MACROPHAGE AEBP1 CONTRIBUTES TO MAMMARY EPITHELIAL CELL HYPERPLASIA AS A NOVEL REGULATOR OF SONIC HEDGEHOG SIGNALLING

by

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ABSTRACT

Chronic inflammation stimulates mammary tumourigenesis by disrupting signalling interactions between the epithelial ducts and the surrounding stromal microenvironment. Adipocyte enhancer-binding protein 1 (AEBP1) promotes mammary epithelial cell hyperplasia as a stromal factor that enhances activity of the proinflammatory transcription factor Nuclear Factor-κB (NF-κB) in macrophages. Aberrant NF-κB activity in macrophages elevates production of proinflammatory signals and the ligand sonic hedgehog (Shh), a significant contributor to tumourigenesis. In this study, Shh expression was elevated in macrophages isolated from transgenic mice ($AEBP1^{TG}$) that overexpress AEBP1. Transient overexpression of AEBP1 in a macrophage cell line resulted in increased Shh expression. Furthermore, hedgehog target genes *Gli1* and *Bmi1* were up-regulated in mammary epithelium of $AEBP1^{TG}$ mice and HC11 mammary epithelial cells co-cultured with $AEBP1^{TG}$ macrophages. Growth of HC11 cells and mammary tumours was enhanced in response to $AEBP1^{TG}$ macrophages. These findings suggest that macrophage AEBP1 overexpression contributes to mammary hyperplasia through enhanced hedgehog signalling.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ACLP	aortic carboxypeptidase-like protein
AE-1	adipocyte enhancer 1
AEBP1	adipocyte enhancer binding protein 1
aP2	adipose P2 (422) gene
APS	ammonium persulfate
ATCC	American Type Culture Collection
Bmi1	B-cell-specific Moloney murine leukaemia virus integration site 1
BSA	bovine serum albumin
CDK	cyclin-dependent kinase
Cdkn2a	cyclin-dependent kinase inhibitor 2A
COS2	costal2
CP	carboxypeptidase
DLD	discoidin I-like domain
ddH2O	double-distilled water
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
E. coli	*Escherichia coli*
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EMT	epithelial–mesenchymal transition

ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FU	fused
Gli	glioma-associated oncogene family zinc finger
GSK-3β	glycogen synthase kinase 3 beta
HRP	horseradish peroxidase
IgG	immunoglobulin G
IκBα	inhibitor of kappa B alpha
IKK	IκB kinase
IL	interleukin
KO	knock-out
LXRα	liver x receptor alpha
MDM2	mouse double minute 2 homolog
MEM	minimum essential medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NF-κB	nuclear factor kappa B
NT	non-transgenic
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDK	3-phosphoinositide dependent protein kinase 1
PcG	polycomb group family of transcriptional repressors
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A

PMSF	phenylmethylsulfonyl fluoride
PPARγ	peroxisome proliferator-activated receptor gamma
PRC	polycomb repressive complex
PTCH	patched
PTEN	phosphatase and tensin homolog
RB	retinoblastoma
SEM	standard error of the mean
SDS	sodium dodecyl sulfate
Shh	sonic hedgehog homologue
Ski	skinny hedgehog
SMO	smoothened
STAT	signal transducer and activator of transcription
SUFU	suppressor of fused
TAE	tris-acetate-EDTA
TBST	tris-buffered saline tween20
TEB	terminal end buds
TEMED	N,N,N′,N′-tetramethylethane-1,2-diamine
TG	transgenic
TGF-β	transforming growth factor beta
TNFα	tumour necrosis factor alpha
WAT	white adipose tissue
WT	wild type

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CHAPTER 1: INTRODUCTION

Hyperplasia is a physiological increase in cell proliferation that typically precedes neoplastic transformation and ultimately tumourigenesis. In the mammary gland, epithelial cell hyperplasia and tumourigenesis often develops as a result of aberrant signalling from the surrounding microenvironment. Immune cells that infiltrate into the microenvironment, primarily macrophages, can promote tumourigenesis by creating a proinflammatory microenvironment that stimulates growth of the adjacent epithelial cells. Adipocyte enhancer-binding protein 1 (AEBP1) is a novel proinflammatory mediator implicated in the development of mammary tumourigenesis by enhancing production of numerous cytokines and chemokines from macrophages within the microenvironment (Holloway *et al.,* 2012). Overexpression of various proinflammatory cytokines has been shown to promote the initiation and growth of tumours, yet more studies are finding that activation of the hedgehog signalling, an essential developmental pathway that stimulates cell proliferation, is also enhanced in association with increased proinflammatory signalling (Nakashima *et al*., 2006; Kasperczyk *et al*., 2009; Cui *et al*., 2010). Enhanced hedgehog signalling activity contributes to the development of cancer in numerous human and murine tissues (Nakashima *et al*., 2006; Kasperczyk *et al*., 2009; Cui *et al*., 2010; Karhadkar *et al*., 2004). Understanding how AEBP1 affects these pathways involved in the crosstalk between the microenvironment and the mammary epithelial cells and what effect(s) this may have on the adjacent epithelial cells in the mammary tissue will facilitate the development of therapeutic strategies that prevent and/or treat breast cancer.

1.1. The Mammary Gland

1.1.1. Mammary Gland Structure

The mammary gland is a distinct type of sweat gland whose function is to produce milk that nourishes newborn offspring (Viacava *et al*., 1997; Wiseman & Werb, 2002). The gland is composed of two main components: the epithelium, which forms a branching ductal structure responsible for milk production and secretion, and the stromal microenvironment (Figure 1; Wiseman & Werb, 2002). The epithelial ductal structure is surrounded by the adipose-rich stromal region that consists of a heterogeneous population of cells, such as fibroblasts, endothelium, adipocytes, and immune cells, within an extracellular matrix (ECM; Wiseman & Werb, 2002). Unlike most organs that are patterned during embryogenesis, the mammary gland is unique in that the patterning of this organ changes throughout the life cycle of females. The epithelial ductal structure initially forms during embryogenesis as a rudimentary population of cells located at the nipple. After birth, this population of epithelial cells develops into a branching network of ducts invading into the stromal compartment through remodelling of the ECM and increased proliferation of mammary epithelial cells. At the onset of puberty, ovarian hormones induce the formation of bulb-like structures at the ends of the epithelial ducts referred to as terminal end buds (TEBs). By 10-weeks of age in mice, the mammary ductal structure elongates and invades to the limits of the stroma. This occurs as new ductal branches form from the bifurcation of the TEBs and increased side-branching arises from mature ducts (Wiseman & Werb, 2002). Growth and migration of the branching epithelial ducts into the stroma is regulated by communication between these two compartments, referred to as the stromal-epithelial crosstalk (Wiseman & Werb,

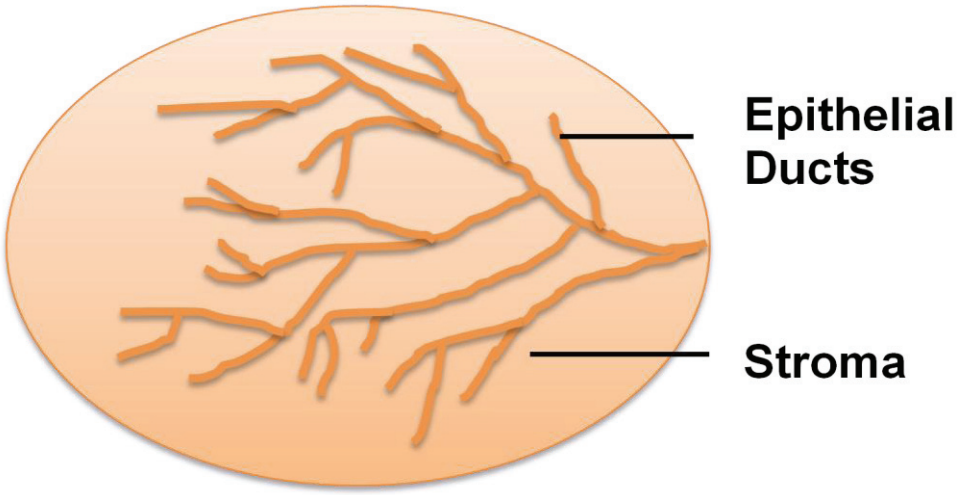

Figure 1. Schematic diagram depicting the structure of the mammary gland. Adapted from Hennighausen & Robinson 2005, *Nat. Rev. Mol. Cell Biol.*

2002). This crosstalk between the epithelial ducts and the stromal compartment is crucial to the normal development of the ductal structure.

Numerous reports have found that irregularities in the composition of the mammary stromal compartment can disrupt the stromal-epithelial crosstalk and cause tumour formation (Coussens & Werb, 2002; reviewed in Ghajar & Bissell, 2008). Abnormal stromal composition often results in dysregulation of factors that remodel the ECM or signalling pathways between the epithelium and stromal microenvironment (Wiseman & Werb, 2002). Inflammatory events are a common factor that disrupts the composition of the stroma by promoting angiogenesis, inflammatory immune cell infiltration, and elevated production of proinflammatory factors and matrix remodelling proteins (Coussens & Werb, 2002). Previous reports have demonstrated that aberrant ECM remodelling resulting from excess production of matrix metalloprotease (MMP)-3 in the mammary gland stroma leads to tumour formation (Radisky *et al.,* 2005; Sternlicht *et al.,* 1999). In addition, aberrant chemokine production contributes to the development of neoplasia and tumour formation by increasing infiltration of proinflammatory immune cells into the stroma (Lin *et al.,* 2001; Soria & Ben-Baruch, 2008). The stromal-epithelial crosstalk is a significant influence on the gene expression and growth of the epithelial ductal structure. However, irregularities in the composition of the stroma provide a microenvironment that allows unrestrained growth of mammary epithelial cells resulting in tumour formation (Bissell & Radisky, 2001).

1.1.2. Role of Stromal Macrophages in The Mammary Gland

Macrophages are a prominent component of the mammary stromal compartment involved in regulating the growth and patterning of the mammary ductal structure as well as promoting tumour initiation, growth, and metastasis. Macrophages originate as $CD34^+$ bone marrow progenitors that release their progeny cells into the bloodstream where they develop into monocytes. Circulating monocytes are recruited into the stromal compartment of the mammary tissue by the release of chemokines from stromal, epithelial, or tumour cells. Once monocytes infiltrate into the stroma, they will differentiate into 'resident' macrophages (Ross & Auger, 2002). The main functions of resident macrophages are to remove microbial invaders, regulate cell turnover and assist in wound healing through phagocytosis and tissue remodelling (Gouon-Evans *et al.,* 2002; Mantovani *et al*., 2002). In addition, resident macrophages are critical in regulating proliferation and survival of ductal epithelial cells of the mammary gland through the release of growth factors, cytokines and chemokines (Gouon-Evans *et al.,* 2002). Resident macrophages and their secreted factors also have a significant contribution to tumour growth or inhibition (Lin & Karin, 2007; Biswas *et al*., 2008).

Stromal and/or epithelial cells will release cytokines in response to injury, inflammation, infection, and/or malignancy (Pollard, 2004; Biswas *et al*., 2008). These cytokines induce resident macrophages to acquire one of two distinct activated phenotypes: Type I classically activated (M1) or Type II alternatively activated (M2) macrophages (Mantovani *et al*., 2002). The M1 macrophages are induced by cytokines such as interferon-gamma (IFNγ) and tumour necrosis factor alpha (TNFα), whereas interleukins (IL)-4 and IL-13 are responsible for the induction of the M2 macrophages (Mantovani *et al*., 2007). M1 macrophages are primarily involved in the destruction of

foreign microbes, tumouricidal activity and tend to produce abundant amounts of proinflammatory cytokines and chemokines. This type of activated macrophage is notable for the production of cytokines TNFα, IL-6, IL-1β and IL-12 (Mantovani *et al*., 2007). In contrast, M2 macrophages are involved in immune-suppression, angiogenesis, tissue remodelling, and secretion of anti-inflammatory cytokines IL-4, IL-10, and transforming growth factor-beta (TGF-β; Mantovani *et al*., 2004, 2007; Martinez *et al*., 2009).

The M1- and M2-activated macrophages have distinctly different effects on tumour initiation and growth. Previous reports have indicated that proinflammatory M1 macrophages present at sites of chronic inflammation are significant contributors to the onset of neoplastic transformation through persistent production of proinflammatory cytokines (Balkwill & Mantovani, 2001; Biswas *et al*., 2008). It has been previously demonstrated that the M1-type cytokine TNFα is significantly up-regulated in inflammatory cells of *Mdr2*-knockout mice, a mouse model that spontaneously develops inflammation-mediated hepatocellular carcinoma (Pikarsky *et al*., 2004). They further demonstrated that inhibition of TNFα using anti-TNFα antibody caused apoptosis of dysplastic hepatocytes with concomitant reduction of the anti-apoptotic factors in *Mdr2*-knockout mice (Pikarsky *et al*., 2004). In addition to these findings, other studies using murine models of cancer have also shown that inhibition of M1-type proinflammatory cytokines diminishes the incidence of neoplastic transformation and tumour progression (Greten *et al*., 2004; Maeda *et al*., 2005). In contrast, M2 macrophages contribute to tumour growth rather than tumour initiation by promoting immunosuppressive activity, IL-10 signalling, angiogenesis, and tissue remodelling (Mantovani *et al*., 2002, 2007;

Biswas *et al*., 2008). In fact, tumour-associated macrophages (TAMs) recruited into the tumour microenvironment, through tumour-derived production of the chemokine monocyte chemotactic protein-1 (MCP-1/CCL2), acquire an M2-like phenotype during tumour progression (Mantovani *et al*., 1992, 2002). M1 macrophages contribute to tumour initiation while the M2-like TAMs support tumour progression; however, redirecting M2-like TAMs to the M1 phenotype can have an inhibitory effect on tumour growth (Saccani *et al*., 2006). Guiducci *et al*. (2005) have demonstrated that mammary tumour growth is reduced when TAMs are switched from the M2 to M1 phenotype by blocking induction of M2 macrophages with anti-IL-10 receptor (anti-IL-10R) antibody combined with a microbial stimulus, the toll-like receptor ligand CpG. In addition, treatment of TAMs with CpG and anti-IL-10R antibody induced the expression of M1-type cytokines IL-12 and TNFα (Guiducci *et al*., 2005). These studies illustrated that the proinflammatory M1 macrophages within the mammary stroma are an important contributor to neoplastic transformation and tumour initiation. Once a tumour is established, it will further recruit macrophages into the stroma and convert M1 macrophages to an M2-like phenotype, which is crucial in tumour progression.

1.1.3. Nuclear Factor-Kappa B (NF-κB) in Mammary Tumourigenesis

Sites of chronic inflammation in the mammary gland generally are where tumours are initiated due to irregular signalling interactions between the ductal epithelium and cells of the stromal microenvironment (Coussens & Werb, 2002; Clevers, 2004). NF-κB is a pivotal proinflammatory transcriptional regulator involved in mammary gland tumourigenesis (Bhat-Nakshatri *et al*., 2002). NF-κB activation

contributes to neoplastic transformation and tumour progression by increasing the production of various cytokines and chemokines that promote aberrant cell proliferation, survival, and invasion (Baud & Karin, 2009). In the canonical pathway, NF-κB is a heterodimer, composed of the subunits p50 and p65, which translocates to the nucleus where it induces numerous target genes (Hayden & Ghosh, 2008). The inhibitor of NF-κB (IκB) proteins interact with and sequester NF-κB in the cytoplasm in quiescent cells, thus inhibiting its activity. Various stimuli may provoke NF-κB nuclear translocation by inducing the multimeric IκB kinase (IKK) complexes to phosphorylate IκB, leading to its ubiquitination and consequent degradation by the proteosome (Hayden & Ghosh, 2008).

The contribution of macrophage NF-κB activation to tumourigenesis differs depending on the stages of tumour progression. High levels of NF-κB activation and proinflammatory cytokines in macrophages have a significant impact on tumour initiation. This was observed in a diethylnitrosamine (DEN)-induced hepatocellular carcinoma model where the ablation of the NF-κB activator IKKβ in liver macrophages, known as Kupffer cells, resulted in the down-regulation of proinflammatory cytokines IL-6 and TNFα. Furthermore, IKKβ ablation in Kupffer cells impairs DEN-mediated induction of hepatocellular carcinoma (Maeda *et al.,* 2005). In contrast to tumour initiation, NF-κB activity and expression of proinflammatory cytokines are reduced in TAMs of established tumours. The transcript levels of NF-κB-induced genes, such as TNFα and IL-12, are drastically down-regulated in lipopolysaccahride (LPS)-treated TAMs from murine fibrosarcomas (Biswas *et al*., 2006) and human ovarian carcinoma (Saccani *et al*., 2006). The reduced expression of these proinflammatory genes is attributed to impaired nuclear translocation of the NF-κB (p50/p65) heterodimer. Saccani

et al. (2006) found that the nuclear translocation of the NF-κB (p65) subunit is reduced in TAMs; however, translocation of NF-κB (p50) subunits to the nucleus is increased. As a result of increased nuclear translocation, NF-κB (p50) subunits homodimerize and inhibit the production of M1-type cytokines. Furthermore, TAMs from NF-κB $(p50)^{-/-}$ mice produce M1-type cytokines and are associated with reduced growth of transplanted tumours (Saccani *et al*., 2006). These studies indicate that although macrophage NF-κB activation is a significant contributor to formation of tumours, it can be inhibitory to tumour growth in later stages of tumour progression.

