INVESTIGATING THE PHOSPHOINOSITIDE 3-KINASE PATHWAY FOR THERAPEUTIC STRATEGIES FOR BREAST CANCER

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Investigating the Phosphoinositide 3-Kinase Pathway for Therapeutic Strategies for Breast Cancer

Abstract

Despite major advances in our understanding of the etiology of breast cancer, it remains a leading cause of cancer death in women worldwide. This warrants the search for novel alternatives for diagnosis and therapeutic intervention. The phosphoinositide 3-kinase (PI3K) pathway regulates all aspects of breast cancer development, from initiation to metastatic dissemination. This is underscored by the high prevalence of somatic mutations in *PIK3CA*, the gene encoding the catalytic subunit of PI3K, in breast cancer. Although targeting the PI3K pathway is a viable therapeutic approach, many PI3K pathway inhibitors have yet to show significant clinical efficacy.

Here we examine the potential chemotherapeutic benefit of aspirin in PI3K-driven breast cancer. We demonstrate that mutant *PIK3CA* breast cancer cells show a dose-dependent decrease in cell viability and anchorage-independent growth in soft agar, when treated with increasing concentrations of aspirin/salicylate. Co-treatment of aspirin sensitizes mutant *PIK3CA* breast cancer cells to PI3K inhibitors to enhance suppression of proliferation. Additionally, immortalized mammary epithelial cells expressing mutant *PIK3CA H1047R* show greater sensitivity to aspirin when compared to cells expressing wild-type *PIK3CA* grown in three-dimensional (3D) Matrigel culture. Mechanistic studies indicate that the growth inhibitory effect of aspirin are due to activation of AMP-activated protein kinase (AMPK), inhibition of mammalian target of rapamycin complex 1

(mTORC1) signaling and induction of autophagy. Importantly, this growth suppression is independent of the activities of cyclooxygenase-2 (COX-2) and nuclear factor kappa B (NF-κB).

We have also investigated the transcriptional responses to PI3K activation in breast cancer cells. By performing microarray and gene ontology analysis, we show that constitutive PI3K pathway activation preferentially affects the gene expression of cytokines, chemokines and other secreted factors. We identify a novel PI3K-regulated gene termed long Pentraxin 3 (*PTX3*), whose functional role in cancer is largely unknown. We demonstrate that the PI3K pathway regulates the expression of *PTX3* in an NF-κB dependent manner. Using functional cell-based assays, we demonstrate a role for PTX3 in PI3K-driven proliferation and survival in two-dimensional (2D) monolayer and three-dimensional (3D) Matrigel culture.

Additionally, we identify a novel large intergenic noncoding RNA (lincRNA) transcript termed c14orf34, which is upregulated in both mutant *PIK3CA* and *Src*-transformed MCF10A cells. By performing gene expression profiling, we show that this transcript is associated with basal-like breast cancer cells and genomically altered in a subset of invasive breast carcinomas from human patients. By employing ChIP-seq analysis, we demonstrate that c14orf34 is regulated by the transcription factor STAT3. Loss-of-function studies suggest a role for this lincRNA in cell invasion and migration. We also identify several genes that are differentially expressed upon overexpression of c14orf34 in MCF10A cells, indicative of a potential role in regulating global gene expression.

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ABBREVIATIONS

4EBP1	4E-Binding Protein
AKT	Protein Kinase B
AMPK	5′ AMP-activated Protein Kinase
ANRIL	Antisense RNA In The INK4 Locus
ARF	ADP Ribosylation Factor
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine Triphosphate
ATR	Ataxia Telangiectasia and Rad3 related
BAD	BCL-2 Agonist of Cell Death
BAK	BCL2-Antagonist/Killer 1
BAX	BCL2-Associated X Protein
BCL-2	B-Cell CLL/Lymphoma 2
BTK	Bruton's Tyrosine Kinase
C-MYC	Cellular v-Myc Myelocytomatosis viral oncogene homolog
CRP	C-Reactive Proteins
DNA-PK	DNA-dependent Protein Kinase
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial- Mesenchymal Transition
ERK	Extracellular-Regulated Kinase
FOXO3a	Forkhead box O 3a
GEF	Guanine nucleotide Exchange Factor
GLUT4	Glucose Transporter Type 4
GPCR	G Protein–coupled Receptor
HER2/ERBB2	Human Epidermal growth factor Receptor 2
HER3/ERBB3	Human Epidermal growth factor Receptor 3
HIFα	Hypoxia-Inducible Factor 1 alpha
HOTAIR	Hox Antisense Intergenic RNA
ILK	Integrin-Linked Kinase
INPP4B	Inositol Polyphosphate-4-Phosphatase type II
IRS1	Insulin Receptor Substrate 1
KTN1	Kinectin 1
LINCRNA	Large Intergenic Non-coding RNA
MAPK	Mitogen-Activated Protein Kinase
MSC	Mesenchymal Stem Cell
MTORC1	mammalian Target of Rapamycin Complex 1
NFAT	Nuclear Factor of activated-T cells
NFκB	Nuclear Factor Kappa-B
PARP	Poly ADP Ribose Polymerase

PDK1	Phosphoinositide-Dependent Kinase-1
PH	Pleckstrin Homology
PHLPP1/2	PH domain and Leucine rich Protein Phosphatase 1/2
PI3K	Phosphoinositide 3-Kinase
PKC	Protein Kinase C
PLCγ	Phospholipase C γ
PRAS40	Proline-Rich Akt Substrate of 40 kDa
PRC1	Polycomb Repressive Complex 1
PTEN	Phosphatase and Tensin Homolog
PTX3	Pentraxin 3, Long
PUMA	p53 Upregulated Modulator of Apoptosis
RAC	Ras-related C3 Botulinum Toxin Substrate
RHO A	Ras Homolog Gene Family, Member A
RTK	Receptor Tyrosine Kinase
SAP	Serum Amyloid P
S6K1	p70 Ribosomal S6 Kinase 1
SCF	Skp, Cullin, F-box containing
SGK3	Serum and Glucocorticoid- Regulated Kinase 3
SH	Src Homology
SKP2	S phase Kinase-associated Protein 2
SMG-1	SMG1 homolog, Phosphoinositol 3-kinase-related kinase
STAT3	Signal Transducer and Activator of Transcription 3
T-ALL	T-cell Acute Lymphoblastic Leukemia
TSC1/2	Tuberous Sclerosis Complex1/2
VPS34	Vacuolar Protein Sorting 34

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CHAPTER I

INTRODUCTION

1. OVERVIEW OF CLASSICAL PI3K SIGNAL TRANSDUCTION

Our understanding of PI3K signaling has expanded exponentially since its discovery in the 1980s as a novel lipid kinase associated with viral oncogenes and activated protein tyrosine kinases (Courtneidge and Heber, 1987; Fukui and Hanafusa, 1989; Whitman et al., 1985). Three classes of PI3Ks have been defined based on primary structure, regulation and substrate specificity (**Figure 1-1**) (Courtney et al., 2010).

Class I PI3K

Class I PI3Ks are the best understood and most clearly implicated in human cancer. *In vitro*, this class of PI3Ks can phosphorylate the 3' position of the inositol ring of PI (phosphoinositol), PI(4)P (phosphoinositol 4-phosphate) and PI(4,5)P2 (phosphoinositol 4, 5-bisphosphate), leading to the generation of PI(3)P (phosphoinositol 3-phosphate), PI(3,4)P2 (phosphoinositol 3, 4-bisphosphate), PI(3,4,5)P3 (phosphoinositol 3,4, 5-triphosphate), respectively. However, PI(4,5)P2 is considered the preferred substrate *in vivo* (Fry, 1994). Class I PI3Ks are further subdivided into Class IA and Class IB.

