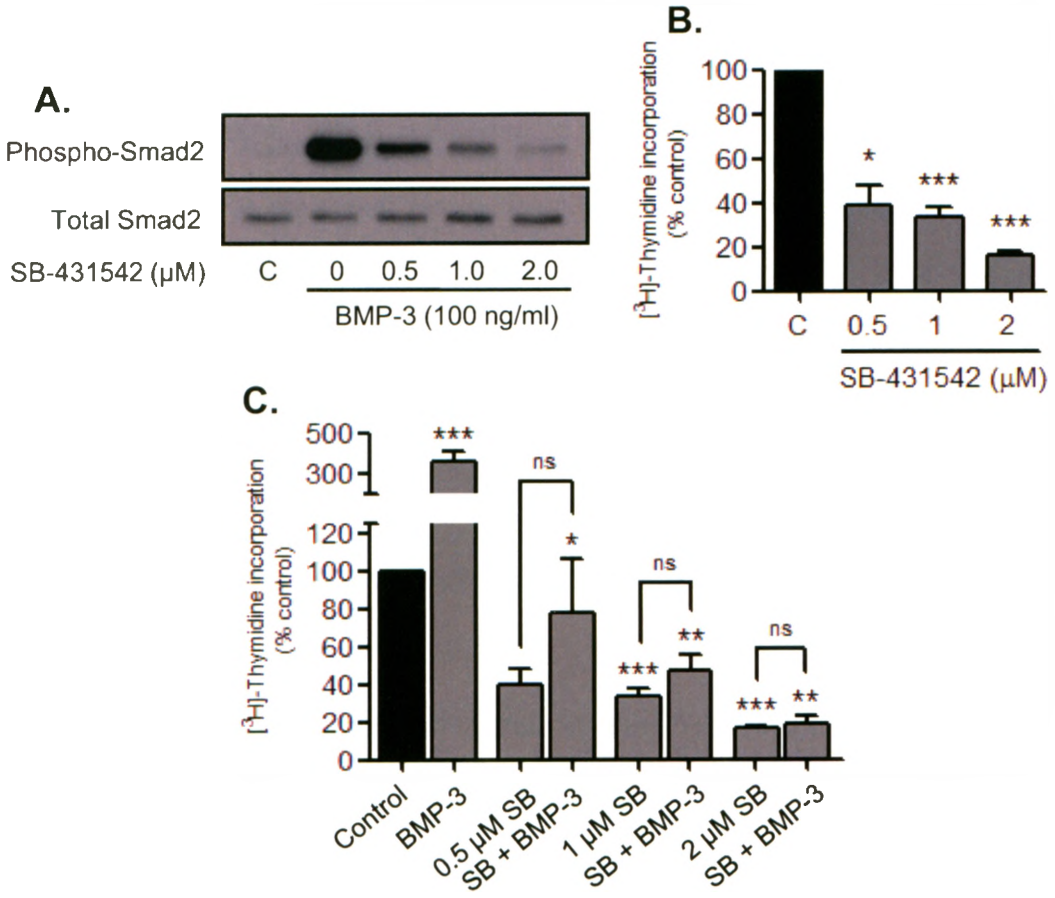


Figure 3.5 Effects of TGF-β/activin pathway inhibitor SB-431542 on basal and BMP-3-stimulated C3H10T1/2 stem cell proliferation. C3H10T1/2 stem cells were cultured in standard growth medium (10% FBS) until 50 – 60% confluence, at which time cells were starved for 24 h in serum-free medium. Cells were then pre-treated with increasing concentrations of SB-431542 (0.5 – 2 μM) for 24 h in serum-free medium **(B)**; or pre-treated without or with 0.5 μM, 1 μM, or 2 μM **(C)** of SB-431542 for 1 h in serum-free medium, followed by treatment with 100 ng/ml BMP-3 in serum-free medium for 24 h. During the last 4 h of treatment, cells were pulse labeled with [^{3}H]-thymidine (0.5 μCi/well) and the rate of [^{3}H]-thymidine incorporation was determined. Data are means ± SEM, $n = 3 – 5$ independent experiments, each performed in triplicate (* $p < 0.05$, *** $p < 0.001$ *vs.* control; *ns* = not significant). **(A)** Cells were treated with increasing concentrations of SB-431542 (0.5 – 2 μM) for 1 h in serum-free medium, followed by treatment with 100 ng/ml BMP-3 for 1 h in serum-free medium. Cell lysates were prepared and subjected to standard western blot analysis with antibodies specific for phosphorylated Smad2 and total Smad2 proteins. Results from one representative western blot are shown. Similar results were obtained from two independent experiments.