1.2. Adipocyte Enhancer-Binding Protein 1 (AEBP1)

1.2.1. Gene Expression and Function of AEBP1

Studies have found that the novel proinflammatory mediator and transcriptional repressor AEBP1 plays a critical role in mammary gland development (Zhang *et al*., 2011) and is overexpressed in the stroma of primary breast tumours (Farmer *et al*., 2009). The murine *Aebp1* gene contains 21 exons over 10 kb of genomic DNA and produces two transcripts, *Aebp1* and *Aclp* (aortic carboxypetidase-like protein; Layne *et al*., 1998), via alternative splicing (Ro *et al*., 2001). Unlike ACLP, AEBP1 lacks the additional 380 amino acids on the N-terminal end containing a signal peptide sequence, the lysine-proline rich 11 amino acid repeating motif (Layne *et al*., 1998). Furthermore, *Aebp1* also retains the 9^{th} intron in its transcript with exon 10 containing the ATG start codon (Ro *et al*., 2001). AEBP1 is an 83-kDa ubiquitous, multifunctional protein that is equally distributed between the cytoplasm and nucleus (Park *et al*., 1999; Majdalawieh *et al*., 2007). ACLP is a 175-kDa secreted protein highly expressed by

smooth muscle cells of the aorta (Layne *et al*., 1998). In contrast, AEBP1 is expressed at high levels in macrophages and preadipocytes (He *et al*., 1995; Ro *et al*., 2001; Zhang *et al*., 2005). Although transcripts for both ACLP and AEBP1 are detected in every mouse tissue examined (Ro *et al*., 2001), ACLP protein is not detected in heart, liver, skeletal muscle, and kidney (Layne *et al*., 1998). AEBP1 was originally identified as a transcriptional repressor that binds to adipocyte enhancer 1 (AE-1) recognition sites in the promoter region of the adipose P2 (*aP2*) gene (He *et al*., 1995). AEBP1 was later discovered to be a modulator of extracellular-signal-regulated kinase 1 and 2 (ERK1/2; Kim *et al*., 2001), IκBα (Majdalawieh *et al*., 2006, 2007), and phosphatase and tensin homolog (PTEN; Zhang *et al*., 2005; Ro *et al*., 2007), mediating these effects through protein-protein interactions. AEBP1 has since been found to play a regulatory role in adipogenesis, inflammation and macrophage cholesterol homeostasis (He *et al*., 1995; Zhang *et al*., 2005; Majdalawieh *et al*., 2006, 2007, 2009).

1.2.2. AEBP1 Protein Structure

The murine AEBP1 protein consists of 748 amino acids and contains three key domains: the N-terminal discoidin-like domain (DLD), the carboxypeptidase domain (CP), and the C-terminal domain composed of basic, serine-threonine-proline (STP), and acidic amino acid rich regions (Figure 2; He *et al*., 1995; Ro *et al*., 2001). The DLD domain of AEBP1 mediates several of its protein-protein interactions and contributes to its transcriptional repression function of genes such as *aP2* (He *et al*., 1995). Unlike full-length AEBP1, a truncated form of AEBP1 lacking the DLD domain ($AEBP1^{\Delta N}$)

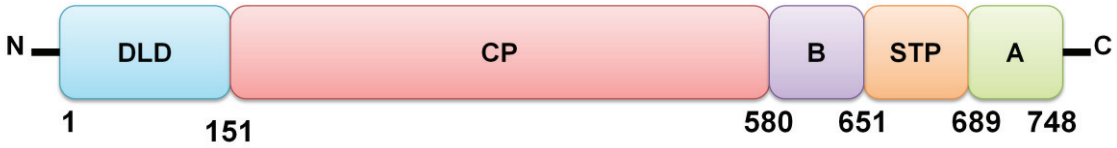

Figure 2. Schematic diagram of the domains of the murine AEBP1 protein. The AEBP1 protein consists of the N-terminal discoidin-like domain (DLD), the central carboxypeptidase (CP) domain, and the C-terminal region containing the basic (B), serine-threonine-proline rich (STP), and acidic (A) subdomains.

expressed in mammalian cells is incapable of repressing a luciferase reporter gene under the control of the aP2 promoter (Lyons *et al*., 2006), demonstrating that the DLD domain contributes to the transcriptional repression ability of AEBP1. The DLD domain of AEBP1 is also required for AEBP1 interactions with ERK1/2 (Kim *et al*., 2001) and IκBα (Majdalawieh *et al*., 2007). The interaction between the DLD domain of AEBP1 and IκBα results in the phosphorylation and degradation of IκBα, which consequently causes the up-regulation of NF-κB activity (Majdalawieh *et al*., 2007). In addition, the interaction between ERK1/2 and AEBP1 increases and maintains ERK1/2 activity by a protective effect towards MAP Kinase Phosphatase 3 (MKP-3; Kim *et al*., 2001). Although the DLD domain is crucial for AEBP1 to physically interact with ERK1/2 and IκBα, the CP domain is essential for AEBP1 interaction with the tumour suppressor PTEN, which results in PTEN degradation and consequently causes the up-regulation of Akt activity (Ro *et al*., 2007). DLD-truncated AEBP1 mutants retain the ability to interact with PTEN; however, a mutant form of AEBP1 lacking only the first N-terminal 70 amino acid residues of the CP domain ($AEBP1^{\Delta NCP}$) does not interact with PTEN (Ro *et al*., 2007). Like the DLD domain, the C-terminal region of AEBP1 also contributes to its transcriptional repression ability. Unlike the full-length AEBP1, a C-terminal truncation mutant form of AEBP1 ($AEBP1^{\Delta STY}$) is unable to bind to DNA containing AE-1 recognition sites (Kim *et al*., 2001; Majdalawieh *et al*., 2006). These findings demonstrate that both the DLD domain and the C-terminal region are required for transcriptional repression by AEBP1.

1.2.3. AEBP1 is a Novel Proinflammatory Mediator in Macrophages

Chronic inflammation is a key contributor to initiation and progression of tumourigenesis. Although AEBP1 modulates the activities of vital factors involved in tumourigenesis such as ERK1/2 and PTEN, AEBP1 may have a significant impact on inflammation-induced tumourigenesis through its role as a proinflammatory mediator. AEBP1 manifests itself as a critical proinflammatory mediator by enhancing NF-κB activity through two distinct means. First, AEBP1 enhances NF-κB (p65) activation by impairing IκBα inhibitory function. As previously discussed, the DLD domain of AEBP1 physically interacts with IκBα to promote its ubiquitination and consequent degradation, therefore allowing the phosphorylation and nuclear translocation of NF-κB (Majdalawieh *et al*., 2006, 2007). Interestingly, AEBP1 is able to inhibit IκBα function independent of any variation in the kinetic activity of the IKK complex (Majdalawieh *et al*., 2007). Secondly, AEBP1 also regulates inflammation by directly repressing the transcription of cholesterol efflux mediators liver X receptor alpha (LXRα) and peroxisome proliferator-activated receptor gamma (PPARγ; Majdalawieh *et al*., 2006, 2010), which also have a suppressive effect on inflammation. PPARγ is capable of impairing inflammation by interfering with NF-κB activity through physical interaction with the NF-κB subunits p50 and p65 in a ligand-independent manner (Chung *et al*., 2000). Furthermore, treatment of macrophages with oxidized low density lipoprotein (oxLDL), an agonist of PPARγ, impaired NF-κB binding to κB recognition sites in target gene promoters. Similarly, LXRα also interferes with NF-κB-mediated signalling. Thioglycollate-elicited macrophages treated with LXRα agonists GW3965 or T1317 display reduced expression of NF-κB target genes including MCP-1/CCL2, IL-1β, and IL-6 (Joseph *et al*., 2003). Joseph *et al*. (2003) further demonstrated that LXRα

activity impairs the ability of NF-κB to activate transcription. The expression of a luciferase reporter gene under the control of a promoter containing multiple κB recognition sites was reduced in response to the activation of either LXRα or LXRβ (Joeseph *et al*., 2003). Although LXRα suppresses NF-κB activity, the precise mechanism remains unclear. AE-1 recognition sites present in the promoters of the *Lxrα* and *Pparγ* genes suggest that AEBP1 directly mediates their repression (Kim *et al*., 1999; Majdalawieh *et al*., 2006). This was confirmed as the expression of luciferase reporter genes under the control of either the *Lxrα* or *Pparγ* promoter was diminished after transfection with an AEBP1 expression plasmid in a dose-dependent manner (Majdalawieh *et al*., 2006). Furthermore, the C-terminal truncated AEBP1 mutant AEBP1$^{\Delta STY}$, which is incapable of binding AE-1 sites (Kim *et al*., 2001), does not repress LXRα or PPARγ expression. The ability of AEBP1 to up-regulate NF-κB activity by promoting degradation of IκBα and transcriptional repression of LXRα and PPARγ (Figure 3) strongly suggests its role as a crucial proinflammatory mediator that contributes to tumourigenesis.

1.2.4. Role of AEBP1 in the Stromal-Epithelial Crosstalk of the Mammary Gland

The expression of AEBP1 in mammary gland tissue is confined to the stromal compartment, and absent in mammary epithelial cells, of adult mice (Zhang *et al*, 2011). AEBP1 expression is particularly abundant in preadipocytes (Kim *et al*., 2001; Ro *et al*., 2001) and macrophages (Majdalawieh *et al*., 2006, 2007), which are both present in the mammary gland stromal compartment. Given that AEBP1 overexpression in macrophages increases production of proinflammatory chemokines and cytokines

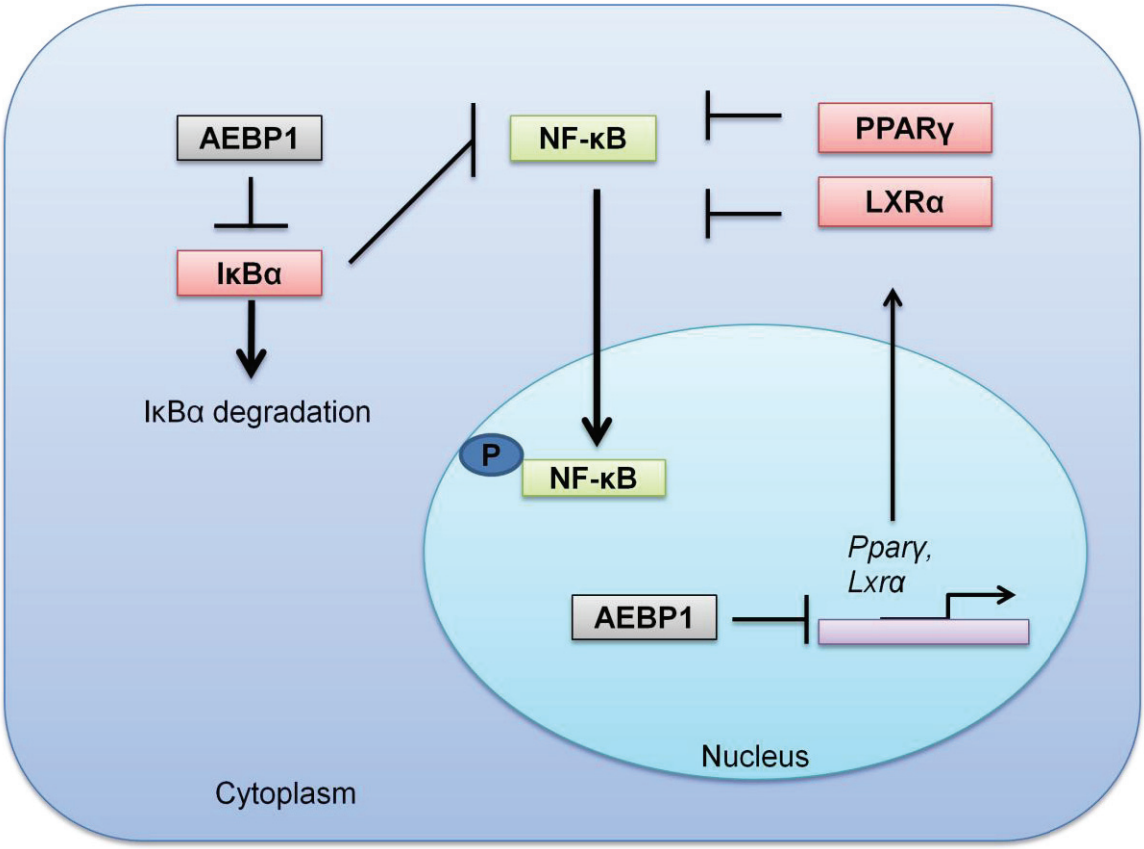

Figure 3. Diagram depicting the involvement of AEBP1 in mediating inflammation by regulation of NF-κB activity and transcriptional repression of *Lxrα* and *Pparγ*. AEBP1 allows the phosphorylation and nuclear translocation of NF-κB by promoting the degradation of its inhibitor IκBα through protein-protein interaction. AEBP1 also allows NF-κB activity by preventing the inhibitory effects of PPARγ and LXRα through transcriptional repression.

involved in tumourigenesis (Wiseman & Werb, 2002; Schwertfeger *et al*., 2006; Majdalawieh *et al*., 2007; Qian *et al*., 2011), it is possible that AEBP1 contributes to mammary tumour initiation and/or progression. The consequence of excess AEBP1 expression in the stromal compartment was observed using transgenic mice ($AEBP1^{TG}$) in which an AEBP1 transgene driven by the fat-specific promoter/enhancer from the fatty acid-binding protein gene *aP2* (Makowski *et al*., 2001; Ross *et al*., 1990) results in overexpression of AEBP1 specifically in macrophages and adipocytes. In 30-32 week old female mice fed on a standard chow diet, whole mount analysis of mammary glands revealed epithelial cell hyperplasia in 29% of $AEBP^{TG}$ mice whereas non-transgenic mice ($AEBP1^{NT}$) did not develop hyperplasia. When the mice were given a 20-week high-fat diet, 100% of 30-week old $AEBP1^{TG}$ mice displayed mammary epithelial cell hyperplasia compared to only 20% in $AEBP1^{NT}$ mice (Holloway *et al*., 2012). Experimental evidence suggests that AEBP1 expression is induced in adipose tissue (Zhang *et al*., 2005), macrophages (Bogachev *et al*., 2011), and mammary tissue (unpublished) in mice while challenged on a high-fat diet. Furthermore, $AEBP1^{TG}$ mice exhibit increased levels of NF-κB activity and TNFα expression in mammary gland tissue and stromal macrophages. To determine whether AEBP1 expression in macrophages was responsible for these proinflammatory effects, $AEBP1^{NT}$ or $AEBP1^{TG}$ bone marrow was transplanted into irradiated $AEBP1^{NT}$ recipient mice. This allowed the expression of AEBP1 to be selectively altered exclusively in stromal macrophages since they are derived from circulating monocytes produced from the bone marrow. Both NF-κB activity and TNFα expression were increased in mammary gland tissue as a result of receiving $AEBP^{TG}$ bone marrow (Holloway *et al*., 2012), thus indicating that AEBP1 expression in

macrophages, but not adipocytes, is responsible for the up-regulation of proinflammatory factors that contribute to mammary epithelial cell hyperplasia. These findings illustrate that AEBP1 is a critical stromal factor that contributes to mammary epithelial cell hyperplasia and possibly tumourigenesis through promoting a proinflammatory microenvironment.