Class IA PI3Ks are heterodimers comprised of a p85 regulatory subunit and a p110 catalytic subunit. In the mammalian genome, the regulatory subunits p85α, p85β, and p55γ are transcribed from the *PIK3R1*, *PIK3R2*, and *PIK3R3* genes respectively, and are collectively referred to as p85. Alternate splicing of these substrates also leads to the generation of additional isoforms of p85 (Fry, 2001). The catalytic isoforms p110α, p110β and p110δ are encoded from the *PIK3CA*, *PIK3CB*, and *PIK3CD* genes

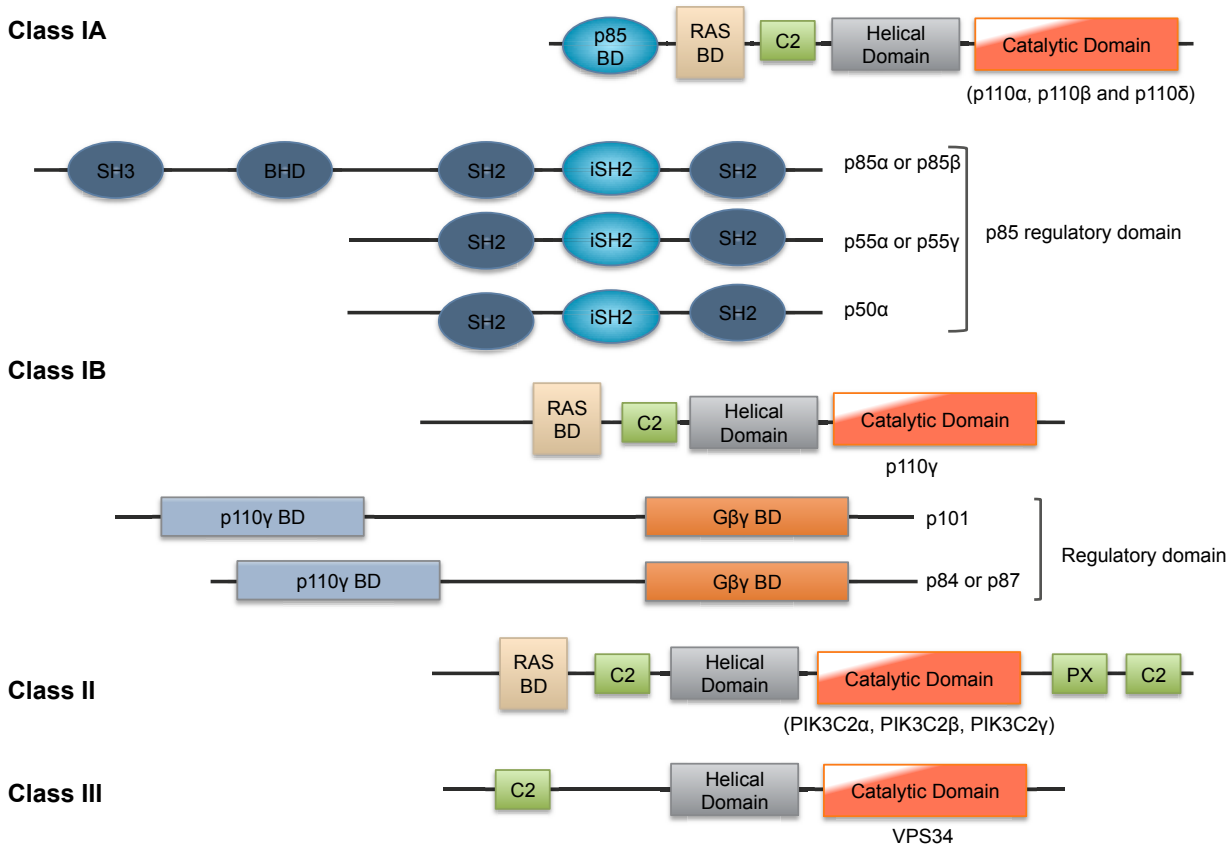

Figure 1-1: Classification of the phosphoinositide 3-kinase (PI3K) family. The PI3K family is divided into three groups based on primary structure, regulation and substrate specificity. Class IA PI3Ks isoforms are highly homologous in their p85 regulatory and catalytic subunits. Isoforms of the p85 regulatory subunit share three core domains including a p110-binding domain (iSH2). Under basal conditions, p85 interacts with the p110 iSH2 domain and inhibits its catalytic activity. Class II and III PI3Ks are monomers and contain a single catalytic subunit. Abbreviations: RAS-binding domain (RAS BD), p85-binding domain (p85 BD), Src-homology 2 (SH2), Src-homology 3 (SH3), inter-SH2 (iSH2), BCR homology domain (BHD). Adapted from (Liu et al., 2009).

respectively (Engelman et al., 2006). Class IA PI3Ks are activated by a multitude of receptor tyrosine kinases (RTK) in response to growth factors and other stimuli (Fry, 1994). Direct interaction between the regulatory subunits and the phosphotyrosine residues on various receptor tyrosine kinases or associated adaptor proteins such as insulin receptor substrate 1, IRS1, leads to the relief of the inhibitory interaction between the SH homology domains of the regulatory subunit and the p110 catalytic subunit (Carpenter et al., 1993; Zhao and Vogt, 2008). This results in subsequent membrane localization of PI3K. Here PI3Ks lies in closer proximity to its lipid substrates and an increase in PI3K lipid kinase activity ensues. Direct association between small G proteins such as RAS and the p110 catalytic subunit, can also lead to activation of Class IA PI3Ks (Rodriguez-Viciana et al., 1996).

Class IB PI3Ks are predominantly activated by G-protein coupled receptors (GPCRs) and form heterodimers composed of a p101 regulatory subunit and a p110γ catalytic subunit (Vanhaesebroeck and Waterfield, 1999). Recently, two new regulator subunits, p84 and p87PIKAP have been documented (Suire et al., 2005; Voigt et al., 2006). Class IB PI3Ks are primarily found in immune cells and function in inflammatory diseases and the immune response (Hawkins et al., 2006; Patrucco et al., 2004; Sasaki et al., 2000).

Class II PI3Ks

Three human Class II PI3Ks have been documented: ubiquitously expressed *PIK3C2α* and *PIK3C2β*, and tissue-specific *PIK3C2γ* (Fry, 2001). These PI3Ks share structural features with Class I PI3Ks but possess additional regulatory domains

including the Phox homology and C2 domains, in lieu of a p85 or p101 binding motif (Vanhaesebroeck and Waterfield, 1999). Class II PI3Ks are also activated by a diverse array of RTKs and can phosphorylate PI, and PI(4)P but not PI(4,5)P2, *in vitro* (Arcaro et al., 2000; Fry, 2001). Functional characterization of this class of PI3Ks requires further investigation.

Class III PI3Ks

Class III PI3Ks specialize in the production of PI(3)P, using PI as its only substrate (Fruman et al., 1998; Vanhaesebroeck et al., 1997). The prototypic class III PI3Ks Vps34p, was identified from yeast studies. Both yeast and the human homolog of Vps34, play an important role in vesicle sorting and intracellular trafficking (Odorizzi et al., 2000; Vanhaesebroeck and Waterfield, 1999). Association of Vps34 with the myristoylated serine/threonine kinase Vps15/p150 leads to its recruitment to the cell membrane and an increase in its lipid kinase activity (Odorizzi et al., 2000).

Major transducers of PI3K signaling – A focus on Akt

Phosphoinositide second messengers propagate PI3K signaling which ultimately leads to promotion of cell growth, survival, motility and metabolism (**Figure 1-2**). These lipid substrates serve as binding sites for proteins containing pleckstrin homology (PH) domains and FYVE-fingers (Leevers et al., 1999; Wiedemann and Cockcroft, 1998). While FYVE motifs appear to preferentially bind to PI(3)P, PH domain effector proteins associate with PI(3,4,)P2 and PI(3,4,5)P3 (Leevers et al., 1999). These include the well-known serine/threonine kinase Akt and the phosphoinositide-dependent kinase