inhibitor reduced levels of phosphorylated Smad2 protein in a concentration-dependent fashion. Having established the effective concentrations of SB-431542, we then used them in [^{3}H]-thymidine incorporation assays to determine if they could block BMP-3 induced cell proliferation. We first determined the effects of the inhibitor alone, in order to gain insight into the molecular mechanisms that underlie proliferation/self renewal of C3H10T1/2 stem cells under basal conditions. As shown in Fig. 3.5B, treatment of C3H10T1/2 stem cells with SB-431542 led to a concentration-dependent decrease in [^{3}H]-thymidine incorporation with a greater than 80% reduction at 2 μM ($p < 0.001$). To determine if the TGF-β/activin signalling pathway mediates the mitogenic effects of BMP-3, C3H10T1/2 stem cells were pre-treated with or without SB-431542, followed by BMP-3 treatment. As shown in Fig. 3.5C, SB-431542 at all three concentrations completely abrogated BMP-3 induced stimulation of C3H10T1/2 stem cell proliferation.

3.7 BMP-3 inhibits ERK1/2 and p38 but not JNK signalling pathways in C3H10T1/2 stem cells

Actions of the TGF-β family are mediated through both Smad-dependent and Smad-independent signalling cascades, such as ERK, JNK, and p38 MAPK (Derynck and Zhang, 2003; Massague, 1998). To determine the effects of BMP-3 on the Smad-independent signalling pathways, we examined changes in the phosphorylation status of ERK1/2, JNK, and p38 MAPK following BMP-3 treatment. As shown in Fig. 3.6A – 3.6C, BMP-3 decreased levels of phosphorylated ERK1/2 and p38 but not JNK proteins in C3H10T1/2 stem cells. Given that phosphorylated JNK protein was undetectable under basal conditions, we used UV radiation to activate the JNK pathway in these cells.