1.3. Sonic Hedgehog (Shh): A Critical Factor in Tumourigenesis

1.3.1. Shh Expression, Processing and Post-translational Modification

Shh is a signalling molecule initially found to be a critical factor in the development of tissues such as the nervous system and limbs (Ingham & McMahon, 2001). Out of the three hedgehog (Hh) ligands (Indian, Desert and Sonic), Shh has been implicated in the development and progression of tumours within various tissues, such as the pancreas, breast and prostate (Nakashima *et al*., 2006; Kasperczyk *et al*., 2009; Cui *et al*., 2010; Karhadkar *et al*., 2004). Furthermore, NF-κB (p65) was shown to transcriptionally activate the gene encoding Shh in inflammatory-stimulated macrophages (Nakashima *et al*., 2006). The Shh ligand is produced from a 45-kDa precursor protein that is cleaved in a cholesterol-dependent manner by proteolytic activity of the 26-kDa C-terminal domain to generate the 19-kDa N-terminal Shh ligand (ShhN). The C-terminal domain of Shh acts as a cholesterol transferase that covalently bonds a cholesterol molecule to the C-terminus of the N-terminal domain. Simultaneously, the covalent bonding of cholesterol releases the N-terminal domain at a conserved Gly-Cys-Phe motif thereby producing a cholesterol-modified ShhN (Porter *et al*., 1995, 1996). Skinny

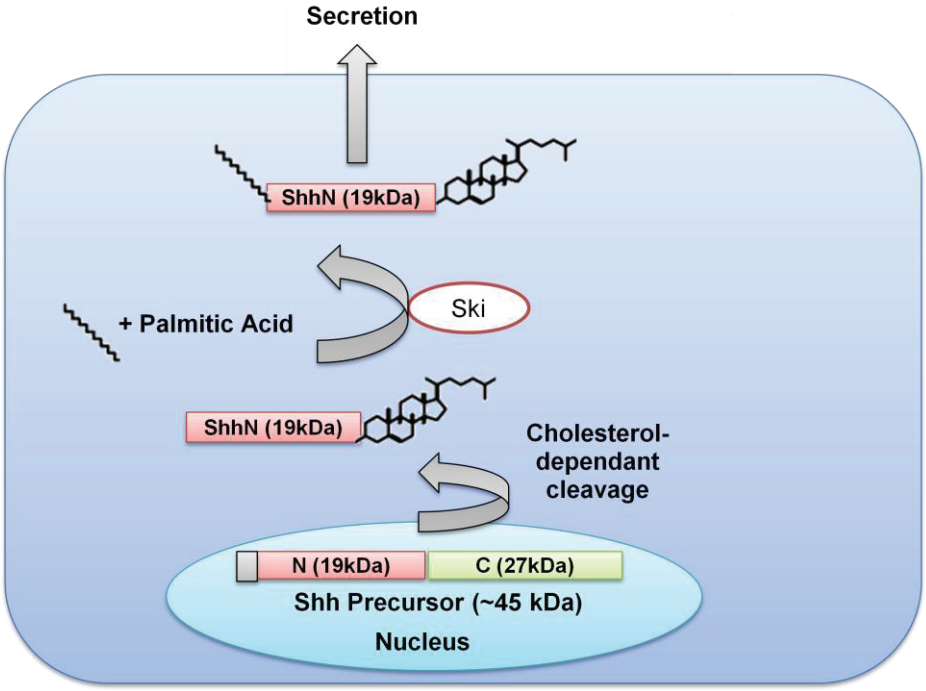

Figure 4. Diagram depicting the processing and post-translational modification of the Shh ligand. The Shh protein is produced as a 45-kDa precursor protein that undergoes cholesterol-dependent processing where the N-terminal domain (ShhN) is simultaneously cleaved off and post-translationally modified by the addition of cholesterol on the C-terminus of the N-terminal domain. The acyltransferase skinny hedgehog (Ski) provides a second post-translational modification by the addition of palmitic acid to the N-terminus of the N-terminal domain. Adapted from Daya-Grosjean & Couvé-Privat 2005, *Cancer Lett*.

hedgehog (Ski), an acyltransferase unrelated to the Hh ligand family, catalyzes a second modification, the addition of palmitic acid to a cysteine residue at the N-terminal end of ShhN (Chamoun *et al*., 2001). Both of these post-translational modifications are essential to the signalling function of ShhN (Farzan *et al*., 2008; Figure 4).

1.3.2. The Shh Signal Transduction Pathway

The Hh signalling pathway is a critical regulator of tissue morphogenesis and tumour progression in various tissues (Nakashima *et al*., 2006; Kasperczyk *et al*., 2009; Cui *et al*., 2010). In the absence of Hh ligands, the transmembrane receptor Patched (PTCH) prevents downstream Hh signalling by inhibiting the activity of the transmembrane hedgehog effector Smoothened (SMO). The Hh signalling pathway is activated in the presence of Hh ligands that bind and inactivate their receptor PTCH allowing activation of SMO. The activation of SMO prompts the release of the glioma-associated oncogene homolog (Gli) transcription factors to the nucleus from the hedgehog signalling complex (HSC) consisting of the serine/threonine kinase fused (FU), suppressor of FU (SUFU) and costal2 (COS2). Although the mechanism of Gli release is not completely understood, some suggest that SMO activation provokes a series of phosphorylation events that may cause FU or some other kinase to phosphorylate SUFU and COS2 (Wang *et al*., 2000; Ruel *et al*., 2007; Ingham *et al*., 2011). Phosphorylation of SUFU and COS2 releases Gli from the HSC and allows its nuclear translocation. Once inside the nucleus, the Gli transcription factors bind to promoters containing the Gli recognition sequence 'GACCACCCA', subsequently activating Hh-responsive genes such as *Gli1, Ptch1, and Hhip* (Figure 5; Kinzler & Vogelstein, 1990; Katoh & Katoh,

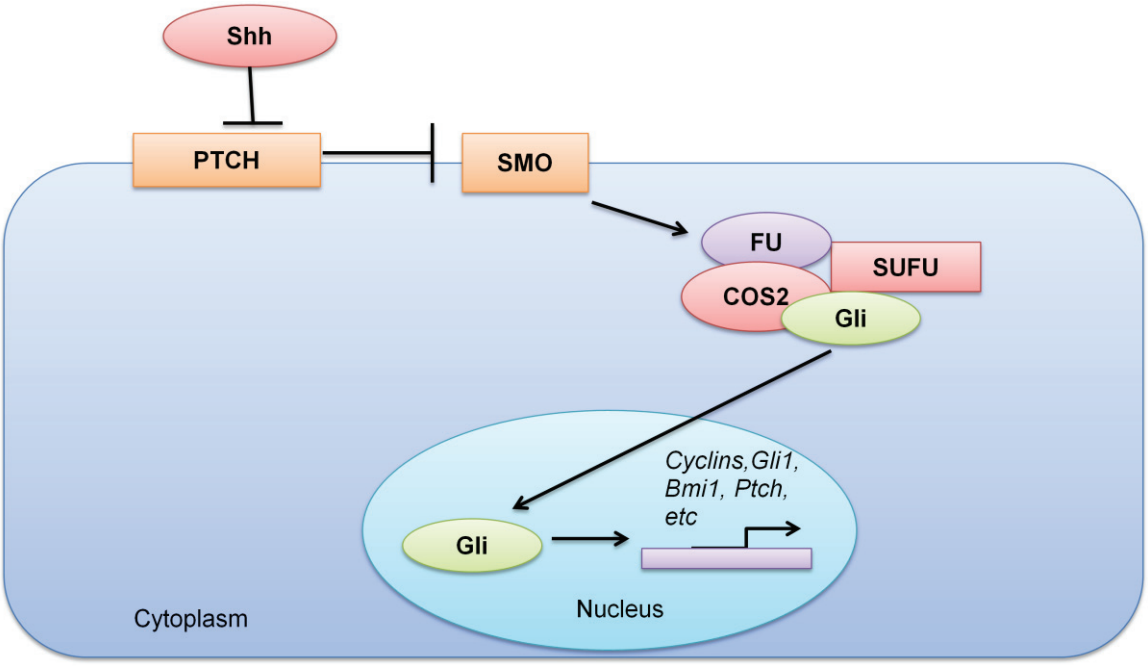

Figure 5. Diagram of the hedgehog signal transduction pathway. Sonic hedgehog (Shh) binds to its receptor patched (PTCH) to alleviate it inhibition of the effector smoothened (SMO). This allows the nuclear translocation of the Gli transcription factors from the hedgehog signalling complex (FU, SUFU, COS2). Once in the nucleus, Gli will activate the transcription of various genes involved in cell proliferation.

2009). Constitutive activation of Hh signalling is essential in tumour progression as it up-regulates genes involved in proliferation (e.g. cyclins), apoptotic resistance (e.g. Bcl-2), and epithelial-to-mesenchymal transition (e.g. Snail; Duman-Scheel *et al*., 2002; Bigelow *et al*., 2004; Pola *et al*., 2001; Li *et al*., 2006a). Although studies have shown that aberrant production of Shh originates from tumour cells (Nakashima *et al*., 2006; Fan *et al*., 2004), the production of Shh from NF-κB-stimulated stromal macrophages has also been reported to promote tumour growth in a paracrine manner (Yamasaki *et al*., 2010).

1.3.3. Gli1 and Bmi1 Induction in Tumourigenesis

The induction of Gli transcriptional activities is essential to tumour initiation and progression caused by aberrant Shh signalling (Dahmane *et al*., 2001; Sanchez *et al*., 2004). Shh-mediated activation of Gli transcription factors induces one of their own members, Gli1. Unlike Gli2 and Gli3, Gli1 expression is induced exclusively by the Hh ligands, thus making it a marker of Hh signalling activity (Ingham & McMahon, 2001). Similar to Shh, Gli1 is also a critical factor involved in development as well as tumour progression (Ruiz i Altaba, 1999; Fiaschi *et al*., 2009; Thomas *et al*., 2011). Overexpression of nuclear Gli1 has been observed in human breast carcinomas and breast cancer cells lines and often correlates with unfavorable prognosis (ten Haaf *et al*., 2009; Xu *et al*., 2012; Thomas *et al*., 2011) as well as Shh overexpression (ten Haaf *et al*., 2009). Fiaschi *et al*. (2009) have demonstrated that the transgenic overexpression of Gli1 directed by the mouse mammary tumour virus (MMTV) promoter in mammary epithelial cells results in defective TEBs, ductal hyperplasia, and tumour formation. Sanchez *et al*. (2004) found that growth of metastatic prostate cancer cell lines is reduced

when Gli1 expression is inhibited by RNA interference or treatment with the Hh pathway inhibitor cyclopamine, thus signifying that the induction of Gli1 is vital to Hh-mediated tumourigenesis.

In addition to Gli1, activation of the Hh signalling pathway also induces the oncogene Bmi1 (B-cell-specific Moloney murine leukaemia virus integration site 1), a member of the polycomb group (PcG) family of transcriptional repressors (Lui *et al*., 2006). The PcG family members are crucial in regulating stem cell self-renewal (Jacobs *et al*., 1999; Lui *et al*., 2006) through repression of cell cycle inhibitors produced from the cyclin-dependent kinase inhibitor 2A (*Cdkn2a*) tumour suppressor locus (Jacobs *et al*., 1999). However, dysregulation of PcG activities can influence tumour development and progression (Lui *et al*., 2006; reviewed in Bracken & Helin, 2009). This family of proteins are involved in the formation of the multi-component complexes polycomb repressive complex 1 and 2 (PRC1 and 2), which are responsible for the epigenetic repression of the *Cdkn2a* locus (Jacobs *et al*., 1999; Satijn *et al*., 2001) and HOX genes (Cao *et al*., 2005). The core of PRC1 usually consists of the proteins Bmi1, RING1a/b, polycomb (HPC), polyhomeotic (HPH), and posterior sex combs (PSC). The core of PRC2 consists of enhancer of zeste-2 (EZH2), ying yang 1 (YY1), suppressor of zeste 12 (SUZ12), and embryonic ectoderm development (EED; Levine *et al*., 2004). In order to repress transcription, PRC2 must first tri-methylate histone 3 on lysine 27 or to a lesser extent lysine 9 (Cao *et al*., 2002; Czermin *et al*., 2002). Histone 3-methylation enables the recruitment of PCR1, which consequently impairs the SWI/SNF chromatin remodelling machinery and obstructs transcriptional initiation (Shao *et al*., 1999; Lavigne *et al*., 2004). The PcG gene Bmi1, a core component of PRC1 (Li *et al*., 2006), is often

overexpressed in human cancers (Vonlanthen *et al*., 2001; Kim *et al*., 2004; reviewed in Valk-Lingbeek *et al*., 2004). Studies have found aberrant Shh signalling responsible for the overexpression of Bmi1 during tumour progression. Lui *et al*. (2006) have demonstrated that the addition of the Shh ligand to clumps of stem cell-like mammary epithelial cells known as mammospheres induces Bmi1 expression and that the addition of the Hh inhibitor cyclopamine prevents this induction. They further showed that the overexpression of Gli1 or Gli2 in mammospheres also results in up-regulation of Bmi1. These studies illustrate that the Shh signalling pathway is vital to the induction of the oncogenes Gli1 and Bmi1, both of which promote tumour initiation and progression.

1.3.4. Repression of The *Cdkn2a* Tumour Suppressor Gene

Dysregulation of proteins involved in cell cycle control and survival are prerequisites for tumour initiation and progression. Often, cells that grow abnormally, as is seen in the case of hyperplasia, have a reduction or loss of cell cycle regulators that normally prevent excess proliferation. In particular, cell cycle inhibitors produced from the *Cdkn2a* locus are often down-regulated in tumours through aberrant repression by the PRC complexes (Jacobs *et al*., 1999). The *Cdkn2a* locus encodes several transcript variants of cell cycle inhibitors known as the INK4 proteins ($p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, $p19^{INK4D}$), which all differ in their first exons (Cánepa *et al*., 2007). *Cdkn2a* also encodes a structurally and functionally different protein from the INK4 family known as $p19^{ARF}$ ($p14^{ARF}$ in humans) via an alternative reading frame. The $p16^{INK4A}$ and $p19^{ARF}$ transcripts from the *Cdkn2a* locus share exons two and three, but the $p19^{ARF}$ transcript contains an alternate first exon with its own promoter located 20-kb upstream of the first

exon of $p16^{INK4A}$ (Quelle *et al*., 1995). The $p16^{IKN4A}$ protein inhibits cell cycle progression in the early G1 phase by physically binding to cyclin-dependent kinase (CDK) 4 and 6, thus preventing them from forming a complex with cyclin D that allows the CDKs to phosphorylate retinoblastoma (Rb; Gil & Peters, 2006). By inhibiting the phosphorylation by CDK 4 and 6, $p16^{INK4A}$ allows Rb to remain associated with the transcription factor E2F, thereby preventing it from activating genes such as cyclin E and CDK2 that progress the cell cycle past the G1 phase (Pavletich, 1999; Gil & Peters, 2006). The ARF protein ($p14/19^{ARF}$) physically interacts with murine-double-minute 2 (MDM2) and sequesters it from promoting the ubiquitination and consequent degradation of p53. The ARF protein allows p53 transcriptional activation of the cell cycle inhibitor $p21^{CIP/WAF1}$ and various pro-apoptotic factors (Gil & Peters, 2006; Michael & Oren, 2003). Interestingly, $p16^{INK4A}$ and $p14^{ARF}$ are concomitantly repressed in primary breast tumours by genetic/epigenetic alterations (Silva *et al*., 2001, 2003, 2006). Overexpression of Bmi1 has been shown to down-regulate products from the *Cdkn2a* locus in prostate epithelial and carcinoma cells and enhance prostate tumour growth *in vivo* (Fan *et al*., 2008). The overexpression of Bmi1 in mouse embryonic fibroblasts has also been found to cause down-regulation of $p16^{INK4A}$ and $p19^{ARF}$ (Jacobs *et al*., 1999). Repression of $p16^{INK4A}$ by Bmi1 consequently allows progression of the cell cycle by hyperphosphorylation of Rb (Jacobs *et al*., 1999; Sherr, 2001). Furthermore, repression of $p19^{ARF}$ promotes MDM-2-mediated degradation of p53, thus impairing apoptosis and cell cycle arrest (Jacobs *et al*., 1999; Sherr, 2001). The effects resulting from the down-regulation of $p16^{INK4A}$ and $p14/19^{ARF}$ contribute to tumourigenesis by promoting aberrant cell proliferation and survival.

1.4. Rationale of Thesis

The role of AEBP1 in mammary gland tumourigenesis is beginning to be revealed as it appears to promote a proinflammatory microenvironment through its positive regulation of NF-κB activity in macrophages (Holloway *et al*., 2012). Shh signalling is associated with proinflammatory signalling during tumourigenesis as the transcription of the *Shh* gene is directly controlled of NF-κB (p65) in macrophages (Nakashima *et al*., 2006; Kasperczyk *et al*., 2009). Although AEBP1 enhances NF-κB activity and up-regulates many of its target genes, a relationship between AEBP1 and Shh has not been examined. Preliminary qPCR array analysis, using a panel of genes involved in transformation and tumourigenesis, revealed that several cell cycle inhibitors known to be repressed by the Shh-target gene Bmi1 were down-regulated in mammary epithelial cells from AEBP1^{TG} mice compared to AEBP1^{NT} mice. Because this suggested that Bmi1 was induced in the mammary epithelial cells of AEBP1^{TG} mice and that AEBP1 positively regulates NF-κB activity, this study set out to investigate whether AEBP1 may be a novel regulator of Shh expression in macrophages. My working hypothesis was that AEBP1-mediated production of Shh from macrophages might contribute to mammary epithelial cell hyperplasia observed in AEBP1^{TG} mice through induction of oncogenes specific to the hedgehog signalling pathway in mammary epithelial cells (Figure 6). Therefore, the purpose of my research has been to investigate the role of AEBP1 as a novel regulator of Shh signalling from macrophages and to analyze the tumourigenic effect(s) this could have on the mammary epithelial cells.