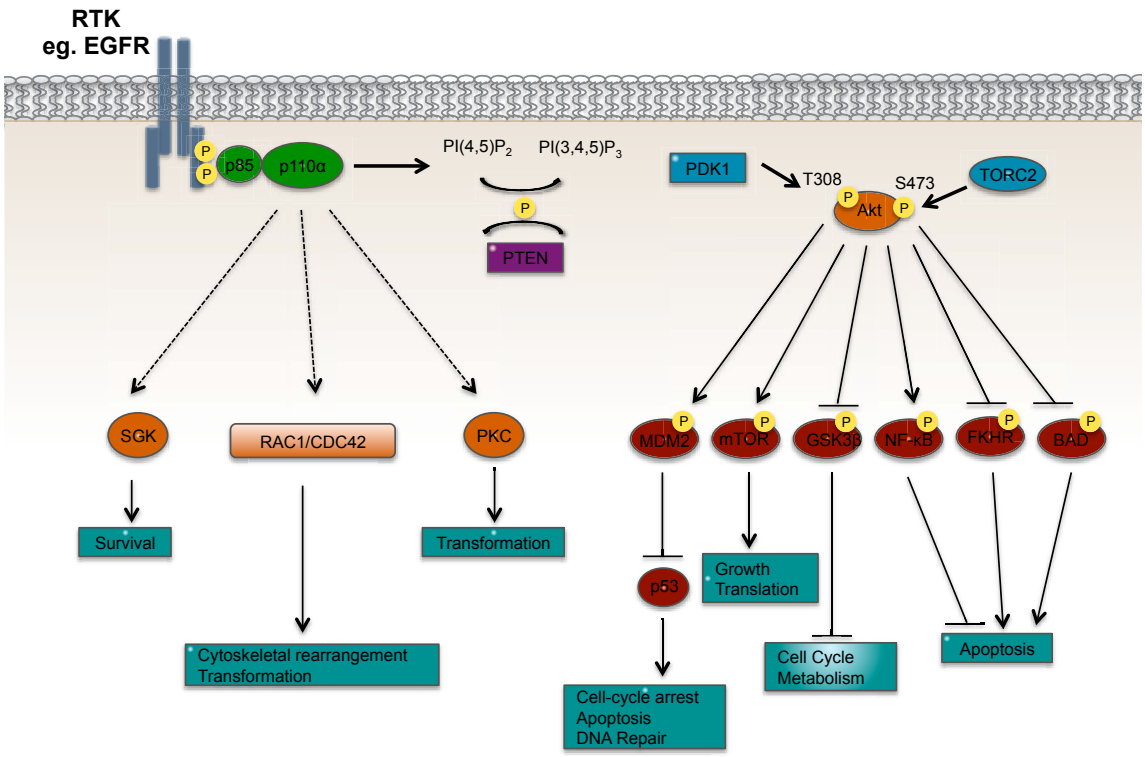

Figure 1-2: Phosphoinositide 3-K (PI3K) signal transduction. Activation of PI3Ks in response to receptor tyrosine kinase (RTK) instigation, leads to the generation of second lipid messengers. In the case of Class I PI3Ks, activation of the p110 catalytic subunit leads to the generation of phosphoinositide-3,4,5-triphosphate (PI(3,4,5)P3) at the membrane. This results in the recruitment and activation of a number of signaling proteins containing pleckstrin homology (PH) domains including the serine/threonine kinase Akt. Activation of Akt leads to the phosphorylation of a broad spectrum of downstream substrates. Ultimately, the PI3K pathway affects several critical cellular processes involved in cell transformation, growth, survival and motility, among others. Adapted from (Vivanco and Sawyers, 2002).

PDK1. Other PH-domain containing effectors include certain guanine nucleotide exchange factors (GEFs) including Rho, Rac and Cdc42, which belong to the Rho family of GTPases (Leevers et al., 1999; Roymans and Slegers, 2001). Many of these GEFs regulate cytoskeletal rearrangement and cell motility, in a PI3K-dependent manner (Hill et al., 2000; King et al., 1997; Posern et al., 2000; Reif et al., 1996; Ridley and Hall, 1992; Roymans and Slegers, 2001). Association of PI(3,4,)P2 and PI(3,4,5)P3 with the PH domain promotes recruitment of the GEFs to their respective GTPases and de-represses the inhibitory interaction between their PH domain and the catalytic region (Cherfils and Zeghouf, 2013). Additionally effectors of PI3K signaling include phospholipase-C (PLC)γ and specific members of the Bruton's tyrosine kinase (Btk) family of tyrosine kinases (Bae et al., 1998; Falasca et al., 1998; Rameh et al., 1997; Rameh et al., 1998; Salim et al., 1996).

Akt

Akt, also called Protein Kinase B, is one of the best-described effectors of PI3K signaling. Following activation of PI3K, Akt is recruited to the plasma membrane through association of its N-terminal PH domain with PI(3,4,5)P3 at the plasma membrane (Franke et al., 1997; Stephens et al., 1998). This leads to a conformational change in Akt that result in exposition of two important phosphorylation sites found in its catalytic domain (**Figure 1-3**). These phosphorylation events are critical for full activation of Akt and involve phosphorylation of Akt at S473 in the activation loop by PDK1, and T308 in the Hydrophobic Motif by mTORC2 (**Figure 1-3**) (Alessi et al., 1997; Sarbassov et al., 2005). As mentioned above, PDK1 also binds to PI(3,4,5)P3 and PI(3,4,)P2 via its PH

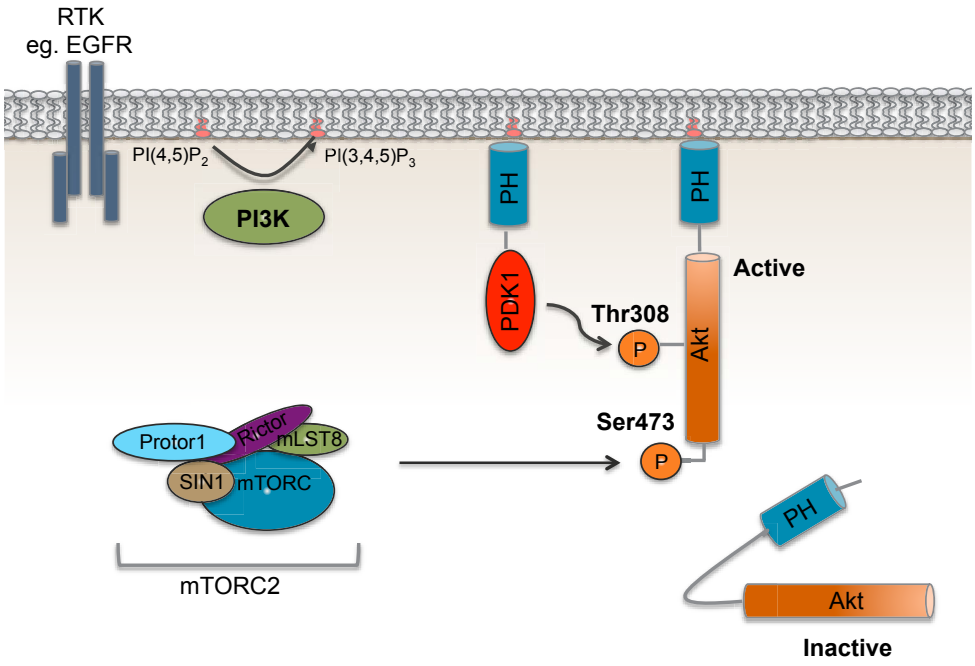

Figure 1-3: Mechanism of activation of Akt. Activation of PI3K results in the translocation of Akt to the plasma membrane through association of its N-terminal PH domain with PI(3,4,5)P3 at the plasma membrane. This promotes an Akt conformational change that results in exposition of two critical phosphorylation sites found in its catalytic domain. This involves phosphorylation of Akt at S473 in the activation loop by PDK1, and T308 in the Hydrophobic Motif by mTORC2. Phosphorylation of these two sites are critical for complete activate of Akt. Abbreviation: Pleckstrin Homology (PH). Adapted from (Pearce et al., 2010).

domain and thus comes in close proximity to its substrate Akt, upon PI3K activation (Anderson et al., 1998). Unlike Akt, PDK-1 is primarily found in a constitutively active state due to autophosphorylation of its activation loop at S241 (Mora et al., 2004). While mTORC2 is considered the primary S473 kinase, other kinases have been proposed to phosphorylate this site. These include autophosphorylation by Akt, Protein Kinase C (PKC), and Integrin-Linked kinase (ILK) (Kawakami et al., 2004; Li et al., 2006; Persad et al., 2001; Toker and Newton, 2000).

The level of PI(3,4,5)P3 is regulated by Phosphatase and Tensin Homolog deleted on chromosome ten (PTEN). PTEN is a phosphatidylinositol 3' phosphatase that catalyzes the conversion of PI(3,4,5)P3 into PI(4,5)P2 and thus terminates PI3K-induced activation of Akt (Maehama and Dixon, 1998; Myers et al., 1998). Given its critical role as a negative regulator of PI3K signaling, loss of function mutations/deletions of PTEN are frequently observed in human cancers (Yuan and Cantley, 2008). Another important negative regulator of Akt is the Inositol polyphosphate 4-phosphatase type II (INPP4B). INPP4B catalyzes the dephosphorylation of PI(3,4)P2 at the 4' position, to yield PI(3)P (Gewinner et al., 2009). Similar to PTEN, genetic aberrations that result in loss-of-function of INPP4B, and thus constitutive Akt activation, are frequently found in human cancers. This is particularly predominant in basal-like breast cancers (Fedele et al., 2010). Once activated, Akt localizes to various cellular compartments where it phosphorylates a large number of downstream substrates that regulate virtually all aspects of cell biology, including cell survival, proliferation, cell motility, and metabolism (Manning and Cantley, 2007).