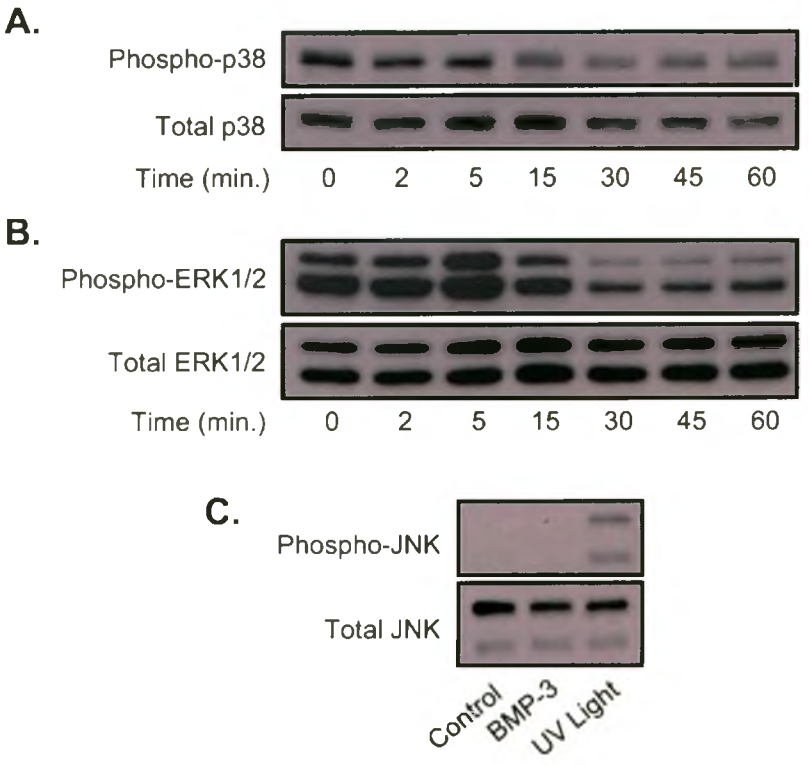


Figure 3.6 Inhibition of Smad-independent signalling pathways by BMP-3 in C3H10T1/2 stem cells. C3H10T1/2 stem cells were cultured in standard growth medium (10% FBS) until 50 – 60% confluence, at which time cells were starved for 24 h in serum-free medium. Cells were then treated with 100 ng/ml BMP-3 in serum-free medium, and at indicated times thereafter, cell lysates were prepared and subjected to standard western blot analysis with antibodies specific for **(A)** phosphorylated and total p38 proteins, **(B)** phosphorylated and total ERK1/2 proteins, and **(C)** phosphorylated and total JNK proteins. Due to the inability of BMP-3 to induce JNK phosphorylation, a positive control (cells were exposed to 40 mJ of UV radiation) was used to show the induction of JNK phosphorylation. Results from one representative western blot are shown. Similar results were obtained from two independent experiments.

Exposure to UV light led to a dramatic increase in phosphorylated but not total JNK protein levels, demonstrating that the JNK signalling pathway is operational in C3H10T1/2 stem cells (Fig. 3.6C).

3.8 The role of Smad-independent signalling pathways in the maintenance of basal C3H10T1/2 stem cell proliferation

Given that there is little information regarding the factors that are responsible for stem cell self-renewal/proliferation, coupled with our observations that C3H10T1/2 stem cells possess considerable ERK1/2 and p38 MAPK activities under basal/non-stimulating conditions, we examined their involvement in maintaining basal C3H10T1/2 stem cell proliferation. Using specific pharmacological inhibitors, we first showed that both the ERK1/2 inhibitor U0126 and the p38 MAPK inhibitor SB-202190 decreased their respective phosphorylated protein levels in a concentration-dependent fashion (Fig. 3.7A and 3.7C). Importantly, the two inhibitors also reduced C3H10T1/2 stem cell proliferation in a concentration-dependent manner under basal conditions, revealing SB-202190 was far more effective than U0126 as they displayed a reduction of 80% and 40%, respectively ($p < 0.001$; Fig. 3.7B and 3.7D).

3.9 Specificity of the pharmacological inhibitors for their respective signalling pathway

Considering that we defined the signal transduction pathways that mediate both basal and BMP-3-stimulated stem cell proliferation using the well-established pharmacological inhibitors, it is important to demonstrate the specificity of these inhibitors. Therefore, we conducted western blot analyses on cell lysates from cells that

were treated with each inhibitor. As shown in Fig. 3.8A – 3.8C, each inhibitor effectively reduced phosphorylation of their intended target protein without non-specifically decreasing phosphorylation of other proteins.