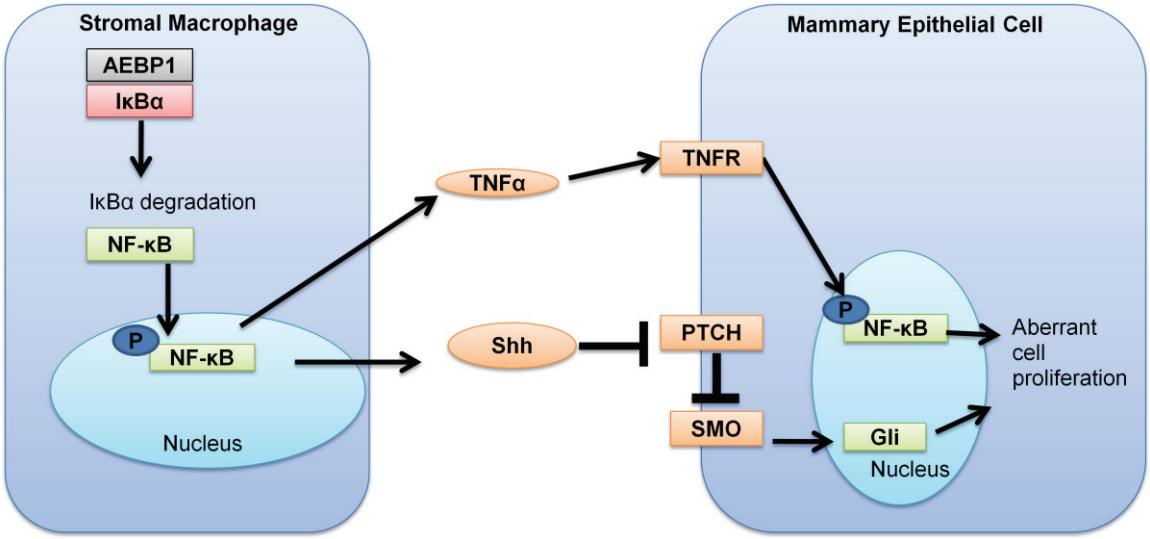

Figure 6. Model depicting the involvement of macrophage AEBP1 in the stromal-epithelial crosstalk in the mammary gland.

CHAPTER 2: MATERIALS AND METHODS

2.1. Materials

2.1.1. Reagents

Brewer's thioglycollate broth medium and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (Oakville, ON, Canada). Luminatata™ Crescendo Western HPR Chemiluminescence Substrate was purchased from Millipore (Billerica, MA, USA). DNA oligonucleotides, EXPRESS SYBR® GreenER™ qPCR SuperMix, high-glucose Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine sera (FBS), trypsin/EDTA, TRIzol® reagent, SYBR® safe DNA stain, MTT reagent, and penicillin-streptomycin were purchased from Invitrogen (Burlington, ON, Canada). All restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA). The 6X Orange DNA Loading dye and 1-kB DNA molecular mass marker were purchased from Thermo Fisher Scientific (Billerica, MA, Canada). GenePORTER3000™ transfection kit was purchased from Genlantis (San Diego, CA, USA). Collagenase Type I and Dispase II were purchased from Life Technologies (Burlington, ON, Canada)

2.1.2. Antisera

The Anti-AEBP1 polyclonal antibody, generated in chickens against recombinant mouse AEBP1 (amino acids 156-596), was purchased from Gallus Immunotech, Inc (Fergus, ON, Canada). Anti-β-actin antibody was purchased from Sigma–Aldrich (Oakville, ON, Canada). Anti-Shh, anti-Gli1 and anti-Bmi1 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Production of Plasmid DNA

2.2.1. Plasmids

Full-length murine AEBP1 cDNA was cloned into the pRC/CMV plasmid (Invitrogen) to generate the pRC/CMV-AEBP1 expression vector that constitutively expresses high levels of AEBP1 under the control of the CMV promoter (Wu, unpublished).

2.2.2. Transformation of Competent *E. coli* Cells

Competent DH5α *E. coli* (Invitrogen) were unthawed on ice. The plasmid to be amplified (200 ng) was added to 50 μL of *E. coli* cells, which were kept on ice for 30 minutes. The *E. coli* cells were heat shocked for 90 seconds at 42°C, immediately placed on ice, transferred to 1 mL of Luria-Bertani (LB) medium [1% (w/v) peptone, 0.5% yeast extract and 1% NaCl] containing 50 μg/mL ampicillin and incubated for 1 hour at 37°C with shaking (225 rpm). Subsequently, bacteria were screened on LB-agar plates (LB medium with 1.5% Bacto Agar) supplemented with 100 μg/mL ampicillin. The bacteria were incubated for 12-16 hours at 37°C to allow colony formation.

2.2.3. Plasmid DNA Preparation and Characterization

E. coli cultures were grown overnight at 37°C with shaking (225 rpm) in 500 mL LB medium supplemented with 100 μg/mL ampicillin. The next day, the bacterial cultures were centrifuged for 10 minutes at 5,000 x g and plasmid DNA was extracted from the cell pellets using the QIAprep® Spin Maxiprep Kit (Qiagen; Valencia, CA, USA) according to manufacturer's protocols. To confirm the identity and yield of

plasmids, plasmid DNA was digested by overnight incubation (at 37°C) with the appropriate restriction enzymes and the digested DNA was subsequently examined by agarose gel electrophoresis. Briefly, 10 μL of each digested DNA sample was combined with 2 μL of 6X Orange DNA Loading dye (Thermo Fisher Scientific) and resolved on a 0.8% (w/v) agarose gel containing SYBR® safe DNA stain (Invitrogen; 8 μL per 100 mL of agarose). A 1-kB DNA molecular mass marker (Thermo Fisher Scientific) was used on the gels to determine the size of the DNA fragments. After electrophoresis, DNA bands from agarose gels were examined using the Versa-Doc imaging system (Bio-Rad).

2.3. Culture and Transfection of Mammalian Cell Lines

2.3.1. Cell Culture

All murine cell lines used in this study (HC11 mammary epithelial cells, 4T1 mammary carcinoma cells and RAW264.7 macrophage cells) were cultured in complete DMEM medium [DMEM medium (Invitrogen) supplemented with 10% FBS (Invitrogen) and 1% penicillin-streptomycin antibiotic cocktail (Invitrogen)] under standard culture conditions (at 37°C in a humidified atmosphere and 5% CO_2). Cells were passaged at 60-80% confluency and maintained at an initial cell density of 1 x 10^6 cells in 10 cm culture plates. RAW264.7 cells were detached using a cell scraper in 5 mL of complete DMEM medium. HC11 and 4T1 cells were detached by coating the cells with 1 ml of 0.05% trypsin-EDTA (Invitrogen) and then immediately aspirating the trypsin/EDTA. The cells were subsequently incubated for 5 minutes under standard culture conditions and rinsed from the plate using 5 mL of complete DMEM medium.

2.3.2. Transient Transfection

RAW264.7 macrophage cells were seeded in 12-well plates (3.0×10^5 cells/well) and transfected the following day using the GenePORTER3000™ transfection kit (Genlantis) in serum-free OPTI-MEM medium (Invitrogen), according to manufacturer's protocols. First, culture medium was replaced with 1 mL of serum-free OPTI-MEM medium. Next, the transfection reagent mix was prepared from 14 μl of the GP3K transfection reagent and 56 μL of serum-free OPTI-MEM (per well). A DNA dilution was then prepared from 1 μg of plasmid DNA in 150 mL of diluent (per well). Both the transfection reagent mix and the DNA dilution were incubated at room temperature for 5 minutes. The transfection reagent mix was then combined with the DNA dilution, incubated for another 5 minutes to form the transfection-DNA complexes. The transfection-DNA mix was then added to the cells with gentle swirling and subsequently incubated under standard culture conditions for 4 hours. Afterwards, each well was supplemented with an equal volume of OPTI-MEM containing 10% FBS and cells were incubated for ~24 hours.

2.4. Mouse Models

2.4.1. Handling and Maintenance

Mice used in this study were FVB/NJ or NOD/SCID mice. Age-matched mice were kept on a 12-hour light cycle in air-conditioned rooms in the Carleton Animal Care Facility at Dalhousie University where they were fed and watered *ad libitum*. Standard rodent chow was used. NOD/SCID mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained in microisolators under specific

pathogen-free conditions. Mice were sacrificed by CO_2 asphyxiation and used to isolate tissues and peritoneal macrophages for protein or RNA analysis. All animal protocols have been approved by the University Committee on Laboratory Animals (UCLA).

2.4.2. Generation of AEBP1 Transgenic (AEBP1^{TG}) Mice

AEBP1^{TG} mice were generated by integrating an AEPB1 transgene, a murine AEBP1 cDNA fragment of 2.6 kb, under the control of the fat-specific promoter/enhancer (Ross *et al*., 1990) from the fatty acid-binding protein gene, *aP2*. The *aP2* promoter/enhancer specifically overexpresses AEBP1 in macrophages and allows persistent AEBP1 expression in adipocytes (Makowski *et al*., 2001). The AEBP1 transgene plasmid was digested using *HindIII* and *NdeI* to release the 8.7-kb aP2-AEBP1 transgene for injection. The construct was injected into the pronucleus of fertilized zygotes from FVB/NJ mice and transferred to pseudo-pregnant females. A total of 35 pups were produced from pseudo-pregnant mice that had blastocysts injected with the AEBP1 transgene construct. Mice positive for the transgene were determined by PCR analysis of tail DNA using the forward primer in exon 18 (5'-GGA CTA CAC CAG CGG CAT GG-3') and the reverse primer from exon 21 (5'-GCG TGA GCT GTC ACA CGG TA-3'). This primer pair amplifies a 360 bp fragment of cDNA and ~900 bp genomic sequence. Out of the 35 pups, PCR analysis of tail DNA indicated that only 8 acquired the transgene.

2.4.3. Genotyping

Approximately 5 mg of mouse ear tissue (collected by ear puncher) was used to extract DNA with the REDextract-N-Amp Tissue PCR Kit (Sigma) according to the manufacturer's recommendation. In a 10 µl reaction volume, a DNA sample (5 μl) was used for amplification using the following primers: GT-sense, 5'-CAA TGG CTA CGA GGA AAT G-3'; GT-anti-sense, 5'-GTG CGT AGT AGC TGT AGA CAG-3'; anti-SK, 5'-GGG GAT CCA CTA GTT CTA AGA-3'. The MJ Research PTC-100 Thermal Cycler (Scientific Support; Hayward, CA, USA) was used for amplification. PCR commenced at 95°C (4 min), followed by 35 cycles at 95°C (30 sec), 54°C (1 min), and 72°C (1 min), and finally 72°C (10 min). Subsequently, 1 μl of 10X loading buffer was added to PCR samples, which were resolved on 1% agarose gel at 70 V. The expected DNA fragments for the different genotypes were obtained: A 400 bp fragment for $AEBP1^{NT}$ and 400 bp and 200 bp fragments for $AEBP1^{TG}$.

2.5. Primary Cell Isolation and Culture

2.5.1. Thioglycollate-Elicited Peritoneal Macrophages

Mice were injected intraperitoneally with 3 ml sterile 4% Brewer's thioglycollate broth medium (Sigma–Aldrich). Five days later, mice were sacrificed and peritoneal cells were isolated by lavage using 10 mL of cold, complete DMEM medium. Lavage fluids were centrifuged at 1,500 rpm for 10 minutes at 4°C and supernatant was removed. The resulting cell pellets were resuspended in complete DMEM. Macrophages were further purified by allowing cells from the lavage fluids to adhere to the culture plate (10 cm) for ~16 hrs under standard culture conditions, at which time media was replaced to remove the non-adherent, non-macrophage cells. Table 1. = removed

2.5.2. Peritoneal Macrophages and Mammary Epithelial Cell Co-Culture

For co-culture experiments, HC11 cells (0.6-1.0 x 10^5 per well) were seeded to 6-well culture plates (bottom chamber) in complete DMEM medium. The following day, culture inserts (transparent/translucent PET membrane, 1 μm pore size; BD Biosciences) were seeded with $AEBP1^{NT}$ or $AEBP1^{TG}$ peritoneal macrophages (2-2.5 x 10^5) and placed onto each well containing HC11 cells. Macrophages and HC11 cells were co-cultured for 24 hours. HC11 cells were subsequently lysed for immunoblot analysis.

2.5.3. Mammary Epithelial Cell Isolation

Mammary gland tissues were excised, minced into small pieces, and then incubated in a filter-sterilized collagenase/dispase (Life Technologies) tissue disassociation solution [2 U/mL Dispase II, 200 U/mL Collagenase Type I, 2.5 mL filter-sterilized FBS, and 5 μg/mL gentamycin within DMEM medium] for 2 hrs at 37°C with shaking (225 rpm). Mammary gland cells were collected by centrifugation at 1500 rpm for 10 minutes at 4°C. Mammary epithelial cells were isolated using the magnetic EasySep Mouse Epithelial Cell Enrichment Kit from StemCell Technologies (Vancouver, BC, Canada) according to manufacturer's protocols.

2.6. AEBP1 Knock-Down in Macrophages Via Morpholino Antisense Oligo

Morpholino antisense oligo constructs were designed to target the translation initiation site of the murine AEBP1 transcript (Ro *et al.*, 2001). AEBP1-

antisense morpholino (AEBP1-MO: 5'-TGT CCT CAA TGC GGT GTG ACT CCA T-3') and the non-specific control morpholino (CONT-MO: 5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3') were synthesized by Gene Tools (Philomath, OR, USA). After removing media, complete DMEM medium containing morpholino oligos (10 μM) was added to peritoneal macrophages from AEBP1^{NT} mice. Endo-Porter (Gene Tools) was subsequently added to cells as the delivery reagent (Final concentration of 6 μM), which were incubated for ~24 hours under standard culture conditions.

2.7. Cell Proliferation Assay of HC11 Cells Co-Cultured with Macrophages

HC11 cells were seeded in 24-well plates at a density of 6.0-8.0 x 10^3 cells/well. The following day, AEBP1$^{NT \text{ and } TG}$ peritoneal macrophages were seeded into cell culture inserts placed into each well (1.0 x 10^4 cells per insert). Growth of the HC11 cells was monitored daily over 4 days by first detaching cells with trypsin/EDTA and then counting them using a haemocytometer.

2.8. MTT Assay

HC11 cells were seeded in 96-well plates at a density of 1.0 x 10^3 cells/well and incubated overnight under standard culture conditions. The following day, culture media of HC11 cells was replaced with 200 μL of conditioned culture media from AEBP1^{NT} or ^{TG} peritoneal macrophages collected 24 hours after seeding (5 x 10^6 cells per 60 mm plate). The proliferation/viability of HC11 cells was assessed over 4 days using the colourimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, where cell viability/proliferation is assessed by their ability to enzymatically

reduce the MTT salt to formazan. Briefly, 20 μL of MTT reagent [25 mg MTT (Invitrogen) in 5 mL 1X PBS (pH 7.4, filter sterilized)] was added to HC11 cells and incubated for 4 hours under standard culture conditions. Afterwards, media was removed and 200 μL of lysis solution [99.4% (v/v) DMSO, 0.6% (v/v) Acetic Acid, 10% (w/v) SDS] was added with mixing. Absorbance of each sample was measured at a wavelength of 595 nm using the using the Model 3550 Microplate Reader (Bio-Rad).

2.9. *In vivo* Mammary Tumour Growth

Peritoneal macrophages from AEBP1$^{NT \text{ and } TG}$ mice were cultured for ~24 hours in complete DMEM medium. The following day, macrophages were harvested, counted and co-injected (5 x 10^5 cells) with 4T1 cells (5 x 10^5 cells) into the subcutaneous flanks of the mammary fat pads of NOD/SCID mice. Tumour growth was assessed every three days for 21 days using caliper measurements to determine tumour volume. The mammary tumours were excised on the 21st day.

2.10. Protein Expression Analysis

2.10.1. Preparation of Cell and Mammary Gland Tissue Lysates

Cells were washed three times with ice cold 1X PBS and lysed in cold RIPA buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.25% sodium deoxycholate, 1% triton X-100, 1 mM phenylmethysulfonyl fluoride (PMSF), 1mM dithiothreitol (DTT), 1 mM Na_3VO_4, 1 mM NaF, 5 mM EDTA, and complete EDTA-free protease inhibitor cocktail (Roche Applied Science; Laval, QC, Canada)]. The lysates were collected from culture plates using a cell scraper and transferred to microcentrifuge tubes, and then snap frozen in liquid nitrogen for future use. After unthawing on ice, cell lysates were

centrifuged at 14,000 rpm for 20 minutes at 4°C. Murine mammary gland tissue extracts were prepared by homogenizing tissues in 500 μL cold high salt buffer [500 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1% triton X-100, 2 mM EDTA, 1mM DTT, 0.1 mM PMSF, 1 mM Na_3VO_4, 1 mM Na_2MoO_4, and complete EDTA-free protease inhibitors cocktail] and then incubating on ice for 30 minutes. The tissue lysates were collected by centrifugation at 14,000 rpm for 30 minutes at 4°C and the supernatant was collected and centrifuged once more.