One of the best-described roles for the PI3K/Akt signaling axis in both normal and pathophysiological conditions involves its promotion of cell growth and survival (Roymans and Slegers, 2001). In fact, one of the first characterized Akt substrates was the pro-apoptotic protein, BCL-2 agonist of cell death (BAD) (Datta et al., 1997; del Peso et al., 1997). Akt phosphorylation of BAD at S136 creates a binding site for the adaptor protein 14-3-3 (Zha et al., 1996). This prevents BAD from interacting with the pro-apoptotic proteins BCL-2 and BCL-X_L, which consequently results in suppression of apoptosis (Zha et al., 1996). Similarly, Akt can phosphorylate members of the forkhead-related transcription factor family to promote association with 14-3-3 (Brunet et al., 1999). This interaction results in the occlusion of these transcription factors from the nucleus and thus a decrease in the transcriptional up-regulation of cell-death promoting genes (Biggs et al., 1999; Kops et al., 1999). Other examples include Akt phosphorylation of the protease caspase 9, which inhibits its protease activity and reduces cell death (Cardone et al., 1998). Akt substrate phosphorylation has also been shown to play an important role in the regulation of cell cycle progression. This is exemplified by the proteasomal degradation of p27 following Akt induced phosphorylation of S phase kinase-associated Protein 2 (SKP2) and formation of the Skp, Cullin, F-box containing (SCF) complex (Gao et al., 2009).

There are three isoforms of Akt: Akt1, Akt2 and Akt3. Each isoform is transcribed from a distinct gene (Vivanco and Sawyers, 2002). Their mechanisms of activation are similar and involve lipid-mediated membrane localization and phosphorylation by PDK1 and mTORC2. In addition, all three isoforms share a consensus Akt substrate motif RXRXXS/T, with a preferred bulky hydrophobic residue at the +1 position relative to the

phospho-acceptor site, and an Arginine at the -3 and -5 positions (Alessi et al., 1996; Hutti et al., 2004; Obata et al., 2000). Despite these similarities, Akt1, Akt2 and Akt3 appear to have both redundant and non-redundant roles, which may be context dependent and tissue specific. For example, Akt1 and Akt2 appear to have opposing roles in cell migration and invasion in breast cancer. While Akt2 promotes breast cancer invasion, Akt1 inhibits breast cancer migration and invasion, *in vitro* and *in vivo* (Arboleda et al., 2003; Hutchinson et al., 2004; Irie et al., 2005; Yoeli-Lerner et al., 2005). This is mediated in part through down-regulation of nuclear factor of activated T cells (NFAT), regulation of the extracellular signal-regulated kinase (ERK) signaling pathway, and phosphorylation and inhibition of the actin-bundling protein, Paladin (Chin and Toker, 2010; Irie et al., 2005; Yoeli-Lerner et al., 2005) . Akt isoform-specific signaling is also dictated by the presence of selective phosphatases that inactivate Akt signaling. For example, it has been shown that the serine/threonine phosphatase, PH domain and Leucine-rich Protein Phosphatases 1 (PHLPP1), preferentially dephosphorylates Akt2 and Akt3 at S474 and S472 respectively. In contrast, PHLPP2 dephosphorylates Akt1 and Akt3 at these cognate sites (Brognard et al., 2007).

The mammalian Target of Rapamycin complex 1, (mTORC1), is another critical signaling node downstream of the PI3K pathway. Its role in cell growth, proliferation and metabolism have been widely documented (Wullschleger et al., 2006). It belongs to the Class IV PI3Ks superfamily, which also consists of ATM (Ataxia Telangiectasia Mutated), ATR (ataxia telangiectasia and Rad3), DNA-PK (DNA-dependent Protein Kinase), and SMG-1 (SMG1 homolog, phosphoinositol 3-kinase-related kinase). Notably, Akt is an important regulator of mTORC1 activation. Phosphorylation of the

tuberous sclerosis complex1/2 (TSC1/2) by Akt, abrogates its inhibitory rheb GTPase activity (Inoki et al., 2002; Manning et al., 2002). Consequently, Akt activation leads to mTORC1 activation. Akt can also facilitate mTORC1 activation directly, through phosphorylation of Proline-rich Akt substrate of 40 KDa, (PRAS40). This phosphorylation event promotes the binding of PRAS40 to 14-3-3, which results in de-repression of mTORC1 inhibition (Vander Haar et al., 2007). Activation of mTORC1 results in phosphorylation of the Eukaryotic Translation Initiation factor (4EBP1) and S6K1 (p70S6 kinase 1) (Brunn et al., 1997a; Brunn et al., 1997b; Gingras et al., 1999; Hara et al., 1997; Heesom and Denton, 1999; Holz et al., 2005). This promotes cap-dependent protein translation, and protein synthesis. mTORC1 can also regulate other downstream substrates to mediate its effect on cell homeostasis (Laplante and Sabatini, 2012). It is noteworthy, that prolonged activation of mTORC1 and its target S6K1, can result in feedback inhibition of insulin or insulin-like growth factor 1-driven PI3K signaling (Harrington et al., 2004; O'Reilly et al., 2006). This results from S6 kinase induced phosphorylation and degradation of IRS1 as well as suppression of IRS1 mRNA (Shah and Hunter, 2006).

Several pan-Akt substrates have been identified over the last couple of years using a combination of bioinformatics and proteomic approaches. To date, it has been proposed that there are over 200 putative Akt substrates, although several of these will require further experimental validation (Manning and Cantley, 2007). Additionally, Akt – isoform specific substrates have also been discovered. For example, Palladin, an actin bundling protein has been described as an Akt1 specific substrate (Chin and Toker, 2010). Phosphorylation of this protein by Akt1, leads to a decrease in breast cancer

invasion and migration. Skp2 is another example of an Akt1 specific substrate (Gao et al., 2009). Akt2 specific substrates have also been documented. Examples of these include AS160 which plays a role in glucose uptake and Myosin5a which functions in glut4 vesicular translocation (Cho et al., 2001; Garofalo et al., 2003; Gonzalez and McGraw, 2009; Yoshizaki et al., 2007). Thus far, no Akt3 specific substrates have been discovered, although research efforts in this area are ongoing. Together, these studies will provide supportive evidence for the non-redundant phenotypic roles of Akt isoforms in various malignancies and cellular context.

Transcriptional responses to PI3K pathway activation

Propagation of PI3K signal transduction ultimately affects many transcriptional events through direct or indirect regulation of the activity of various transcription factors. As described earlier, Akt directly regulates the activity of the Forkhead family of transcription factors. Akt can also modulate p53-mediated transcription via phosphorylation of MDM2, an important ubiquitin ligase of p53. Direct phosphorylation of MDM2 by Akt at S186 promotes MDM2 ubiquitinating-function and subsequent degradation of p53 (Ogawara et al., 2002). Activation of pS6K by the PI3K/mTORC1 signaling axis has also been showed to affect gene expression (Hannan et al., 2003). PI3K signaling can also lead to the indirect activation of other important transcription factors including HIF1α, STAT3 and NF-κB (Hennessy et al., 2005). This often occurs through PI3K-dependent production of cytokines and secretory factors that eventually activate signaling pathways which directly regulates these transcription factors (Hart et al., 2011; Hutti et al., 2012). Additionally, the PI3K pathway may modulate gene

expression through the employment of Akt in the regulation of miRNA expression and epigenetic modifications (Cha et al., 2005; Iliopoulos et al., 2009; Zuo et al., 2011). Ultimately, these transcriptional events dictate the cells' fate under various conditions.

2. ONCOGENIC MUTATIONS IN THE PI3K PATHWAY

The significance of PI3K signaling in breast cancer is underscored by the high frequency of genetic aberrations found in genes that constitute this pathway. These mutations/amplifications often lead to hyperactivation of PI3K signaling and occur at the level of activators and regulators, PI3K isoforms, and transducers of PI3K signaling (**Table 1-1**).

Currently, class IA PI3K is most clearly implicated in human cancer (Yuan and Cantley, 2008). Activating mutations of the *PIK3CA* gene, encoding for the catalytic isoform p110α, have been identified in 18% - 40% of breast cancers (Cancer Genome Atlas Research, 2008; Samuels et al., 2004; Thomas et al., 2007). The mutations are mainly single amino acid substitutions with the majority occurring in two hotspot regions, including the central helical domain encoded by exon nine, and the carboxyl terminal kinase domain, encoded by exon twenty (Yuan and Cantley, 2008). Although the exact mechanism by which these non-synonymous missense mutations facilitate increase lipid kinase activity is not clearly defined, it is postulated that they promote the de-repression of the inhibitory interaction between p110α with p85 (Huang et al., 2008; Huang et al., 2007; Lee et al., 2007; Miled et al., 2007; Zhao et al., 2005). Overexpression of the two most common *PIK3CA* mutations, *E545K* (helical domain) and *H1047R* (kinase domain), in chicken embryo fibroblasts and NIH3T3 cells result in increased PI3K activity, Akt activation and cellular transformation (Bader et al., 2006). Moreover, these *PIK3CA* mutations have been shown to induce oncogenic transformation of mammary epithelial cells and form heterogeneous mammary tumors *in vivo* (Isakoff et al., 2005; Liu et al., 2011). No oncogenic mutations have been

Table 1-1: Frequency of genetic alterations in the PI3K pathway in breast cancer. Table shown was adapted from (Liu et al., 2009).