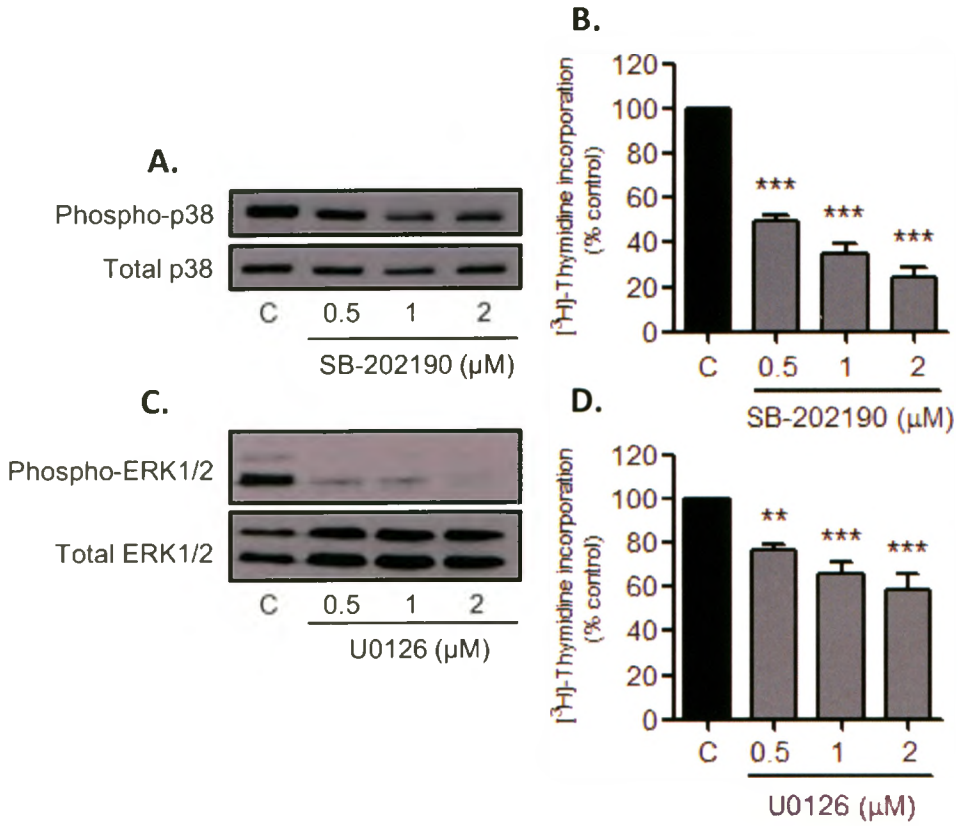


Figure 3.7 Effects of Smad-independent pathway inhibitors on C3H10T1/2 stem cell proliferation under basal/non-stimulating conditions. C3H10T1/2 stem cells were cultured in standard growth medium (10% FBS) until 50 – 60% confluence, at which time cells were starved for 24 h in serum-free medium. Cells were then treated for 1 h in serum-free medium with increasing concentration (0.5 – 2 μM) of the p38 MAPK inhibitor SB-202190 or the ERK1/2 inhibitor U0126. At the end of treatment, cell lysates were prepared and subjected to standard western blot analysis with antibodies specific for **(A)** phosphorylated and total p38 proteins, and **(C)** phosphorylated and total ERK1/2 proteins. Results from one representative western blot are shown. Similar results were obtained from two independent experiments. Alternatively, cells were treated for 24 h in serum-free medium with increasing concentration (0.5 – 2 μM) of the p38 MAPK inhibitor SB-202190 **(B)**, or the ERK1/2 inhibitor U0126 **(D)**. During the last 4 h of treatment, cells were pulse labeled with [^{3}H]-thymidine (0.5 μCi/well) and the rate of [^{3}H]-thymidine incorporation was determined. Data are means ± SEM, n = 4 – 6 independent experiments, each performed in triplicate (** $p < 0.01$, *** $p < 0.001$ *vs.* control).

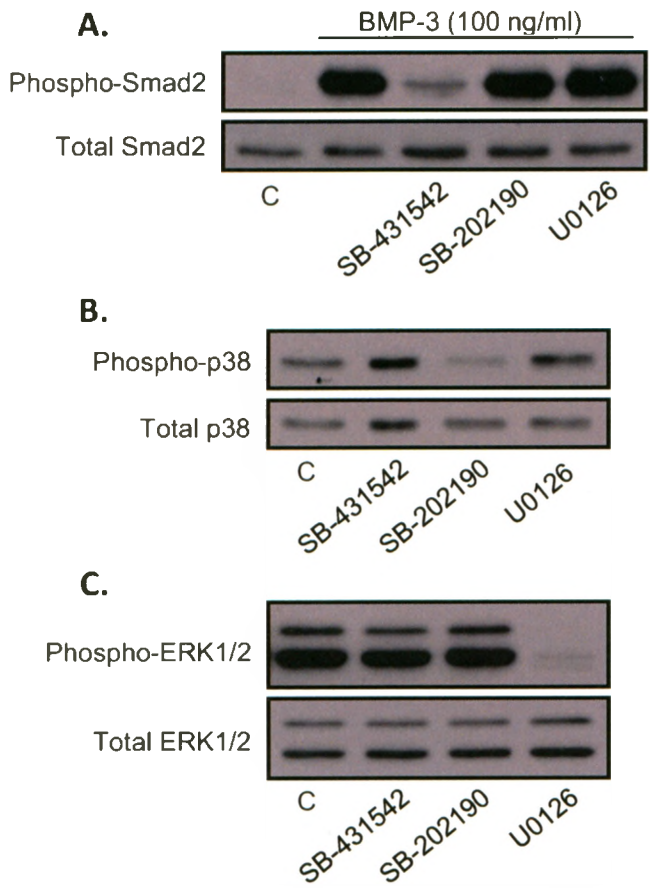


Figure 3.8 Specificity of the pharmacological inhibitors for their targeted signalling pathways. C3H10T1/2 stem cells were cultured in standard growth medium (10% FBS) until 50 – 60% confluence, at which time cells were starved for 24 h in serum-free medium. Cells were then pre-treated for 1 h with 2 μM of SB-431542, SB-202190, or U0126 prior to treatment for 1 h with 100 ng/ml BMP-3 in serum-free medium **(A)**. Alternatively, cells were treated for 1 h with 2 μM of SB-431542, SB-202190, or U0126 in serum-free medium **(B & C)**. Cell lysates were prepared, and subjected to standard western blot analysis with antibodies specific for phosphorylated and total Smad2 **(A)**, p38 **(B)**, and ERK1/2 **(C)** proteins. Results from one representative western blot are shown. Similar results were obtained from three independent experiments.