2.10.2. Protein Concentration Determination

Protein concentration was determined by the Bio-Rad Protein Assay (Bradford, 1976) where 1 μL of protein lysate was added to 799 μL ddH_2O and 200 μL of Bradford protein assay dye (Bio-Rad). A negative control was prepared from 800 mL ddH2O and 200 μl of protein assay dye. Absorbance of the samples was measured at A_{595} using the SmartSpec™ 3000 Spectrophotometer (Bio-Rad). Protein concentration was calculated from a protein standard curve obtained based on known concentrations of BSA (Sigma-Aldrich).

2.10.3. SDS-PAGE and Immunoblot Analysis

Whole cell protein extracts (~20-40 μg) were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the Mini-PROTEAN® 3 Electrophoresis Cell (Bio-Rad). First, protein extracts were mixed with sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, 0.01% bromphenol blue] and denatured by heating at ~95°C for 4 minutes. The samples were

resolved on 8.5% or 10% polyacrylamide gels with the Full-Range Rainbow™ Molecular Weight Marker (GE Healthcare, Munich, Germany) to determine the molecular weights. Electrophoresis was performed at 100 V for approximately 1.5-2 hours in 1X Electrode (Running) Buffer [25 mM Tris base, 192 mM glycine, 0.1 % SDS, pH 8.3].

Following SDS-PAGE electrophoresis, proteins were transferred onto PVDF membranes purchased from VWR (Mississauga, ON, Canada) using the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad). First, PVDF membranes were briefly immersed in 100% methanol and then both the gel and PVDF membrane were soaked in cold transfer buffer [25 mM Tris-HCl, 192 mM glycine, 20% w/v methanol, pH 8.3] for 15 minutes. Proteins were electro-transferred onto the membranes at 100 V for one hour in cold transfer buffer. After the transfer was complete, membranes were briefly washed twice with Tris-buffered saline Tween-20 [1X TBST; 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Tween-20], and then incubated for one hour at room temperature in blocking buffer [5% Carnation® fat-free milk powder (Smucker Foods of Canada Corp., ON, Canada) in 1X TBST] with gentle shaking. The membranes were subsequently washed for 5 minutes in 1X TBST three times and then incubated overnight with shaking at 4°C using specific antibodies in either blocking buffer or 3% BSA in 1X TBST. Table 2 provides a list of the primary antibodies used for immunoblot analysis. The following day, membranes were washed three times with 1X TBST for 5 minutes each and then incubated using the appropriate HRP-linked secondary antibody (at the appropriate dilution in blocking buffer) for one hour at room temperature with gentle agitation. Afterward, the membranes were washed three times with 1X TBST for 5

Table 1. A List of the Primary Antibodies used in Immunoblot Analysis.

Antibody	Working Dilution	Company
Anti-AEBP1	1: 200,000	Gallus Immunotech
Anti-Shh	1: 500	Santa Cruz
Anti-Gli1	1: 1000	Santa Cruz
Anti-Bmi1	1: 1000	Santa Cruz
Anti-β-actin	1: 10,000	Sigma

minutes each and then incubated for 3 minutes using the Luminata™ Crescendo Western HRP substrate (Millipore). The chemiluminescent signals were captured by exposure to KODAK scientific imaging film (Eastman KODAK Company, Rochester, NY) for an appropriate length of time. The bands appearing on the film were quantified based on densitometric analysis using the program ImageJ (www.rsb.info.nih.gov/ij/), normalized to β-actin and represented as a bar graph. Blots that were analyzed using several different antibodies were first washed in mild stripping buffer [200 mM glycine, 0.1% SDS, 1% w/v Tween-20, pH 2.2] twice for 10 minutes each, then in 1X phosphate-buffered saline solution [137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, and 2 mM KH_2PO_4, pH 7.4] twice for 10 minutes each, then in 1X TBST twice for 5 minutes each, and then the blots were incubated for one hour in blocking buffer. After re-blocking, the membranes were washed three times in 1X TBST for 5 minutes each before incubating with other primary antibodies.

2.11. Quantitative PCR (qPCR)

2.11.1. Total RNA isolation and cDNA synthesis

Total RNA was extracted from mammary epithelial cells isolated from murine mammary tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction. Briefly, cells were lysed by resuspending cell pellets in TRIzol (1 mL per 1.0 x 10^7cells, 800 μL for less than 1.0 x 10^7 cells). Cell lysates were subsequently passed through a 25-gauge needle 3-4 times. Chloroform was added to the cell lysates (200 μL for every 1 mL of TRIzol, 160 μL for 800 μL of TRIzol), shaken vigorously by hand, kept for 3 minutes at room temperature, and then

centrifuged for 10 min at 13,000 x g at 4°C. Total RNA was purified from the top aqueous phase of TRIzol extracts using the RNeasy Mini Kit (Qiagen). Total RNA concentration was determined by using a 1:100 dilution in ddH2O, and measured using the SmartSpec™ 3000 spectrophotometer (Bio-Rad). Purity of total RNA was determined by the A260/A280 ratio. The cDNA was directly synthesized from total RNA (1 μg) using the QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer's instruction.

2.11.2. Quantitative Polymerase Chain Reaction (qPCR) Analysis

Using 30 ng/μl of cDNA, the SYBR GreenER qPCR master mix (Invitrogen) and the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA), the genes of interest were amplified with the following primer sets: murine *Gli1*, 5'-TTC GTG TGC CAT TGG GGA GGT T-3' and 5'-TCT TCA CGT GTT TGC GGA GCG A-3'; *Bmi1*, 5'-GCC GCT TGG CTC GCA TTC ATT T-3' and 5'-ACC CTC CAC ACA GGA CAC ACA T-3'; $p16^{INK4A}$, 5'-TCA ACT ACG GTG CAG ATT CG-3' and 5'-AAA GCC ACA TGC TAG ACA CG-3'; $p19^{ARF}$, 5'- TTC TTG GTG AAG TTC GTG CG -3' and 5'- AAT CTG CAC CGT AGT TGA GC-3'; murine *β-actin*, 5'-GAC GGC CAG GTC ATC ACT AT-3' and 5'-GAA AGG GTG TAA AAC GCA GC-3'. All primers were validated though temperature gradient analysis, determining the primer efficiencies from a standard curve using serial dilutions of a DNA template, and the amplicons were verified using melting curve analysis (Bustin *et al.,* 2009). Table 2 provides a list of the primers used for qPCR analysis. Relative gene expression was

Table 2. List of Forward (Fwd; 5' to 3') and Reverse (Rvs; 5' to 3') Primers used for qPCR Analysis.

Target Gene & Reference Sequence	**Primer Set**	**Amplicon Size (bp)**	**Primer Efficiency**
Gli1 (NM_010296.2)	Fwd - TTCGTGTGCCATTGGGGAGGTT	352	100.9%
	Rvs - TCTTCACGTGTTTGCGGAGCGA		
Bmi1 (NM_007552.4)	Fwd - GCCGCTTGGCTCGCATTCATTT	185	85%
	Rvs - ACCCTCCACACAGGACACACAT		
$p16^{INK4A}$ (NM_010296.2)	Fwd - TCAACTACGGTGCAGATTCG	413	71.6%
	Rvs - AAAGCCACATGCTAGACACG		
$p19^{ARF}$ (NM_009877.2)	Fwd - TTCTTGGTGAAGTTCGTGCG	182	89.4%
	Rvs - AATCTGCACCGTAGTTGAGC		
β-actin (NM_007393.3)	Fwd – GACGGCCAGGTCATCACTAT	434	91.1%
	Rvs – GAAAGGGTGTAAAACGCAGC		

evaluated using the comparative Ct method ($^{\Delta\Delta}$Ct method; Livak & Schmittgen, 2001) and normalized to β-actin expression.

2.12. Statistical analysis

Data is expressed as the mean ± S.E.M of the indicated number of samples. Statistical significance was determined using the Student *t*-test for un-paired observations, where $*P < 0.05$ and $***P < 0.001$ are considered statistically significant.

CHAPTER 3: RESULTS

3.1. AEBP1 Positively Regulates Shh Expression in Macrophages

3.1.1. Shh Expression is Up-Regulated in Macrophages From AEBP1^{TG} Mice

Transcription of the *Shh* gene is directly activated by NF-κB (p65) in macrophages (Nakashima *et al*., 2006; Kasperczyk *et al*., 2009). Since AEBP1 enhances NF-κB activity in macrophages (Majdalawieh *et al*., 2006, 2007), elevated AEBP1 expression might therefore cause an increase in Shh expression. To determine if there was a positive correlation between AEBP1 and Shh expression, the level of AEBP1 and Shh proteins in peritoneal macrophages harvested from nulliparous AEBP1$^{NT \text{ and } TG}$ female mice (~8-10 weeks old) was assessed. Mice received an intraperitoneal injection of 4% Brewer's thioglycollate (3 mL/ mouse) and peritoneal exudates were harvested 5 days later by lavage using cold, complete medium (10 ml/ mouse). Each of the peritoneal exudates was incubated (10 cm dish/ mouse) under standard culture conditions for ~16 hrs, and culture media was subsequently replaced to remove non-adherent, non-macrophage cells. Protein extracted from the adherent macrophages was subjected to SDS-PAGE followed by immunoblotting for AEBP1, Shh, and β-actin. Densitometric analysis of immunoblots indicated that the level of the Shh precursor (~45-kDa) was ~5-fold higher in the thioglycollate-elicited peritoneal macrophages from AEBP1^{TG} mice compared to those isolated from AEBP1^{NT} mice (Figure 7). The increased level of Shh protein in AEBP1^{TG} macrophages suggests that AEBP1 is a positive regulator of Shh expression.

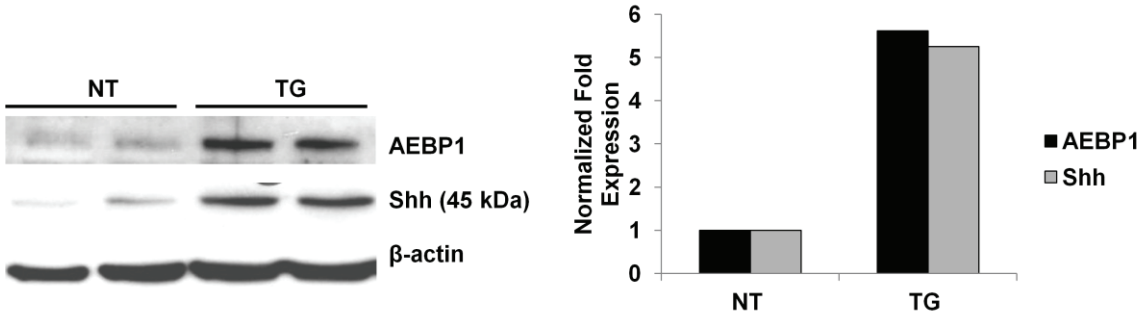

Figure 7. Elevated Shh protein levels in AEBP1^{TG} thioglycollate-elicited peritoneal macrophages. Whole cell protein extracts (20 μg) from peritoneal macrophages of 8-10 week old AEBP1$^{NT\ and\ TG}$ female mice were isolated, resolved on 10% polyacrylamide gels and subjected to immunoblot analysis for AEBP1, Shh, and β-actin. Two samples from each genotype are shown. The expression of the indicated proteins was normalized based on β-actin expression.

3.1.2. Shh Expression is Up-Regulated in RAW264.7 Macrophage Cells Transiently Overexpressing AEBP1

Although constitutive overexpression of AEBP1 in peritoneal macrophages appears to cause an up-regulation of Shh protein levels, the effect of transient AEBP1 overexpression on Shh expression was also examined using a macrophage cell line. RAW264.7 macrophage cells were transiently transfected in 12-well plates with the GenePorter3000™ transfection reagent using equal DNA amounts of the AEBP1-expression vector pRc/CMV-AEBP1 or an empty vector control (pRc/CMV). Protein extracts isolated from transfected RAW264.7 macrophage cells were subjected to SDS-PAGE followed by immunoblotting for AEBP1, Shh, and β-actin. Consistent with the data from peritoneal macrophages, densitometric analysis of immunoblots indicated that the overexpression of AEBP1 (~4-fold) in RAW264.7 macrophage cells results in a ~3-fold increase of Shh precursor protein steady state levels compared to the level in control cells (Figure 8). The increase in Shh protein levels resulting from AEBP1 overexpression further indicates that AEBP1 is a positive regulator of Shh expression in macrophages.

3.1.3. Knock-Down of AEBP1 using Morpholino Antisense Oligos Reduces Shh Expression in Macrophages

If AEBP1 is essential for activating Shh expression in macrophages, then reducing AEBP1 expression should lead to lower Shh protein levels. To determine whether AEBP1 is an essential mediator of Shh expression, AEBP1 gene expression in peritoneal macrophages isolated from nulliparous $AEBP1^{NT}$ female mice (~8-10 weeks old) was knocked-down by addition of 10 μM of AEBP1-specific morpholino antisense

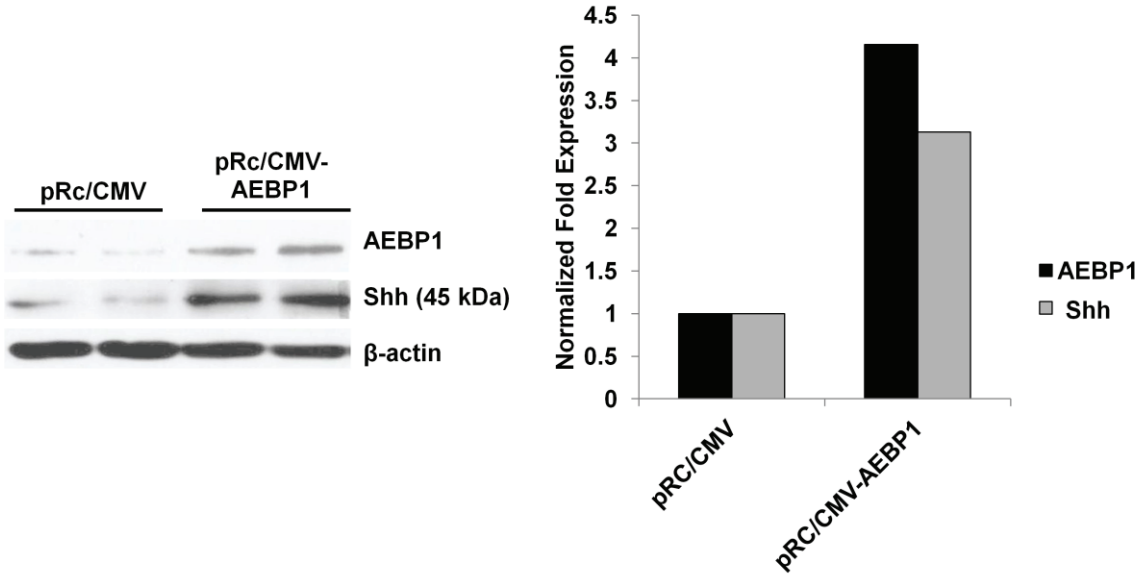

Figure 8. Elevated Shh expression in RAW264.7 macrophage cells transiently overexpressing AEBP1. RAW264.7 macrophage cells were transfected in 12-well plates using 0.5 μg/well of pRc/CMV-AEBP1 or pRc/CMV-empty vector as the control. Four hours after adding the transfection reagent, an equal volume of OPTI-MEM medium containing 10% FBS was added to each well and cells were incubated for 24 hrs under standard culture conditions. Whole cell protein extracts (30 μg) were isolated, resolved on 8.5% polyacrylamide gels and subjected to immunoblot analysis for AEBP1, Shh, and β-actin. Two samples from each transfection are shown. The expression of the indicated proteins was normalized based on β-actin expression.

oligos or a non-specific control morpholino using Endo-Porter as the delivery agent. After ~24 hr, protein was isolated from the morpholino-treated peritoneal macrophages and subjected to SDS-PAGE followed by immunoblotting for AEBP1, Shh, and β-actin. Densitometric analysis of immunoblots revealed that Shh precursor protein levels were decreased (~5-fold) in peritoneal macrophages when endogenous AEBP1 levels were reduced (~2.5-fold) by morpholino knock-down (Figure 9). The down-regulation of Shh resulting from reduced AEBP1 expression suggests that AEBP1 may be an essential mediator of Shh expression in macrophages.