Genetic alteration	Cancer type	Incidence of alterations
p110α (PIK3CA)		
Mutations	Breast	27%
	Endometrial	24%
	Colon	15%
	Upper Digestive tract	11%
	Gastric	8%
	Pancreas	8%
	Ovarian	8%
	Liver	6%
	Brain	5.9%
	Esophageal	5%
	Melanoma	9%
	Urinary tract	17%
Amplifications	Lung (Squamous Cell)	53%
	Lung (Non-Small Cell)	12%
	Cervical	69%
	Breast	8.7%
	Head and Neck	32.2%
	Gastric	36%
	Cervical	9%
	Endometrial	10%
	Ovarian	11.9%
	Glioblastoma	6.1%
p110β (PIK3CB)		
Amplifications	Ovarian	5%
	Breast	5%
Increase in activity and expression	Colon	70%
	Bladder	89%
PDK1		
Amplification and overexpression	Breast	20%

Table 1-1 Continued: Frequency of genetic alterations in the PI3K pathway in breast cancer. Table shown was adapted from (Liu et al., 2009).

Genetic alteration	Cancer type	Incidence of alterations
AKT		
AKT1 mutation (E17K)	Breast	3.7%
	Colon	2.8%
	Ovarian	2%
	Lung	1.9%
AKT1 amplifications	Gastric	20%
AKT2 amplifications	Ovarian	14.1%
	Pancreas	20%
	Head and Neck	30%
	Breast	3%
AKT3 mutation (E17K)	Skin	1.5%
AKT3 amplifications	Glioblastoma	2%
p85α (PIK3R1)		
Mutations	Glioblastoma	9.9%
	Ovarian	4%
	Colon	2%
PTEN		
Loss of heterozygosity	Gastric	25.3%
Mutations	Breast	24.9%
	Melanoma	37%
	Prostate	30%
	Glioblastoma	28%
Mutations	Endometrial	38%
	Brain	21%
	Skin	17%
	Prostate	14%
	Large Intestine	13%
	Ovary	9%
	Breast	6%
	Stomach	6%
	Liver	5%
	Kidney	5%

observed in the PI3K isoforms p110β, p110δ, p110γ, although their oncogenic potential has been demonstrated (Zhao et al., 2005) (Kang et al., 2006). On the other hand, amplification of these isoforms have been detected in various primary tumors and cancer cell lines (Courtney et al., 2010).

Mutations in *PIK3R1* gene encoding for p85α, have also been found in many human cancers including glioblastomas, ovarian cancers and colorectal cancers (Mizoguchi et al., 2004; Philp et al., 2001). Most of these mutations arise from truncations, or in-frame deletions found in the inter-SH2 domain. Similar to *PIK3CA*, it is hypothesized that these mutations result in constitutive PI3K signaling due to disruption of the inhibitory interaction between p85α with p110α (Philp et al., 2001; Shekar et al., 2005).

Akt1 amplification and somatic mutations have also been observed in various cancers including breast, colorectal, endometrial, ovarian and melanoma (Courtney et al., 2010). In breast cancer, Akt1 E17K was mutated at a frequency of ~2-8% (Yuan and Cantley, 2008). This mutation is located in the lipid binding PH domain of Akt1 and likely facilitates constitutive growth-factor independent membrane localization even in the absence of PI(3,4,5)P3 (Carpten et al., 2007; Stemke-Hale et al., 2008). The analogous mutation has also been observed for Akt2 and Akt3 albeit at a much lower frequency to Akt1 E17K (Davies et al., 2008; Parsons et al., 2005).

Signaling through RTKs is one of the most common mechanisms for PI3K activation. The frequent mutation, amplification or overexpression of RTKs in human cancer, results in hyperactivation of PI3K signaling. In particular, amplification or overexpression of the human epidermal growth factor receptor 2, HER2, is found in

about 20% of breast cancer and appears to coexist with *PIK3CA* mutations and PTEN loss (Yuan and Cantley, 2008). In lung cancer, somatic mutations in epidermal growth factor receptor, EGFR, is also very frequent. Upon stimulation, both EGFR and HER2 interact with the kinase-dead HER3, which leads to direct recruitment and subsequent activation of PI3K (Engelman and Settleman, 2008; Holbro et al., 2003; Moasser et al., 2001). Because of their critical role as activators of PI3K, as well other important signaling cascades, drugs targeting these receptors are under clinical evaluation for the treatment of cancer. Overexpression of the non-receptor protein tyrosine kinase, c-Src, is also observed in a large percentage of breast cancers (Biscardi et al., 1998; Verbeek et al., 1996). C-Src plays a functional role in EGFR-mediated PI3K activation and various Src inhibitors are currently being developed and tested (Biscardi et al., 2000).

RAS mutations occur in ~30% of lung adenocarcinomas and are found in almost all pancreatic ductal adenocarcinoma (Cox et al., 2014). In contrast, they occur with a lower frequency of ~5% in breast cancer (Cox et al., 2014). PI3K signaling plays a fundamental role in the initiation of many RAS-driven tumors. However in some instances, these tumors become less reliant on PI3K during tumor maintenance and progression (Yuan and Cantley, 2008). In the case of endometrial cancers, RAS mutations are mutually exclusive with mutant *PIK3CA* whereas they are found to co-exist, in colorectal cancers (Yuan and Cantley, 2008). These differences may be indicative of the evolution of the tumors and while *PIK3CA* may offer a fitness advantage in one type of cancer, in another it may be detrimental. This is possible in a scenario where co-existence of oncogenic RAS and mutant *PIK3CA* leads to oncogenic-induced senescence.

In human cancers, inactivation of the tumor suppressor PTEN is the most frequent genetic alteration in the PI3K pathway (Courtney et al., 2010). Loss-of-function of PTEN leads to unrestrained PI3K activity due to the accumulation of PI(3,4,5)P3 (Haas-Kogan et al., 1998; Myers et al., 1998). Several modes of PTEN inactivation have been documented. Somatic mutations, which result in a truncated PTEN protein, and loss-of-function missense mutations, which render PTEN phosphatase inactive, have been described (Han et al., 2000). Although PTEN mutations are predominantly somatic, germ line mutations in PTEN are causal in autosomal dominant hamartoma tumor syndromes such as Cowden disease (Liaw et al., 1997). Homozygous and hemizygous loss of PTEN has also been documented (Li et al., 1997; Sun et al., 2009). More recently, transcriptional silencing and epigenetic regulation of PTEN has been shown to promote PTEN inactivation (Garcia et al., 2004; Goel et al., 2004). Recently, it was observed that p110β is the predominant PI3K isoform in PTEN-deficient tumors (Wee et al., 2008).

3. PI3K PATHWAY INHIBITORS IN CLINICAL DEVELOPMENT FOR CANCER

Activation of the PI3K signaling pathway contributes to virtually all aspect of cancer development from tumor initiation to metastasis. Given the critical role of PI3K in cancer pathogenesis, several pharmacological inhibitors targeting various nodes of the PI3K pathway are currently being evaluated in preclinical studies and early human clinical trials (**Figure 1-4**).

PI3K inhibitors

Early attempts at inhibiting the PI3K pathway involved targeting PI3K itself using first generation PI3K inhibitors such as wortmannin and LY294002. While these inhibitors were very instrumental in furthering our understanding of PI3K signaling and provided a blueprint for future PI3K inhibitors, they lack PI3K isoform specificity and display high toxicity levels in animal studies (Liu et al., 2009). Wortmannin derivatives with improved pharmacologic properties were consequently developed (Blois et al., 2008). One such example is the Pan-Class IA PI3K inhibitors, PX-866. Both PTEN and *PIK3CA* mutation status appear to correlate with improved sensitivity to PX-866 (Oncothyreon) (Ihle et al., 2009). Despite the cytostatic antitumor effects observed *in vivo*, animals treated with PX-866 develop hyperglycemia (Ihle et al., 2005). However, this may be managed by administration of anti-diabetic drugs.