Chapter 4

Discussion

4.1 General Discussion

BMP-3 is a divergent member of the BMP family because it signals through the TGF-β/activin, rather than the BMP, signal transduction pathway. BMP-3 is a negative regulator of bone density (Daluiski *et al*, 2001); however, its role in other organ systems is unknown. Given the reciprocal relationship between osteogenesis and adipogenesis (Jeon *et al*, 2003; Lecka-Czernik *et al*, 2002), coupled with our recent demonstration that BMP-3 expression is increased in visceral adipose tissue of an early-life programmed rat model of increased visceral adiposity (Guan *et al*, 2005), we hypothesized that BMP-3 promotes adipogenesis. Our hypothesis is also supported by a recent independent study, reporting a similar increase in BMP-3 expression in a diet-induced mouse model of obesity (Koza *et al*, 2006). Importantly, the enhanced BMP-3 expression proceeded increases in fat mass, suggesting causation rather than a consequence of obesity. Therefore, the present study was undertaken to test this hypothesis using the well-characterized *in vitro* model systems, C3H10T1/2 MSCs and 3T3-L1 preadipocytes, which have been shown to recapitulate many of the physiological events that occur *in vivo* (Jasuja *et al*, 2005; Katagiri *et al*, 1990; Student *et al*, 1980; Tang *et al*, 2004; Zhang and Stott, 2004). We demonstrated for the first time that BMP-3 promotes proliferation of MSCs and preadipocytes. We also provided evidence that p38 MAPK, ERK1/2, and TGF-β/activin signalling pathways are involved in the maintenance of basal MSC proliferation; however, the latter mediates the mitogenic effects of BMP-3.

Accelerated adipogenesis is a hallmark of obesity, but the factors and the molecular mechanisms that orchestrate this important event are poorly understood. Adipogenesis begins with the commitment of MSCs to the adipocyte lineage, followed by terminal differentiation of preadipocytes to lipid-filled mature adipocytes (Rosen and

MacDougald, 2006). Several transcription factors have been identified to play a key role in controlling adipocyte differentiation, which include PPARγ and members of the C/EBP family (Farmer, 2006). However, the molecular determinants of MSCs to adipocyte precursor cells remain largely unknown. MSCs can give rise to four distinct cell types, including adipocytes, chondrocytes, myocytes, and osteocytes (Rosen and MacDougald, 2006). Among the four cell lineages, there is evidence for an intimate relationship between the formation of adipocytes and osteocytes. For instance, a number of *in vitro* studies using bone marrow-derived MSCs have demonstrated that factors that induce adipogenesis inhibit osteoblast formation and, conversely, factors that promote osteogenesis inhibit adipocyte formation (Jeon *et al*, 2003; Lecka-Czernik *et al*, 2002).

Considering that BMP-3 is a negative regulator of osteogenesis (Daluiski *et al*, 2001), we first examined the possibility that BMP-3 may promote the commitment of MSCs to the adipocyte lineage. To do so, we utilized an established protocol in which C3H10T1/2 MSCs were treated with recombinant BMP-3 during their proliferative phase until two days post-confluence. At this time, cells were induced for adipogenesis. We used BMP-4 as a positive control because it is known to drive MSCs toward the adipocyte lineage (Tang *et al*, 2004). Our results revealed that BMP-3 treatment did not result in adipocyte formation. We also explored, but found no evidence to support, the contention that BMP-3 may act in concert with BMP-4 to promote the commitment of MSCs to the adipocyte lineage. Furthermore, we determined the effects of BMP-3 on adipocyte differentiation, and showed that BMP-3 did not alter the differentiation of 3T3-L1 preadipocytes. Together, our data indicate that BMP-3 has no effect on the commitment or the differentiation steps of adipogenesis.