3.2. Macrophage AEBP1 Promotes Shh Pathway Activity in Mammary Epithelium

3.2.1. Mammary Epithelial Cells of AEBP1^{TG} Mice Have Increased mRNA Levels of *Gli1* and *Bmi1*

The findings above show that the precursor protein level of Shh is increased when AEBP1 is overexpressed in macrophages; however, this does not indicate that AEBP1 promotes Shh signalling activity via increased secretion of the processed form of Shh (ShhN). If the overexpression of AEBP1 in macrophages does increase ShhN secretion, there should be induction of Shh-specific genes in the target cells. Since Gli1 is induced exclusively by Shh, it can be used as an indicator of Shh signalling activity (Ingham & McMahon, 2001). To investigate whether AEBP1 promotes Shh signalling activity in the mammary gland, total RNA was isolated from mammary epithelial cells of nulliparous AEBP1$^{NT \text{ and } TG}$ female mice (~8-10 weeks old) and transcript levels of *Gli1* and *β-actin* were analyzed using qPCR. Compared to AEBP1^{NT} mice, qPCR analysis revealed that the level of *Gli1* mRNA in mammary epithelial cells from AEBP1^{TG} mice was up-regulated by ~4-fold (Figure 10A). Up-regulation of *Gli1*

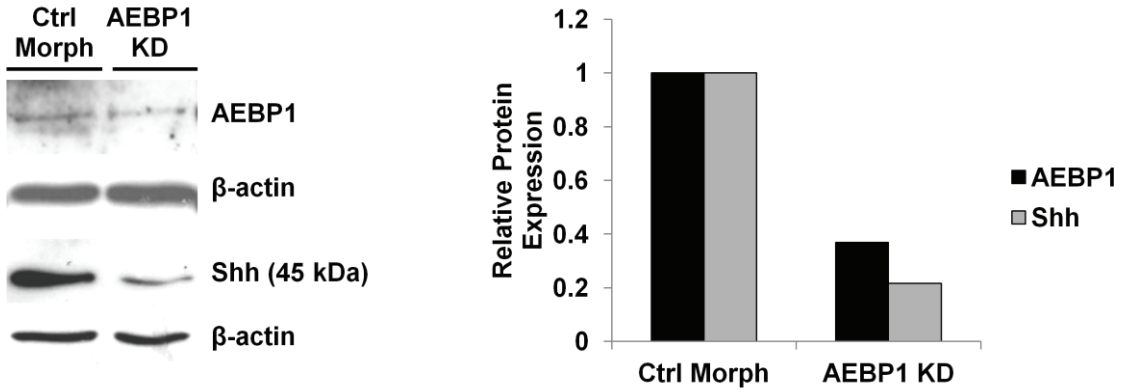

Figure 9. Morpholino knock-down of AEBP1 expression in thioglycollate-elicited peritoneal macrophages reduces Shh expression. Peritoneal macrophages from 8-10 week old AEBP1^{NT} female mice were cultured for 24 hrs in serum-free media containing 6 μM of Endo-Porter and 10 μM of AEBP1-antisense morpholino (AEBP1 KD) or control morpholino (Ctrl Morph). Whole cell protein extracts (20 μg) were isolated, resolved on 10% polyacrylamide gels and subjected to immunoblot analysis for AEBP1, Shh, and β-actin. One sample from each treatment is shown. The expression of the indicated proteins was normalized based on β-actin expression.

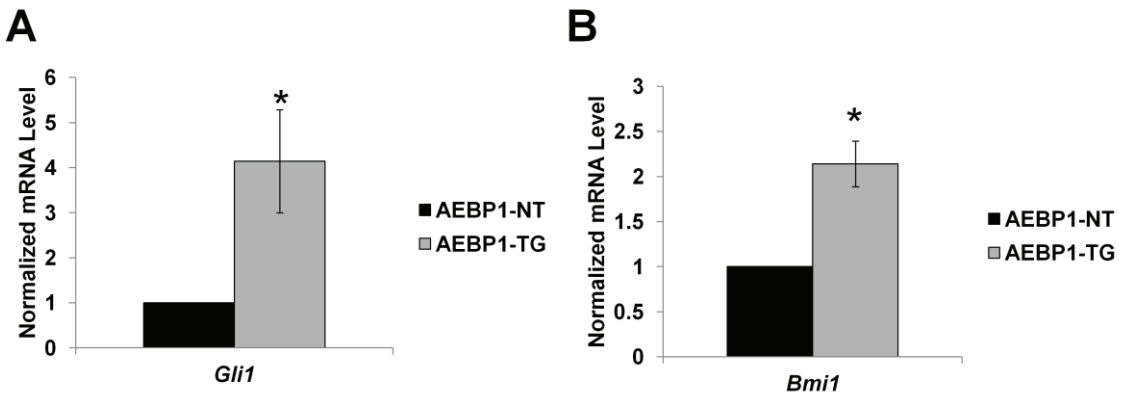

Figure 10. Elevated *Gli1* and *Bmi1* mRNA levels in mammary epithelial cells isolated from AEBP1^{TG} mice. Total RNA from mammary epithelial cells isolated from pooled mammary tissue of 8-10 week old AEBP1$^{NT\ and\ TG}$ mice (n = 4-5) was extracted and used for cDNA synthesis. The relative expression of (A) *Gli1* and (B) *Bmi1* mRNA levels was determined from cDNA by qPCR analysis and normalized to β-actin levels. Data are represented as mean ± SEM (n = 3), where *P < 0.05 is considered statistically significant

mRNA in AEBP1^{TG} mammary epithelial cells strongly suggests that AEBP1 expression in macrophages enhances Shh signalling.

To further assess whether AEBP1 expression in macrophages modulates Shh signalling, expression of another Shh-target gene *Bmi1* (Lui *et al*., 2006) in mammary epithelial cells was also examined. The qPCR analysis revealed a ~2-fold increase of *Bmi1* mRNA in AEBP1^{TG} mammary epithelial cells compared to the AEBP1^{NT} control (Figure 10B). Together, these results show the up-regulation of genes downstream of the Shh signalling pathway in mammary epithelial cells from mice that overexpress AEBP1 in macrophages. The up-regulation of Gli1 and Bmi1 in mammary epithelial cells suggests that AEBP1-overexpressing macrophages contribute to mammary epithelial cell hyperplasia through enhancing Shh signalling activities.

3.2.2. Mammary Epithelial Cells from AEBP1^{TG} Mice Have Reduced mRNA Levels of Tumour Suppressors *p16^{INK4A}* and *p19^{ARF}*

AEBP1^{TG} mice display elevated Shh expression in macrophages along with an up-regulation of the Shh-target gene Bmi1 in mammary epithelial cells. Furthermore, previous studies found that Shh-induction of Bmi1 expression in mammary epithelial cells leads to the repression of the p16^{INK4A} and p19^{ARF} tumour suppressor genes (Jacobs *et al*., 1999; Bishop *et al*., 2010). These findings suggest that induction of Bmi1 in AEBP1^{TG} mammary epithelial cells may cause a down-regulation of p16^{INK4A} and p19^{ARF}. To examine whether p16^{INK4A} and p19^{ARF} are down-regulated in mammary epithelial cells of AEBP1^{TG} mice relative to the levels in AEBP1^{NT} mice, total RNA was isolated from mammary epithelial cells and transcript levels of *p16^{INK4A}*, *p19^{ARF}*, and *β-actin* were analyzed using qPCR. In comparison to AEBP1^{NT} mammary epithelial cells,

qPCR analysis revealed ~2- and ~1.5-fold decreases in *p16*^{INK4A} and *p19*^{ARF} mRNA levels, respectively, in AEBP1^{TG} mammary epithelial cells (Figure 11). This indicates that AEBP1-specific overexpression in macrophages promotes p16^{INK4A} and p19^{ARF} down-regulation in mammary epithelial cells, potentially through Bmi1 induction. The down-regulation of these tumour suppressors in response to AEBP1-overexpression in macrophages may contribute to the development of mammary epithelial cell hyperplasia observed in AEBP1^{TG} mice (Holloway *et al*., 2012).

3.2.3. Gli1 and Bmi1 Expression is Induced in HC11 Mammary Epithelial Cells Co-Cultured with AEBP1^{TG} Macrophages

The up-regulation of *Gli1* and *Bmi1* mRNA levels in mammary epithelial cells of AEBP1^{TG} mice strongly suggests that AEBP1-overexpression in macrophages is involved in the induction of Shh signalling activity in mammary epithelial cells *in vivo*. If the induction of these Shh-target genes is due to crosstalk between these two cell types, Gli1 and Bmi1 expression should be induced in mammary epithelial cells cultured in the presence of AEBP1-overexpressing macrophages. To test this, Shh-target genes were assessed in HC11 mammary epithelial cells co-cultured with peritoneal macrophages from AEBP1^{NT} or AEBP1^{TG} female mice (~8-10 weeks old). HC11 cells seeded into 6-wells plates were cultured in the presence of AEBP1$^{NT\ and\ TG}$ macrophages contained in co-culture inserts placed into the wells. The inserts had a porous membrane that kept the HC11 cells separate but would allow passage of signalling factors released from the macrophages. Protein isolated from HC11 mammary epithelial cells co-cultured with peritoneal macrophages was subjected to SDS-PAGE and Gli1, Bmi1, and β-actin expression was assessed by immunoblot analysis. Densitometric analysis of immunoblots

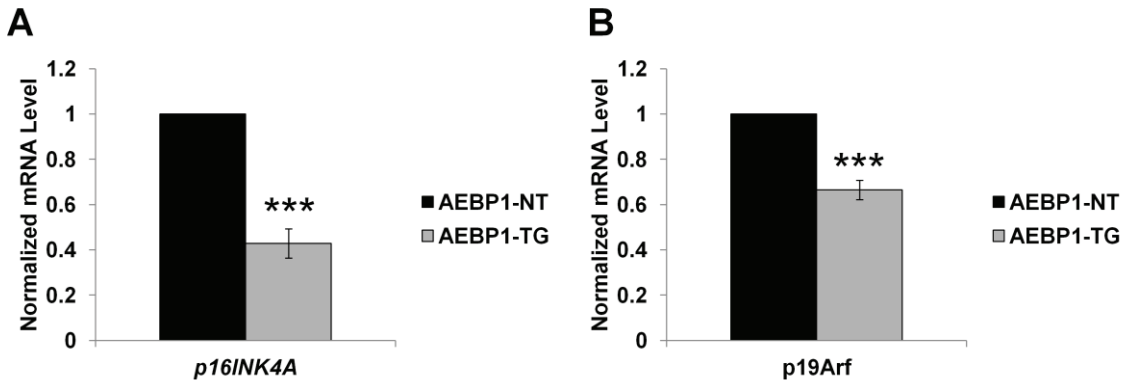

Figure 11. Reduced mRNA levels of tumour-suppressors $p16^{INK4A}$ and $p19^{ARF}$ in mammary epithelial cells isolated from $AEBP1^{TG}$ mice. Total RNA of mammary epithelial cells from pooled mammary tissue of 8-10 week old $AEBP1^{NT}$ or $AEBP1^{TG}$ mice (n = 4-5) was extracted and used for cDNA synthesis. The relative expression of (A) $p16^{INK4A}$ and (B) $p19^{ARF}$ mRNA levels was determined from cDNA by qPCR analysis and normalized to β-actin levels. (A, B) Data are represented as mean ± SEM (n = 3), where $***P < 0.001$ is considered statistically significant.

indicates that HC11 cells co-cultured with AEBP1^{TG} macrophages exhibited a ~1.5- and a ~2-fold up-regulation of Gli1 and Bmi1, respectively, compared to HC11 cells co-cultured with AEBP1^{NT} macrophages (Figure 12). The induction of Gli1 and Bmi1 expression in HC11 cells co-cultured with AEBP1^{TG} macrophages demonstrates that overexpression of AEBP1 in macrophages can induce the up-regulation of Shh-target genes in mammary epithelial cells in a paracrine manner.

3.2.4. Gli1 Expression is Induced in 4T1 Mammary Carcinoma Cells Treated with Conditioned Culture Media from AEBP1^{TG} Macrophages

Macrophages overexpressing AEBP1 exhibit higher levels of Shh protein and are able to induce the Shh-target genes Gli1 and Bmi1 in mammary epithelial cells. Previous reports have found that Shh signalling promotes tumour progression through induction of Gli1 (Dahmane *et al.,* 2001; Sanchez *et al.*, 2004) as well as Bmi1 (Leung *et al.,* 2004; Lui *et al.*, 2006; Wang *et al.*, 2012). These findings suggest that AEBP1-overexpressing macrophages may also promote mammary tumour growth, in addition to hyperplasia, by inducing Shh-target genes in transformed cells. To determine whether AEBP1-overexpressing macrophages induce the Shh-target gene Gli1 in transformed cells, 4T1 murine mammary carcinoma cells were cultured in the presence of conditioned culture media from AEBP1$^{NT \text{ and } TG}$ peritoneal macrophages for ~24 hours. Protein was then extracted from the 4T1 cells and subjected to SDS-PAGE followed by immunoblotting for Gli1 and β-actin. Densitometric analysis of immunoblots indicated that Gli1 expression was up-regulated by ~1.5-fold in 4T1 cells treated with AEBP1^{TG}

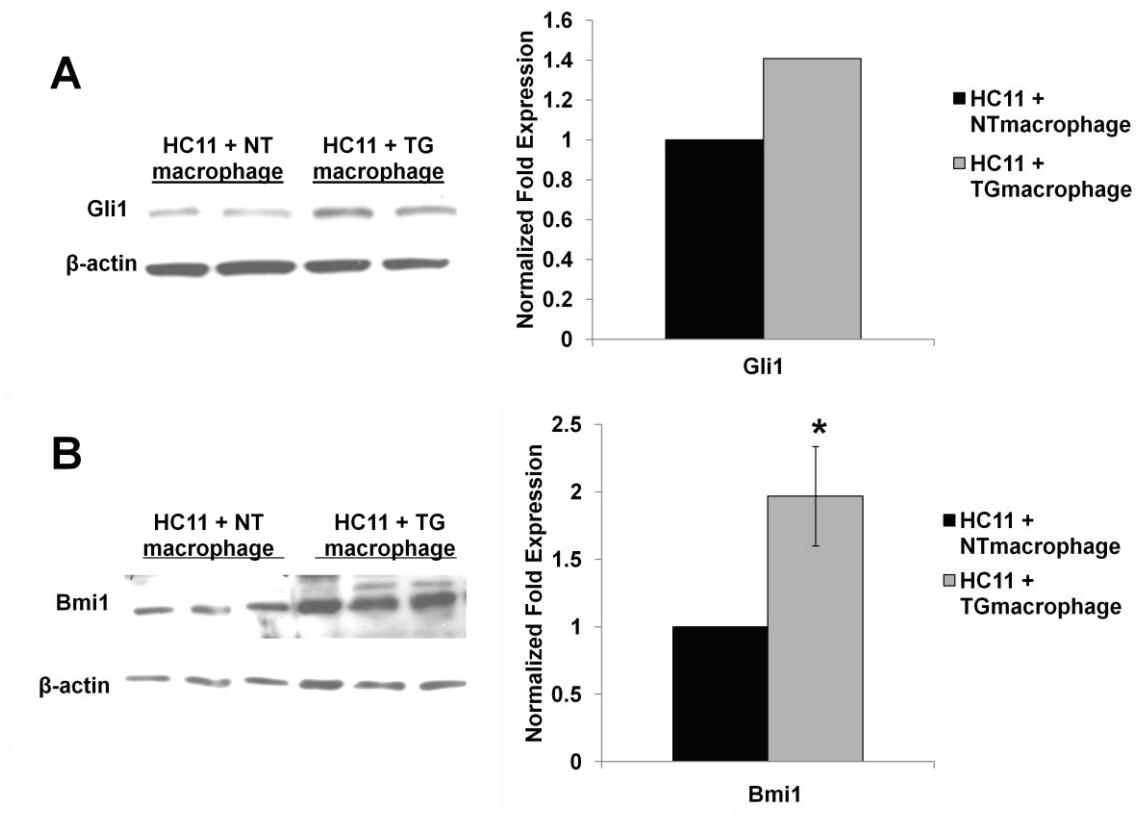

Figure 12. Gli1 and Bmi1 protein levels increased in HC11 mammary epithelial cells co-cultured with peritoneal macrophages from AEBP1^{TG} mice. HC11 cells were cultured for 24 hrs in the presence of pooled peritoneal macrophages from AEBP1^{NT} or AEBP1^{TG} mice (n = 4-5) contained in well-inserts. Whole cell protein extracts (30 μg) were resolved on 8.5% polyacrylamide gels and subject to immunoblot analysis for the indicated proteins. (A) Two samples from each treatment are shown. The expression of the Gli1 in these samples was normalized based on β-actin expression. (B) Data for Bmi1 detected from immunoblots are represented as ±SEM (n = 3), where *P < 0.05 is considered statistically significant. The expression of the Bmi1 in these samples was normalized based on β-actin expression.