To date, a number of Pan-PI3K inhibitors are being tested in clinical trials. BKM120 (Norvatis), a class I PI3K ATP-competitive inhibitor has entered phase I clinical trial (Liu et al., 2009). The quinoxaline derivative, XL147 (Exelixis) is another Class I PI3K inhibitor that is being assessed in phase I clinical trials for the treatment of solid

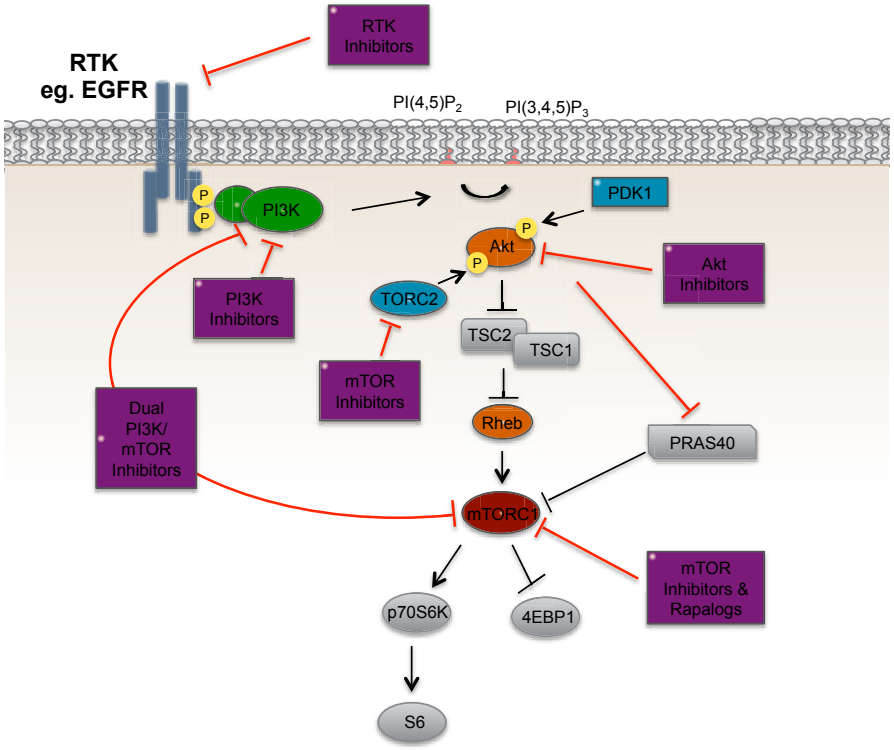

Figure 1-4: Strategies for targeting the PI3K pathway for cancer treatment. Pharmacological inhibitors designed to block the activity of critical PI3K signaling proteins are being evaluated for the treatment of breast cancer. These include RTK inhibitors such as HER2/EGFR inhibitors, pan/isoform-specific PI3K inhibitors, Akt inhibitors, mTOR inhibitors, rapalogs, and dual PI3K/mTORC inhibitors. Adapted from (van der Heijden and Bernards, 2010).

tumors. So far, this inhibitor has resulted in stable disease control in 6 of 39 patients; with minimal effect on plasma glucose levels although increases in food-induced plasma insulin levels were observed (Shapiro et al., 2009). GDC-0941 (Piramed/Genentech) is another promising Class I PI3K inhibitor that can inhibit PI3K activity in the nanomolar range (Liu et al., 2009). This drug displayed robust antitumor effects in preclinical xenograft models and is currently in phase I clinical trial for the treatment of advanced solid tumors or lymphomas (Wagner et al., 2009). Other Pan-PI3K inhibitors not mentioned above have entered clinical trials (Liu et al., 2009).

PI3K isoform-specific inhibitors have also been developed and are currently being examined in preclinical studies. One postulated rationale for the use of these inhibitors is that more effective pathway inhibition maybe achieved at tolerable doses with less severe side effects (Courtney et al., 2010). For example, the Class IA PI3K inhibitor PX-866 (Oncothyreon) shows *in vitro* potency at low nanomolar concentrations (Ihle et al., 2005). Furthermore, a p110δ-specific inhibitor CAL-101 (Calistoga) under clinical evaluation for refractory hematological cancers, shows promising responses in patients (Flinn et al., 2009).

Akt inhibitors

Given that Akt is a critical mediator of PI3K-driven malignancies, drugs targeting this kinase are actively pursued. Akt inhibitors can be classified into two major groups based on their mode of action. This includes (1) ATP mimetics and (2) non-catalytic site inhibitors, which includes allosteric inhibitors and lipid-based phosphatidylinositol (PI) analogs. Many Akt ATP competitive inhibitors lack isoform specificity (Liu et al., 2009).

This includes the pan-Akt inhibitor GSK690693 (GlaxoSmithKline), which shows inhibitory effects in the nanomolar range (Rhodes et al., 2008). Cancers harboring oncogenic Akt genetic aberrations are expected to display greater sensitivity to Akt inhibitors (Courtney et al., 2010).

One of the most clinically advanced Akt inhibitor is Perifosine (Hilgard et al., 1997). This drug is a PI analog that disrupts the interaction between the PH domain of Akt and PI(3,4,5)P3. This prevents the translocation of Akt to the plasma membrane and thus inhibits Akt activation, in response to PI3K. Additional Akt PH domain inhibitors have shown promising signs in preclinical studies. These include PX316 (Pro1X Pharmaceutical) and phosphatidylinositol ether lipid analog (PIAs) (National Cancer Institute/Georgetown University) (Gills and Dennis, 2004; Meuillet et al., 2004). The Pan-Akt allosteric inhibitor MK2206, displayed potent antitumor activity in xenograft studies and has advanced to phase I clinical trial for advanced and metastatic solid tumors (Liu et al., 2009). Recent reports indicate early signs of stable disease in six of the 19 ovarian cancer patients (Tolcher et al., 2009). Similar to Pan-PI3K inhibitors, adverse side effects like hyperglycemia and skin disorders are observed.

In addition, Akt isoform-specific inhibitors are currently being developed. This may be beneficial in light of recent findings indicating non-redundant and sometimes opposing roles of Akt isoforms in various cellular context and cancer-type.

While targeting Akt may be an effective approach for some cancers dependent of Akt signaling, it may prove to be less efficacious in others. This may be the case in cancers that rely on other important Akt-independent PI3K activated effectors such as SGK3 (Vasudevan et al., 2009). Furthermore, long term inhibition of Akt may result in

relief of negative feedback loops that lead to reactivation of PI3K signaling (Courtney et al., 2010).

mTORC inhibitors

Rapamycin is the prototypic mTORC1 inhibitor. It is a fungicide and immunosuppressant, originally isolated from *Streptomyces hygroscopius* (Vezina et al., 1975; Yatscoff et al., 1993). Rapamycin binds directly to the intracellular receptor FK506-binding protein-12 (FKBP12). Interaction of the Rapamycin-FKBP12 complex with mTORC1 leads to suppression of mTORC1 activity (Guertin and Sabatini, 2009). Consequently, treatment with rapamycin inhibits cell growth (size), causes cell cycle arrest, and induces cell death through activation of autophagy or apoptosis (Sabatini, 2006). It has also been shown to inhibit tumor angiogenesis and affects the tumor microenvironment (Sabatini, 2006). Other more advanced rapalogs (derivatives of rapamycin) have been synthesized. These agents display more favorable pharmacokinetic properties and show effective antitumor activity in preclinical models. These include analogs like temsirolimus (Torisel ,Wyeth), everolimus (Novartis) and deforolimus (Merck/Ariad) (Liu et al., 2009). Animal studies and clinical trials have shown that single-agent administration of rapalogs produces a cytostatic response resulting in effective disease stabilization but not tumor regression (Courtney et al., 2010). One reason for the limited efficacy of rapalogs may be due to their incomplete inhibition of mTORC1. Despite these findings, several rapalogs have been FDA-approved for renal cell carcinoma, mantle cell lymphoma and neuroendocrine tumors (Atkins et al., 2004; Faivre et al., 2006; Fruman and Rommel, 2014).

Second generation ATP-competitive inhibitors that target both mTORC1 and mTORC2 have also been developed. In addition to their improved potency, these agents may prove advantageous by also suppressing Akt activation by mTORC2. This becomes particularly important in light of the fact that long suppression of mTORC1 leads to pS6/IRS1-mediated re-activation of Akt signaling. While this rationale is theoretically sound, studies with dual mTORC1/mTORC2 inhibitors such as Torin and PP242 appear to display improved antitumor effects due to better inhibition of mTORC1 rather than additional mTORC2 inhibition (Feldman et al., 2009; Thoreen et al., 2009).