A key, but poorly understood, provision for adipogenesis is proliferation of MSCs and preadipocytes. Using a standard [^{3}H]-thymidine incorporation assay, we demonstrated that treatment of both C3H10T1/2 stem cells and 3T3-L1 preadipocytes led to a 3-fold increase in DNA synthesis, a surrogate marker of cell proliferation. Therefore, these results demonstrate that BMP-3 is a potent mitogen for both MSC and preadipocyte self-renewal. Given the similar effects of BMP-3 on C3H10T1/2 stem cells and 3T3-L1 preadipocytes, and because little is known about the regulation of MSC proliferation, in the present study we investigated the molecular mechanisms that underlie BMP-3 stimulation of MSCs.

Members of the BMP family typically signal through the BMP signalling pathway. BMP ligands bind to the BMP type II receptor (BMPRII), which then binds and activates the type I receptor (BMPRI). The activated type I receptor phosphorylates Smad1, Smad5, and/or Smad8. The receptor-activated Smads form a trimeric complex with the common Smad, Smad4, which then translocates into the nucleus where it regulates the expression of its target genes (Kitisin *et al*, 2007). In contrast, several lines of evidence suggest that actions of BMP-3 are mediated through the TGF-β/activin signalling pathway (Allendorph *et al*, 2007; Bahamonde and Lyons, 2001). First, BMP-3 activates expression of a TGF-β/activin-responsive reporter but not the BMP-responsive reporter (Daluiski *et al*, 2001). Second, BMP-3 displays a high affinity for ActRIIB, the type II receptor for ligands that activate the TGF-β/activin pathway (Allendorph *et al*, 2007). This pathway consists of the type II receptor ActRIIB and the type I receptor ALK-4. Together, this receptor complex activates Smad2/Smad3 (Kitisin *et al*, 2007).

As a first step in examining the involvement of the TGF-β/activin pathway in mediating the mitogenic effects of BMP-3 on MSCs, we determined the expression of the

major components of the TGF-β/activin signalling pathway with standard RT-PCR. Our results showed that all the major components were highly expressed in C3H10T1/2 stem cells, including ActRIIB, ALK-4, Smad2, Smad3, and Smad4. This suggested that these cells are capable of responding to factors that activate the TGF-β/activin signalling pathway. Indeed, BMP-3 dramatically increased levels of phospho-Smad2 protein. Importantly, the TGF-β/activin inhibitor SB-431542 blocked BMP-3 stimulation of MSC proliferation. Furthermore, SB-431542 profoundly inhibited basal proliferation. Together, these results suggest that the TGF-β/activin signalling pathway not only maintains basal, but also mediates BMP-3 stimulated C3H10T1/2 stem cell proliferation. It is noteworthy that we empirically determined the effective concentrations of the inhibitor to be in the lower micromolar range (i.e., 0.5, 1 and 2 μM), as shown by their concentration-dependent inhibition of BMP-3-induced Smad2 phosphorylation. These concentrations of SB-431542 were much lower than those (≥10 μM) reported by the majority of others in the literature. This is an important issue because at higher concentrations, SB-431542 could non-specifically inhibit Smad-independent pathways, such as p38 MAPK, ERK1/2, and JNK.

In addition to activating the BMP pathway, several BMPs have been shown to signal through MAPK signalling pathways, namely p38, ERK, and JNK. For example, BMP-4 has been shown to activate both p38 MAPK (Fiori *et al*, 2006) and ERK1/2 (Yang *et al*, 2007), and BMP-2 has also been shown to activate ERK1/2 (Lou *et al*, 2000; Xing *et al*, 2002). In comparison, the JNK pathway is less defined in relation to BMP signalling (Massague, 1998). Since TGF-β family members are known to activate Smad-independent pathways, we also investigated the effect of BMP-3 on p38 MAPK, ERK1/2, and/or JNK signalling pathways. We showed that BMP-3 induced a decrease in levels of

phosphorylated ERK1/2 and p38 proteins. In contrast, BMP-3 had no effect on JNK protein phosphorylation. It is noteworthy that phosphorylated JNK protein was undetectable in C3H10T1/2 stem cells under basal conditions. Consequently, we used UV radiation, a known activator of JNK, to show that our cells were capable of responding to JNK activation, and perhaps more importantly to demonstrate that our inability to detect JNK phosphorylation under both basal and BMP-3-stimulated conditions was not attributed to any technical difficulties.