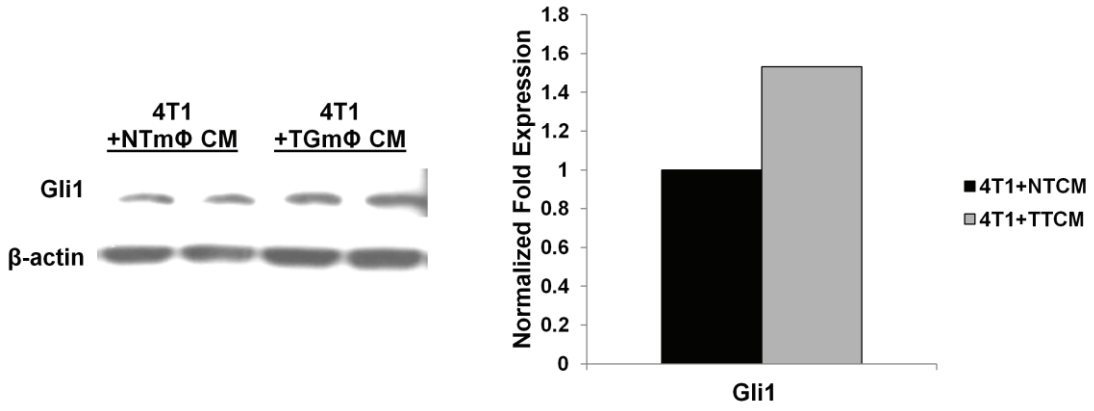

Figure 13. Gli1 protein levels are increased in 4T1 mammary carcinoma cells treated with conditioned culture medium (CM) of peritoneal macrophage from AEBP1^{TG} mice. 4T1 cells were cultured for 24 hrs in the presence of conditioned culture media of pooled AEBP1^{NT} (NTmϕ) or AEBP1^{TG} (TGmϕ) peritoneal macrophages (n = 4-5). Whole cell protein extracts (40 μg) were isolated, resolved on 8.5% polyacrylamide gels and subject to immunoblot analysis for Gli1 and β-actin. Two samples from each treatment are shown. The expression of the indicated proteins in these samples was normalized based on β-actin expression.

macrophage-conditioned medium compared to AEBP1^{NT} treatment (Figure 13). This demonstrates that macrophages overexpressing AEBP1 are able to induce Gli1 expression in mammary carcinoma cells as well as in normal mammary epithelial cells, which suggests that overexpression of AEBP1 in macrophages could promote tumour growth via Shh signalling.

3.3. Overexpression of AEBP1 in Macrophages Promotes Mammary Epithelial Cell Proliferation

3.3.1. Conditioned Culture Medium of AEBP1^{TG} Macrophages Increases the Viability/Proliferation of HC11 Cells

The expression or activities of various proliferative factors, such as TNFα, Akt, and NF-κB, have previously been shown to be up-regulated in the mammary tissue of AEBP1^{TG} mice (Holloway *et al*., 2012). Here, oncogenes involved in cell proliferation, Gli1 and Bmi1 have also been found to be induced in mammary epithelial cells cultured with AEBP1^{TG} macrophages. The up-regulation of these proliferative factors suggests that the overexpression of AEBP1 in macrophages can enhance proliferation of mammary epithelial cells. To examine the effect of AEPB1 overexpression in macrophages on mammary epithelial cell proliferation, HC11 epithelial cells were treated with conditioned culture medium in which either AEBP1^{NT} or AEBP1^{TG} peritoneal macrophages had been cultured. HC11 cells were seeded in 96-well plates on day 1 and 24 hours later (day 2) conditioned media from macrophages was added. The colourimetric MTT assay was used to monitor cell viability/proliferation at 24 hr intervals. Although no difference was detected in the first 3 days, a significant increase in the viability/proliferation of HC11 cells treated with conditioned medium from AEBP1^{TG}

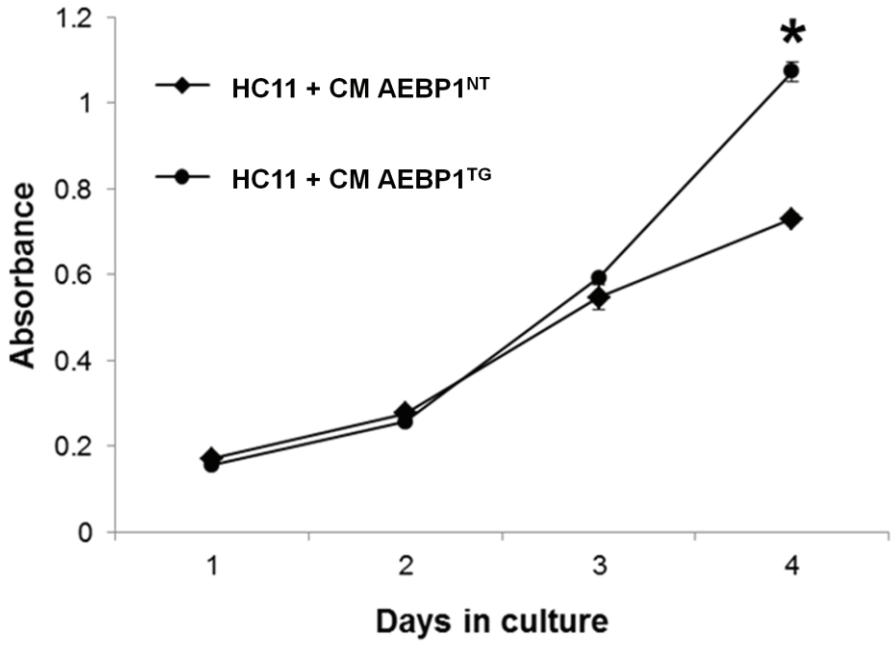

Figure 14. AEBP1^{TG} macrophage-conditioned culture media (CM) increases the viability/proliferation of HC11 mammary epithelial cells. HC11 cells were seeded in 96-well plates on day 1. Starting on day 2, HC11 cells were cultured in the presence of conditioned culture media of pooled peritoneal macrophages from AEBP1$^{NT \text{ and } TG}$ mice (n = 4-5). Cell proliferation/viability was assessed up to day 4 using the MTT assay. Data are represented as mean ± SEM (n = 3), where *P < 0.05 is considered statistically significant.

macrophages relative to AEBP1^{NT} macrophage-conditioned medium treatment was observed by day 4 (Figure 14). The enhanced proliferation of mammary epithelial cells in presence of AEBP1^{TG} macrophage-conditioned culture medium indicates that overexpression of AEBP1 in macrophages could contribute to mammary epithelial hyperplasia.

3.3.2. Proliferation of HC11 Cells is Increased in Co-Culture with AEBP1^{TG} Macrophages

To provide further evidence that macrophages overexpressing AEBP1 are able to enhance mammary epithelial cell proliferation *in vitro*, the growth of HC11 cells co-cultured with AEBP1$^{NT \text{ and } TG}$ peritoneal macrophages in well-inserts was monitored over 4 days. HC11 cells were seeded in 24-well plates on day 1. On day 2, macrophages contained in well-inserts were introduced and cell growth was monitored up till day 4. Each day, HC11 cells were detached and cell counts were obtained using a haemocytometer. Consistent with the result of the MTT assay, there was no difference in HC11 cell growth between the co-cultures in the first 3 days. Yet by day 4, HC11 cells co-cultured with AEBP1^{TG} macrophages displayed higher cell counts compared to those co-cultured with AEBP1^{NT} macrophages (Figure 15). This further indicates that overexpression of AEBP1 in macrophages could contribute to hyperplasia by enhancing mammary epithelial cell proliferation.

3.4. NOD/SCID Mice Co-Injected with 4T1 Mammary Carcinoma Cells and AEBP1^{TG} Macrophages Display Increased Tumour Growth *In vivo*

Both the induction of Gli1 in mammary tumour cells and Shh signalling have been shown to be essential in supporting tumour growth (Dahmane *et al*., 2001;

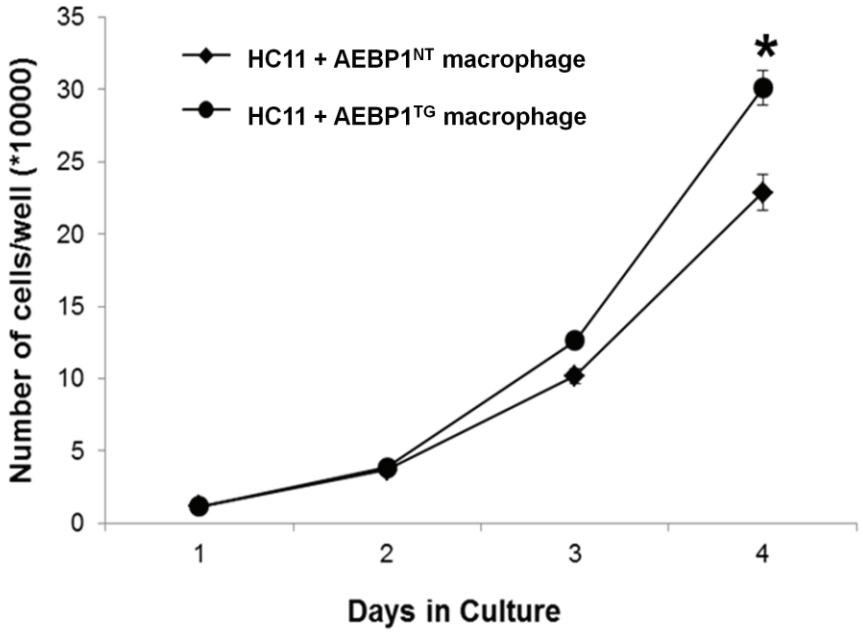

Figure 15. AEBP1^{TG} macrophages increase HC11 mammary epithelial cell proliferation in co-culture. HC11 cells seeded to 24-well plates on day 1. Starting on day 2, HC11 cells were cultured in the presence of pooled peritoneal macrophages from AEBP1^{NT} and AEBP1^{TG} mice (n = 4-5) contained in well-inserts. Cell proliferation was assessed up to day 4 by counting with a haemocytometer. Data are represented as mean ± SEM (n = 3), where *$P < 0.05$ is considered statistically significant.

Sanchez *et al.,* 2004). The observation that conditioned culture media from AEBP1^{TG} macrophages was able to induce Gli1 expression in 4T1 mammary carcinoma cells, suggested that overexpression of AEBP1 in macrophages could contribute to tumour growth. To directly test this, peritoneal macrophages from AEBP1$^{NT\ or\ TG}$ mice were co-injected with 4T1 cells into the subcutaneous flanks of the mammary fat pads of NOD/SCID mice, an established immune-compromised mouse model for monitoring tumour growth (Williams *et al.,* 1993). After day 5, tumour growth was monitored every 3 days for 21 days. From days 14-21, tumours in mice that received AEBP1^{TG} macrophages were significantly larger than to the tumours in mice that received AEBP1^{NT} macrophages (Figure 16). This demonstrates that overexpression of AEBP1 in macrophages influences tumour growth in addition to mammary epithelial cell hyperplasia (Holloway *et al*., 2012). Given that Gli1 is induced in 4T1 cells treated with AEBP1^{TG} macrophage-conditioned media, these results suggest that AEBP1-overexpressing macrophages contribute to tumour growth through the Shh signalling pathway.

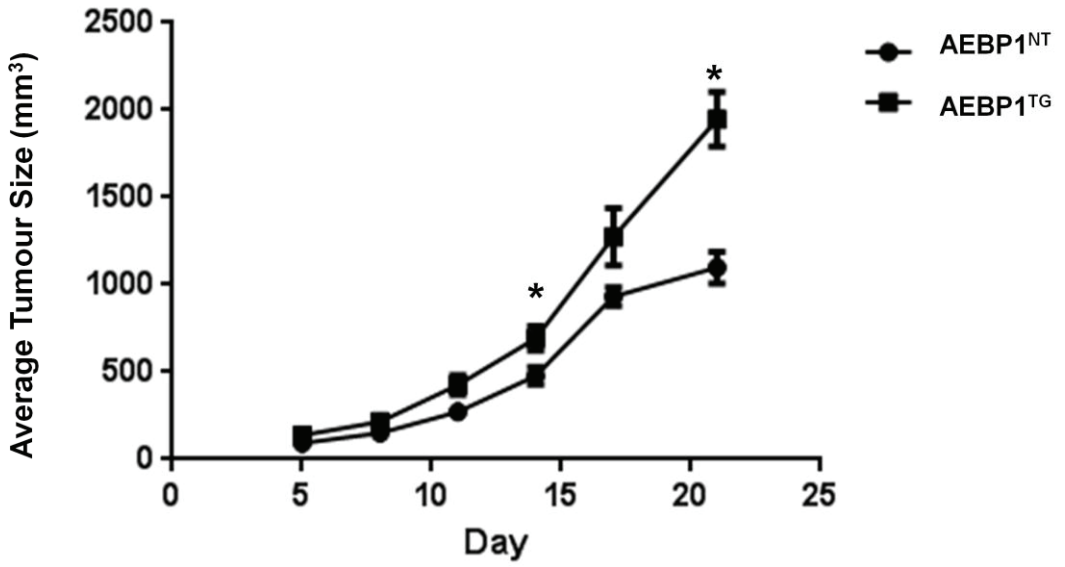

Figure 16. NOD/SCID mice co-injected with 4T1 mammary carcinoma cells and AEBP1^{TG} macrophages display increased tumour growth. NOD/SCID mice were co-injected with 4T1 cells and AEBP1^{NT} (n = 4) or AEBP1^{TG} (n = 6) peritoneal macrophages subcutaneously injected to the flanks of the mammary glands. After day 5, tumour growth was evaluated every three days until day 21 using caliper measurements. Tumours were excised at the end of day 21. Data are represented as mean ± SEM (n = 4-6), where *$P < 0.05$ is considered statistically significant.

CHAPTER 4: DISCUSSION

4.1. AEBP1 is a Novel Regulator of Shh in Macrophages

Previous reports have established that the *Shh* gene is a direct transcriptional target of NF-κB (p65) in macrophages (Nakashima *et al*., 2006; Kasperczyk *et al*., 2009). AEBP1 is a positive regulator of NF-κB (p65) activity in macrophages by interacting with the NF-κB inhibitor IκBα to promote its degradation and by repressing transcription of genes encoding LXRα and PPARγ (Majdalawieh *et al*., 2006, 2007), two proteins known to impede NF-κB activity. In this study, I have investigated whether there is a positive correlation between AEBP1 and Shh expression in macrophages as would be expected since AEBP1 promotes NF-κB activation. Shh protein levels in peritoneal macrophages and a macrophage cell line were found to positively correlate with the level of AEBP1 expression. Since AEBP1 is capable of binding to DNA, AEBP1 might directly activate the *Shh* gene. However, examination of the *Shh* gene promoter did not reveal any AE-1 sites, the sequence recognized by AEBP1 (He *et al*., 1995). Immunoblot analysis showed that the level of the Shh precursor protein was increased in AEBP1-overexpressing macrophages, but this might not necessarily reflect an increase of the ShhN ligand, which is produced by post-translational processing of the Shh precursor. Although NF-κB (p65) plays a significant role in regulating Shh expression, processing of the Shh precursor protein is dependent on cellular cholesterol levels. Guy (2000) demonstrated that processing of the Shh precursor was prevented by depletion of cellular cholesterol using β-methyl cyclodextrin combined with treatment of cholesterol synthesis inhibitors. Interestingly, AEBP1 regulates cholesterol homeostasis by transcriptional repression of cholesterol efflux mediators LXRα and PPARγ

(Majdalawieh *et al*., 2006; Majdalawieh & Ro, 2010a). Kim *et al*. (2009) discovered that LXRα activity negatively regulates Shh-induced Gli1 and PTCH expression in bone marrow stem cells and mouse embryonic fibroblasts. Although ligand-dependent LXRα activation reduced the expression Gli1 and PTCH in the presence of exogenous Shh ligand, LXRα activity had no inhibitory effect on Shh-target gene when the Hh-effector SMO was directly activated using purmorphamine. This suggests LXRα activity may affect endogenous Shh production. They proposed that LXRα plays an inhibitory role in Shh signalling possibly by depleting cellular cholesterol levels resulting in a reduction of cholesterol-dependent cleavage and post-translational modification of Shh (Kim *et al*., 2009). The ability of AEBP1 to regulate cholesterol homeostasis via LXRα repression may contribute to the cholesterol-dependent processing of Shh. This suggests that AEBP1 has a novel dual role in the regulation of Shh signalling, mediating Shh expression and cholesterol-dependent cleavage/post-translational modification.

One issue with the immunoblot analysis of protein isolated from peritoneal macrophages and RAW264.7 macrophage cells is that processed ShhN was not detected. However, AEBP1^{TG} mammary epithelial cells and HC11 cell co-cultured with AEBP1^{TG} macrophages both display up-regulation of Gli1 and Bmi1, which are induced through ShhN secretion (Ingham & McMahon, 2001; Lui *et al*., 2006). Preliminary immunoblot analysis data has indicated that ShhN is present in mammary tissue homogenates from 8-10 week old, female mice. Furthermore, AEBP1^{TG} mammary tissues do display higher ShhN expression compared to AEBP1^{NT} mice, although AEBP1 expression for these samples has yet to be examined (Appendix A). If AEBP1 overexpression in macrophages does increase ShhN production, the level of ShhN should be assessed from the

conditioned culture media of peritoneal macrophages or transfected macrophage cell lines as the relative levels will be secreted from cells.