Dual PI3K-mTORC inhibitors

The catalytic domain of PI3K and mTORC share structural features that render many PI3K or mTORC (1/2) ATP-competitive inhibitors effective in suppressing the catalytic activity of both kinases (Fry, 2001). Theoretically, these dual PI3K-inhibitors might be quite beneficial and effective in shutting down PI3K signaling, as they will be capable of targeting all PI3K isoforms and both mTORC1 and mTORC2. However, it remains to be determined whether the dosage required for complete inhibition of each of these signaling nodes by these dual inhibitors will be achievable at tolerable doses in human cancer patients.

SF-1126, a derivative of LY294002, is an example of a dual PI3K-mTOR inhibitor under evaluation in clinical trials. Although tumor regression was not observed, tumor stasis was observed in many patients with minimal toxicity issues (Chiorean et al., 2009; Garlich et al., 2008). Likewise, another dual PI3K-mTOR inhibitor, BEZ235 also displayed tumor stasis with tolerable side effects in PTEN-deficient human cancer cell

line xenograft studies (Maira et al., 2008). Notably, treatment of BEZ235 in estrogen-deprived estrogen receptor-positive breast cancer cells containing either *PIK3CA* or *PIK3CB* amplification resulted in apoptosis (Crowder et al., 2009). This finding underscores the need for stratifying patients, to determine which ones are most like to benefit from these various PI3K-pathway inhibitors. Other PI3K-mTORC inhibitors in clinical trials include: NVP-BGT226 (Novartis) and XL765 (Exelixis) (Courtney et al., 2010).

4. ALTERNATIVE DRUG COMBINATIONS WITH PI3K PATHWAY INHIBITORS

Because most single agent PI3K pathway inhibitors result in a cytostatic effect resulting in tumor stasis instead of overt tumor shrinkage, combination of PI3K pathway inhibitors with other important survival pathways are being evaluated. These combination strategies should be rationally designed to take into account whether the expected therapeutic benefit outweighs any toxicity issues, cost and complexity of the drug treatment. Below are some proposed cancer treatment strategies that involve PI3K pathway inhibitors.

PI3K and tyrosine kinase inhibitors (TKI)

Several mechanistic studies have provided justification for combining PI3K pathway inhibitors with various tyrosine kinase inhibitors. While many tyrosine kinase inhibitors including EGFR and HER2 inhibitors, can initially display impressive therapeutic outcomes, these are often stymied by the emergence of resistance mechanisms that often involve activation of the PI3K pathway (Rexer and Arteaga, 2013). For example, breast cancer cell lines resistant to HER2 tyrosine kinase inhibitor lapatinib have been shown to acquire hotspot mutations in *PIK3CA* that contributes to uncoupling of PI3K signaling with the HER2 receptor (Rexer et al., 2014). Furthermore, HER2+ xenograft studies showed that co-treatment with BKM120 and trastuzumab and lapatinib result in improved tumor regression and decreases tumor relapse (Rexer et al., 2014).

The converse is also true – resistance to PI3K inhibitors can result from Foxo-mediated overexpression of various tyrosine kinases. In this scenario, co-administration

of PI3K pathway inhibitor with the respective tyrosine kinase inhibitor should circumvent this resistance mechanism and lead to improve therapeutic outcome. Supporting this rationale is one study, which demonstrated that resistance to the dual PI3K/mTORC1 inhibitor PF-04691502 can be attributed to FOXO3a-mediated transcriptional induction of the ERBB family members (EGFR, HER2 and HER3) in a KRAS mutant colorectal cancer model (Belmont et al., 2014). Moreover, treatment of Pan-ERBB inhibitor, Dacomitinib restored sensitivity to PF-04691502 and resulted in tumor regression in PF-04691502 resistant allografts in mice (Belmont et al., 2014). Combination of PI3K inhibitors with TKIs may prove beneficial. However, the challenge will be in determining which TKIs should be targeted, as many TKs are often overexpressed/re-activated in PI3K resistant tumors (Liu et al., 2009).

PI3K and STAT3 pathway inhibitors

Combined inhibition of the PI3K/Akt/mTORC1 signaling pathway with agents targeting the STAT3 signaling axis has also been proposed in light of emerging studies illustrating their interdependency. For example, STAT3 plays an essential role in *PIK3CA*-induced oncogenic transformation in immortalized mammary epithelial cells (Hart et al., 2011). Furthermore, re-activation of STAT3 can contribute to resistance to PI3K inhibition and agents targeting STAT3 or its upstream activators can lead to improved sensitivity to PI3K inhibitors (Vogt and Hart, 2011).

PI3Ks and MEK/ERK pathway inhibitors

There is extensive cross talk between the PI3Ks pathway and the RAS/MEK/ERK signaling pathway, which also plays a critical role in cancer cell growth and survival. For instance, long-term inhibition of mTORC1 can lead to phosphorylation of ERK and inhibition of MEK can abrogate PTEN membrane recruitment and increase Akt activity (Carracedo et al., 2008; Kinkade et al., 2008; Zmajkovicova et al., 2013). The observation that the MAPK pathway is often re-activated upon PI3K inhibition underscores the potential therapeutic benefit of targeting these two important pathways. In fact, several studies have shown that PI3K inhibitors can synergize with MEK/ERK inhibitors in various cancer models including KRAS-driven lung cancer and NRAS mutant melanoma (Engelman et al., 2008; Posch et al., 2013). One proposed mechanism for this synergistic effect involves the stabilization and transcriptional up-regulation of pro-apoptotic proteins such as BIM, PUMA and BAD (Liu et al., 2009). Although dual PI3K/MAPK inhibition might have great therapeutic potential, both pathways play an essential role in normal physiology and achieving a therapeutic window might prove challenging. However, optimization of dosing and scheduling regimens might help to mitigate any arising toxicity effects. Furthermore, using combinations with inhibitors targeting other effectors of these pathways might provide more tolerable avenues.

Targeting Myc in combination with the PI3K pathway

Amplification of the oncogene Myc has also been attributed to resistance to PI3K inhibitors. In a mouse model of mutant-*PIK3CA* breast cancer, amplification of *c-Myc*

was shown to play a causal role in resistance against the PI3K inhibitor GDC-0941 (Liu et al., 2011). Cooperation between Myc and PI3K signaling is also evident in various hematological malignancies such as Burkitt lymphoma (Sander et al., 2012; Schmitz et al., 2012). Given that BET (bromo and extra terminal) proteins are transcriptional regulators of Myc, combination of BET inhibitors with PI3K pathway inhibitors might be beneficial (Liu et al., 2009). Additionally, use of NOTCH inhibitors to decrease Myc activity, along with a PI3K pathway inhibitor, might be useful in T-cell acute lymphoblastic leukemia (T-ALL) (Liu et al., 2009). This cancer might be particularly sensitive to this drug combination given the high frequency of PTEN loss and NOTCH mutations (Grabher et al., 2006; Guo et al., 2011).

PI3K and DNA damaging drugs

The combination of PI3K inhibitors with DNA-damaging agents is also another attractive therapeutic strategy. PI3K inhibitors have been shown to promote DNA damage, and improve the efficacy of PARP inhibitors in triple negative breast cancers (Ibrahim et al., 2012; Kao et al., 2007; Kumar et al., 2010). Consequently early clinical trials with PARP inhibitor Olaparib and BKM120 are underway for triple negative breast cancer patients and high-grade serous ovarian cancer patients (Liu et al., 2009). PTEN-deficient tumors may also benefit from this drug combination as evident by preclinical studies (Bassi et al., 2013). While the DNA-repair enzymes ATM, ATR and DNA-PK are part of the PI3K kinase related family, it is worth considering whether inhibition of these kinases would synergize with DNA-damaging drugs. Although many of the PI3K

pathway inhibitors show improved specificity, they can display off-target effects at higher concentrations and consequently inhibit members of this pathway.

One promising approach to improve the efficacy of PI3K inhibitors while minimizing toxicity, would be to combine PI3K inhibitors with BCL2 family antagonists. BCL2 family members suppress the activity of pro-apoptotic proteins such as BAX and BAK and maintain mitochondrial integrity (Davids and Letai, 2012). Thus, treatment with the BCL2 antagonist should theoretically lower the level required for cell death in response to PI3K inhibition (Davids and Letai, 2012).