Given that there were considerable levels of phosphorylated p38 MAPK and ERK1/2 proteins in C3H10T1/2 stem cells under basal conditions, we explored their potential involvement in the maintenance of stem cell self-renewal/proliferation. Using well-established pharmacological inhibitors, we demonstrated that both p38 MAPK and ERK1/2 signalling pathways play important roles in maintaining C3H10T1/2 stem cell basal proliferation, revealing that the former has a more prominent role because the p38 MAPK inhibitor SB-202190 caused a far greater reduction (80%) in DNA synthesis than the ERK1/2 inhibitor U0126 (40%). Because of the lack of detectable levels of phosphorylated JNK protein under both basal and BMP-3-stimulated conditions, we did not pursue this pathway further.

Knowing that there is potential for the inhibitors to non-specifically target other signalling pathways, coupled with the fact that our study relied heavily on the interpretation of data obtained from using these pharmacological inhibitors, it is imperative that the specificity as well as the effective concentrations of these inhibitors be empirically determined and demonstrated in our cell model system. Indeed, we conducted such experiments, and our results showed that each inhibitor was able to attenuate phosphorylation of its target protein in a concentration-dependent fashion.

Importantly, they also demonstrated that there was no cross-inhibition among the inhibitors. Thus, each inhibitor used in the present study specifically targeted its corresponding signalling pathway.

4.2 Summary and Conclusions

In summary, the present study demonstrates that BMP-3 promotes proliferation of both MSCs and preadipocytes *in vitro*. Moreover, it provides evidence that the mitogenic effects of BMP-3 on MSCs are mediated through the TGF-β/activin signalling pathway. In addition, this pathway along with p38 MAPK and ERK1/2 signalling pathways are involved in maintaining basal MSC proliferation. Thus, our present study not only provides insight into the role of BMP-3 in providing a source for adipogenesis, but also reveals a previously unappreciated role for the TGF-β/activin, ERK1/2, and p38 MAPK signalling pathways in regulating MSC self-renewal.

4.3 Future Directions

Having concluded that BMP-3 is a potent mitogenic factor in both C3H10T1/2 and 3T3-L1 cell systems, further investigation is required to understand its effects. To continue the analysis of BMP-3, there are several avenues in which the study could pursue. To begin, investigating the effect of BMP-3 on apoptosis would be of value to expand this study. Given that proliferation and apoptosis are two important mechanisms which often work in concert with one another, it would be valuable to investigate the possibility of BMP-3 inhibiting cell death. Through use of TUNEL staining techniques, the DNA fragmentation that occurs during apoptosis can be tagged and visualised under a

fluorescence microscope. Upon determining the percent of apoptotic cells, the effect of BMP-3 on apoptosis would be apparent.

TGF-β family signalling is remarkably complex with the immense number of receptor combinations. Given that BMP-3 signals through the TGF-β/activin pathway through activation of Smad2, confirming the receptors using siRNA would further support the specific pathway through which BMP-3 exerts its effects. In order to confirm the type I and type II receptors, the knockdown of ActRIIB and ALK-4 receptors can be achieved using siRNA to interfere with of their expression.

Additionally, investigating cell cycle elements would assist in elucidating the effects of BMP-3. Cyclin and cyclin-dependent kinase (CDK) complexes regulate each phase of the cell cycle. Cells in G_1 phase are triggered by the D-type cyclins, which form a complex with CDK4 and CDK6. This complex progresses the cell into S phase where DNA synthesis occurs. Once the cell has completed DNA synthesis and progressed into G_2 phase, the CDK1-cyclin B complex is responsible for driving the cell through mitosis (Malumbres and Barbacid, 2009). By performing a cell cycle analysis, detecting the alteration of expression of these cyclins would assist in understanding the downstream effects of BMP-3.

To further understand the downstream targets that underlie the effects of BMP-3, future directions should be aimed at identifying its target genes. Using a DNA microarray, the change in expression of thousands of genes can be detected. As a result, the identification of target genes will aid in elucidating the downstream effects of BMP-3.

Given that the current literature on BMP-3 is lacking, BMP-3 generally remains poorly understood. As a result, multiple avenues exist to be pursued in order to better understand its location, function, expression, and mechanisms.

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