While ShhN was not detected in immunoblots of proteins extracted from peritoneal macrophages of mice fed on a standard chow diet, preliminary immunoblot analysis of protein extracted from peritoneal macrophages of mice fed on a high-fat diet do display detectable levels of ShhN (Appendix B). This may indicate that cholesterol-mediated processing of Shh is reduced in macrophages from mice on standard chow-diet compared to mice on a high-fat diet because of lower cellular cholesterol levels. Guy (2000) demonstrated that newly synthesized Shh precursor is rapidly converted to the processed ShhN, but cholesterol deprivation inhibits processing of the precursor. This may suggest that the level of cellular cholesterol is rate-limiting to the cholesterol-dependent processing of Shh. Since Shh-target genes were induced in HC11 cells co-cultured with peritoneal macrophages from AEBP1^{TG} mice fed on a standard diet, ShhN might not have been detectable in macrophages due to a slow rate of Shh processing combined with rapid secretion of low ShhN quantities. However, macrophages from AEBP1^{TG} mice on a high-fat diet may have high steady-state levels of ShhN because of faster rates of Shh processing and higher quantities of ShhN within the cell. Since a high-fat diet induces AEBP1 in macrophages (Bogachev *et al.*, 2011), an increase of ShhN in macrophages due to the high-fat diet could be facilitated through the ability of AEBP1 to increase cellular cholesterol levels via transcriptional repression of cholesterol efflux genes LXRα and PPARγ (Majdalawieh *et al.*, 2006, 2010). This may be further evidence for the involvement of AEBP1 in the cholesterol-dependent processing of Shh.

4.2. Macrophage AEBP1 Overexpression Contributes to Mammary Epithelial Cell Hyperplasia Through Gli1 and Bmi1 Induction

Transgenic overexpression of AEBP1 in macrophages promotes the development of mammary epithelial cell hyperplasia in adult female mice (Holloway *et al*., 2012). Furthermore, macrophages overexpressing AEBP1 display an increase in NF-κB activity and TNFα expression. In addition, my results indicate that macrophages overexpressing AEBP1 also display increased steady-state levels of Shh protein. Previous reports have found that Shh signalling is an essential mediator of hyperplasia and tumourigenesis through induction of the oncogenes Gli1 (Dahmane *et al*., 2001; Sanchez *et al*., 2004; Fiaschi *et al*., 2009) and Bmi1 (Leung *et al*., 2004; Lui *et al*., 2006; Wang *et al*., 2012). Hence, I examined the possibility that the up-regulation of Shh in AEBP1-overexpressing macrophages contributes to the occurrence of mammary epithelial cell hyperplasia observed in AEBP1^{TG} mice through the induction of Gli1 and Bmi1. The expression of both Gli1 and Bmi1 was found to be up-regulated in mammary epithelial cells of AEBP1^{TG} female mice as well as HC11 epithelial cells co-cultured with AEBP1^{TG} macrophages. The induction of Gli1 and Bmi1 in mammary epithelial cells is fairly modest in response to macrophages from 8-10 week old AEBP1^{TG} mice on a standard chow diet. However, standard chow diet-fed AEBP1^{TG} mice do not develop hyperplasia until ~30 weeks of age, whereas AEBP1^{TG} mice on a high-fat diet develop hyperplasia as early as 10-weeks of age (Holloway *et al*., 2012). Since Gli1 and Bmi1 are overexpressed during hyperplasia (Dahmane *et al*., 2001; Karhadkar *et al*., 2004), the expression of these Shh-target genes may be considerably increased in mammary epithelial cells from mice on high-fat diet or 30-week old mice on standard chow diet as compared to the 8-10-week old mice on standard chow diet.

Interestingly, various reports have found that the overexpression of Bmi1 in tumours is responsible for repressing the $p16^{INK4A}$ and $p19^{ARF}$ tumour suppressors (Jacobs *et al.*, 1999; Kim *et al.*, 2004; Fan *et al.*, 2008). Here, I examined whether $p16^{INK4A}$ and $p19^{ARF}$ were down-regulated as a result of up-regulated Bmi1 expression induced by AEBP1 overexpression in macrophages. As expected, the induction of Bmi1 does correlate with the down-regulation of $p16^{INK4A}$ and $p19^{ARF}$ transcripts in the mammary epithelial cells of $AEBP1^{TG}$ mice. Repression of the cell cycle inhibitor $p16^{INK4A}$ and the positive p53-regulator $p19^{ARF}$ in $AEBP1^{TG}$ mammary epithelial cells may contribute to mammary epithelial cell hyperplasia observed when AEBP1 is overexpressed in macrophages.

Although various reports have shown that Shh is responsible for induction of both Gli1 and Bmi1, my results do not confirm that AEBP1 overexpression in macrophages is responsible for Gli1 and Bmi1 up-regulation in mammary epithelial cells due to Shh secretion. To verify that macrophage AEBP1-mediated Shh signalling is responsible for Gli1 and Bmi1 induction, secreted Shh should be neutralized in the HC11/macrophage co-cultures or macrophage-conditioned media treatments using an anti-Shh antibody and the results compared to neutralization with anti-IgG controls. Inhibition of Shh should prevent AEBP1-overexpressing macrophages from inducing genes downstream of the Shh signalling pathway in mammary epithelial cells, otherwise it is plausible that AEBP1 regulates other members of the Hh ligand family.

4.3. Macrophage AEBP1 Potentially Promotes Mammary Epithelial Cell Hyperplasia Via Synergy Between TNFα and Shh Signalling Pathways

Previous reports have found a link between chronic inflammation and enhanced hedgehog signalling (Nakashima *et al.,* 2006; Kasperczyk *et al.,* 2009; Yamasaki *et al.,* 2010). AEBP1-mediated TNFα signalling in macrophages induces the activities of Akt and NF-κB in mammary epithelial cells (Holloway *et al.*, 2012). Furthermore, I have demonstrated that AEBP1-overexpression in macrophages also promotes Shh signalling activity in mammary epithelial cells. Interestingly, PI3K-dependent activation of Akt supports Shh signalling activity by antagonizing PKA-mediated Gli-inactivation (Riobó *et al.,* 2006). Macrophage AEBP1-mediated induction of Akt activity in mammary epithelial cells could allow Gli transcriptional activities by inactivating PKA. This suggests that AEBP1 overexpression in macrophages could contribute to the development of epithelial cell hyperplasia in the mammary gland through a synergistic effect between the PI3K/Akt and Shh pathways that enhance Gli transcription activity in mammary epithelial cells.

4.4. Macrophage AEBP1 May Influence Other Stages of Mammary Tumour Progression Via Shh Signalling

Activity of the Shh signalling pathway is critically involved in hyperplasia and tumour initiation in various tissues (Dahmane *et al.,* 2001; Karhadkar *et al.,* 2004; Sanchez *et al.*, 2004; Kasperczyk *et al.,* 2009). Stromal AEBP1 expression in macrophages also appears to mediate mammary epithelial cell hyperplasia by promoting a proinflammatory microenvironment (Holloway *et al.,* 2012). The findings I have presented here suggest that AEBP1 overexpression in macrophages contributes to mammary epithelial cell hyperplasia through Shh signalling, yet this particular signalling pathway is also involved in the latter stages of tumour progression and metastasis

(Taipale & Beachy, 2001; Karhadkar *et al.,* 2004; Yamasaki *et al.,* 2010). Hence AEBP1-mediated Shh signalling may contribute to mammary tumour and cancer development in addition to mammary epithelial cell hyperplasia. Shh mediates tumour growth (Dahmane *et al.,* 2001; Kasperczyk *et al.,* 2009), angiogenesis (Hsieh *et al.,* 2011), and migration (Mori *et al.*, 2006; Thomas *et al.,* 2011) predominately through the induction of Gli1 expression/activation in tumour cells. Since AEBP1-overexpressing macrophages are able to induce the Shh-target gene Gli1 in mammary epithelial cells, I also examined the ability of macrophage AEBP1 to induce Gli1 expression in mammary carcinoma cells. Gli1 was induced in both 4T1 mammary carcinoma cells and HC11 cells in response to AEBP1-overexpressing macrophages, suggesting that macrophage AEBP1-overexpression may affect the growth and perhaps progression of mammary tumours through Shh signalling. To further investigate the contribution of AEBP1-overexpressing macrophages to tumour growth, mammary tumour growth in NOD/SCID mice co-injected with 4T1 cells and peritoneal macrophages from $AEBP1^{NT \text{ and } TG}$ mice was monitored over 21 days. Remarkably, mice that received $AEBP1^{TG}$ macrophages displayed significantly larger tumours than mice that received $AEBP1^{NT}$ macrophages. The result from the macrophage/4T1 co-injection experiment certainly indicates that AEBP1-overexpressing macrophages promote mammary tumour growth *in vivo*. Although AEBP1 overexpression in macrophages enhances both Shh and proinflammatory signalling activities in mammary tissue, it is not clear whether the ability of AEBP1-overexpressing macrophages to promote tumour growth is due to enhanced production of Shh, proinflammatory signals, or some other pathway that has yet to be identified. Inhibition of Shh signalling activity in this macrophage/4T1 co-injection

experiment, via treatment with anti-Shh antibody or the Hh pathway inhibitor cyclopamine, would address whether Shh signalling is a significant contributor to macrophage AEBP1-mediated tumour growth.

AEBP1 is known to promote production of proinflammatory cytokines such as TNFα, IL-6, and IL-1β (Majdalawieh *et al*., 2006, 2007) that are associated with M1 activated macrophages (Mantovani *et al*., 2007). Shh signalling is associated with M1-type cytokine production in inflammatory-stimulated macrophages, yet Yamasaki *et al*. (2010) suggest that TAMs, which have a M2-like activated phenotype (Mantovani *et al*., 1992, 2002), are capable of producing Shh. The precise role of AEBP1 in M1- and M2-activated macrophages is not understood and must be further investigated to determine if AEBP1 is expressed in TAMs and if so, whether this expression contributes to tumour progression.

4.5. The Impact of Aberrant AEBP1 Induction in Mammary Epithelial Cells

AEBP1 protein is not detected in untransformed mammary epithelial cells (Zhang *et al*., 2011); however, several reports have observed the aberrant induction of AEBP1 in different epithelial-derived cancers such as malignant breast cells (Grigoriadis *et al*., 2006), prostate cancer (Li *et al*., 2006b), and primary glioblastoma multiforme (Reddy *et al*., 2008). Preliminary immunoblot analysis has revealed an induction of AEBP1 expression in pooled mammary epithelial cells from a group of AEBP1^{TG} female mice (n = 5) challenged on a 20-week high-fat diet, yet AEBP1 is low or not detected in three different pools of mammary epithelial cells isolated from AEBP1^{NT} mice (Appendix C). Interestingly, one out of the five AEBP1^{TG} mice used for mammary

epithelial cell isolation exhibited mammary epithelial cell hyperplasia whereas none of the AEBP1^{NT} mammary tissues displayed hyperplasia (unpublished). Furthermore, AEBP1 expression is induced in murine mammary epithelial cells and breast cancer cells treated with TNFα (unpublished; McCluskey and Ro, personal communication). These results may suggest that AEBP1 induction in mammary epithelial cells is involved in mammary epithelial cell hyperplasia and possibly tumourigenesis. Further investigation will be needed to determine whether the aberrant induction of AEBP1 expression in mammary epithelial cells contributes to neoplastic transformation as well as growth, invasiveness, and metastasis of tumours.

CHAPTER 5: CONCLUSIONS

AEBP1 is a proinflammatory mediator, positively regulating NF-κB activity in macrophages by promoting the degradation of its inhibitor IκBα and repressing expression of anti-inflammatory genes LXRα and PPARγ. NF-κB is a crucial regulator of the production of various proinflammatory cytokines and chemokines as well as the mitogen Shh. Given the role of AEBP1 in NF-κB regulation, I hypothesized that AEBP1 is a novel regulator of Shh signalling in macrophages and this regulation might contribute to the dramatic incidence of mammary epithelial cell hyperplasia observed in AEBP1^{TG} mice that overexpress AEBP1 specifically in macrophages (Holloway *et al*., 2012). As expected, overexpression of AEBP1 in macrophages up-regulates Shh expression. Furthermore, AEBP1-overexpressing macrophages are able to induce the Shh-targets genes Gli1 and Bmi1 in mammary epithelial and carcinoma cells, two oncogenic transcriptional regulators that are essential to the development of hyperplasia and tumour progression in a variety of tissues. Remarkably, overexpression of AEBP1 in macrophages not only increases proliferation of mammary epithelial cells *in vitro*, it also increased mammary tumour growth *in vivo*. Together, these findings indicate that AEBP1 overexpression in stromal macrophages is a critical contributor to mammary epithelial cell hyperplasia and perhaps tumour development by enhancing Shh signalling activity in the mammary gland.

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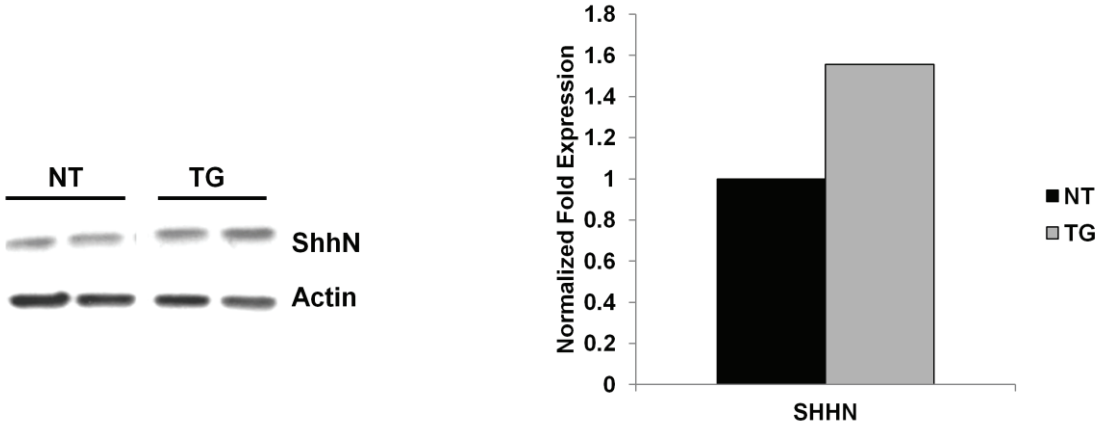
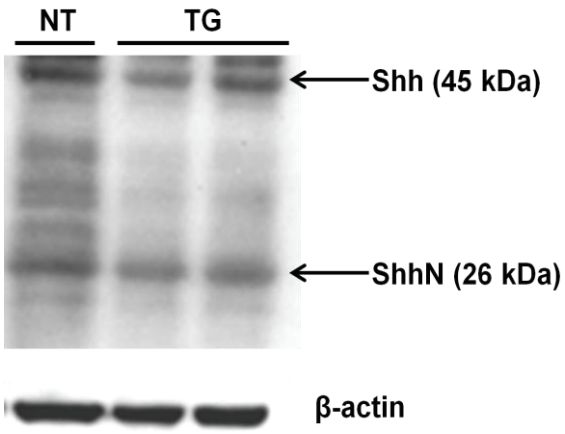
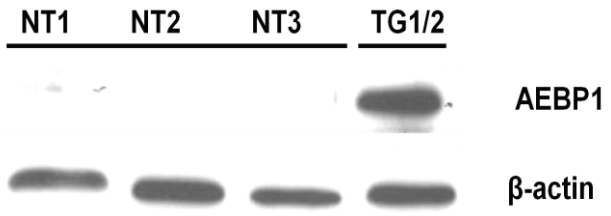


APPENDIX A: Elevated ShhN Protein Levels in Mammary Tissue from AEBP1^{TG} mice. Protein extracts (30 μg) from mammary tissue homogenates of AEBP1^{NT} and AEBP1^{TG} female mice were isolated, resolved on 12.5% polyacrylamide gels and subjected to immunoblot analysis for Shh and β-actin. Two samples from each genotype are shown. The expression of the indicated proteins was normalized based on β-actin expression.



APPENDIX B: ShhN is Detected in Marcophages from Mice on 20-Week High Diet. Whole cell protein extracts (40 μg) from peritoneal macrophages of AEBP1^{NT} and AEBP1^{TG} female mice on a 20-week high fat diet were isolated, resolved on 10% polyacrylamide gels and subjected to immunoblot analysis for Shh and β-actin. One sample from each AEBP1^{NT} and two samples from AEBP1^{TG} macrophages are shown.



APPENDIX C: AEBP1 Expression is Induced in Mammary Epithelial Cells of AEBP1^{TG} Mice on 20-Week High Fat Diet. Whole cell protein extracts (35 μg) from pooled mammary epithelial cells isolated from mammary tissues of AEBP1^{NT} (3-4 mice per pooled sample) and AEBP1^{TG} (*5* mice per pooled sample) female mice on a 20-week high fat diet were isolated, resolved on 10% polyacrylamide gels and subjected to immunoblot analysis for AEBP1 and β-actin. Three samples from AEBP1^{NT} mice and one from AEBP1^{TG} mice are shown.

APPENDIX D:

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December 12, 2012

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