Alternative approaches with PI3K inhibitors

The importance of the immune system and the tumor microenvironment in cancer pathogenesis is a well-known phenomenon. Chronic inflammation resulting in the recruitment of immune cells such as macrophages, dendritic cells, T cells, mast cells and natural killer cells occurs during tumor initiation (Yuan and Cantley, 2008). While the immune system initially responds in restraining tumorigenesis, a reprogramming of the tumor cells and the tumor microenvironment, that favors immune evasion and tumor tolerance, eventually ensues (Chen and Mellman, 2013; Motz and Coukos, 2013). Thus, it is conceivable that drug combinations comprising of PI3K inhibitors with immunotherapies might be efficacious. In theory, this drug combination should promote immune rejection of the tumor, while simultaneously providing direct anti-tumor effects (Liu et al., 2009). However, the complexity of the relationship between the PI3K pathway and the immune system must be carefully considered. Inhibition of the PI3K/Akt/mTOR network can enhance or suppress the efficacy of immunotherapies

or cancer vaccines (Fruman and Bismuth, 2009; Thomson et al., 2009). In these cases, isoform-specific PI3K inhibitors might be even more relevant. In addition, drug combinations that include PI3K pathway inhibitors with anti-angiogenic agents or small molecule antibodies against various cytokines and hormone receptors, may be beneficial.

Summary

The drug combinations discussed above hold great potential for cancer treatment. However, patient stratification, dosing schedule and the type of PI3K inhibitors (Pan-PI3K, PI3K-isoform or dual PI3K/mTORC) employed in early clinical trials, should be carefully considered. In this thesis, we examine the efficacy of aspirin in combination with various PI3K inhibitors in PI3K-addicted breast cancer. We also explore novel effectors of the PI3K pathway that might facilitate malignant transformation.

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CHAPTER II

INVESTIGATING THE THERAPEUTIC EFFECTS OF ASPIRIN IN PI3K-DRIVEN BREAST CANCER

Some of the material described in this chapter will be submitted as part of a manuscript for the peer-reviewed journal, *Cancer Discovery*.

1. ABSTRACT

The phosphoinositide 3-kinase (PI3K) pathway plays a critical role in breast cancer malignancy. This is underlined by the high incidence of mutation in *PIK3CA*, the gene encoding the catalytic subunit of PI3K. While targeting the PI3K pathway is a viable strategy, many PI3K pathway inhibitors have yet to show significant clinical outcome. Here we investigate the potential chemotherapeutic benefit of aspirin in PI3K-driven breast cancer. We demonstrate that mutant *PIK3CA* breast cancer cells show a dose-dependent decrease in cell viability and anchorage-independent growth in soft agar, when treated with increasing concentrations of aspirin. Co-treatment of aspirin sensitizes mutant *PIK3CA* breast cancer cells to PI3K inhibitors to enhance suppression of proliferation. Additionally, immortalized mammary epithelial cells expressing mutant *PIK3CA H1047R* show greater sensitivity to aspirin when compared to cells expressing wild-type *PIK3CA* grown in three-dimensional (3D) Matrigel culture. Mechanistic studies indicate that the cell-autonomous growth inhibitory effect of aspirin can be ascribed to activation of AMP-activated protein kinase (AMPK), inhibition of mammalian target of rapamycin complex 1 (mTORC1) signaling and induction of autophagy. Importantly, this growth suppression is independent of the activities of cyclooxygenase-2 (COX-2) and nuclear factor kappa B (NF-κB). Together, our study supports the evaluation of aspirin in combination with PI3K pathway inhibitors in pre-clinical *in vivo* models and in human clinical trials, for the treatment of PI3K-driven breast cancer.

2. INTRODUCTION

The phosphoinositide 3-kinase (PI3K) signaling pathway plays a critical role in cell growth, survival, motility, and metabolism (Engelman, 2009). Deregulated PI3K signaling is observed in numerous human pathophysiologies, including cancer. In breast cancer, somatic mutations in genes that encode proteins that activate, terminate or transduce PI3K signaling are highly prevalent. Specifically, somatic mutations in *PIK3CA*, the gene encoding the catalytic subunit p110α, occur with a frequency of approximately 40% across all breast cancer molecular subtypes (Engelman et al., 2006; Zhao and Vogt, 2008). The two most frequent mutations comprise single amino acid substitutions in two hotspot regions, His1047Arg and Gln545Lys (Zhao and Vogt, 2008). Expression of these two mutations leads to elevated PI3K activity, downstream Akt activation, oncogenic transformation of mammary epithelial cells and formation of heterogeneous mammary tumors *in vivo* (Kang et al., 2005; Liu et al., 2011; Samuels et al., 2004; Yuan et al., 2013). Similarly, the lipid phosphatase PTEN that terminates PI3K signaling is one of the most frequently mutated tumor suppressors in human cancers. Mutation or loss of at least one copy of *PTEN* occurs in approximately 50% of breast cancer patients, leading to hyperactivation of PI3K/Akt signaling (Pandolfi, 2004). In addition, amplification and mutation of Akt genes have also been identified in breast cancer, albeit with lower frequencies (Carpten et al., 2007; Stephens et al., 2012).

Given the frequency of mutations in the PI3K/PTEN/Akt pathway in breast cancer, numerous small molecule inhibitors have been developed for targeted therapy and are under clinical evaluation. These include pan- and p110 isoform-specific inhibitors, compounds that inhibit both PI3K and the downstream effector mTOR, and

also pan-Akt inhibitors. To date, most of these inhibitors have shown limited efficacy in clinical trials due to dose-limiting toxicities as well as the emergence of resistance mechanisms. However, combination therapies that target both PI3K/PTEN/Akt and other key survival pathways may achieve better therapeutic responses.

Aspirin (acetylsalicylic acid) is one the most frequently administered non-steroidal anti-inflammatory drugs (NSAID) (Fuster and Sweeny, 2011; Norn et al., 2009). Its medicinal use for the treatment of pains, fevers and inflammatory ailment dates back to the time of Hippocrates. Aspirin is also widely used as an antiplatelet drug for the prevention of heart attacks and strokes (Hennekens et al., 1997). Recently, a number of observational and randomized clinical trials have suggested that regular use of aspirin has a chemotherapeutic effect in several cancers, including breast cancer (Baron et al., 2003; Rothwell et al., 2011; Rothwell et al., 2012). Although the effect of aspirin on breast cancer incidence remains poorly understood, recent studies from the Nurses Health Study indicate that aspirin use is associated with a reduced risk of breast cancer distant recurrence and death (Holmes et al., 2010). Additional independent observational studies have also shown that aspirin use is associated with a significant improvement in survival in mutant *PIK3CA* colorectal cancer but not among those with wild-type *PIK3CA* (Domingo et al., 2013; Liao et al., 2012). Despite these observations, the molecular basis underlying the benefit of aspirin use in *PIK3CA*-mutant cancer remains undefined.

Aspirin and its metabolite salicylate inhibit the activity of multiple signaling pathways associated with malignancy. Aspirin is primarily known as an inhibitor of the cyclooxygenases COX-1 and COX-2 (Vane, 1971), that catalyze the biosynthesis of

thromboxanes and prostanoids and consequently play a key role in the inflammatory response and cancer (Greenhough et al., 2009). Aspirin irreversibly inactivates the COX enzyme by acetylating a serine residue near the active site (Lecomte et al., 1994; Roth and Majerus, 1975). Aspirin/salicylate also inhibits the phosphorylation of protein kinase IκB Kinase β (IKKβ) and NF-κB signaling (Kopp and Ghosh, 1994; Yin et al., 1998). NF-κB in turn controls the transcription of COX-2 in an IKK-dependent manner (Plummer et al., 1999; St-Germain et al., 2004). More recently, aspirin was shown to target two main regulators of intracellular energy homeostasis and metabolism through direct activation of adenosine monophosphate-activated protein kinase (AMPK) and inhibition of mammalian target of rapamycin (mTOR) signaling (Din et al., 2012; Hawley et al., 2012). Whether any of these mechanisms contribute to the protective effects of aspirin in *PIK3CA*-mutant breast cancer has not been explored.

Here we evaluate the efficacy of aspirin either as a single agent, or in combination with PI3K inhibitors, in PI3K-driven breast cancer. We also investigate the mechanism by which aspirin may elicit a therapeutic effect in this disease.

3. MATERIALS AND METHODS

Antibodies

Anti-p110α (#4249), anti-phospho-Akt Ser473 (#4060), anti-phospho-Akt Thr308 (#2965), anti-Akt (#4691), anti-phospho-Pras40 Thr246 (#2997), anti-Pras40 (#2691), anti-phospho-GSK3β Ser9 (#9336), anti-GSK3β (#9315S), anti-βactin (#4970), anti-phospho-IKKα/β Ser176/180 (#2697), anti-phospho-IκBα Ser32/36 (#9246), anti-IκBα (#9247), anti-phospho NF-Kappa-B p65 Ser536 (#3033), anti-NF-Kappa-B p65 (#8242), anti-AMPKα (#2532S), anti-phospho-AMPKα Thr172 (#2535), anti-ACC (#3676), anti-phospho-ACC Ser79 (#3661), anti-S6K (#2708), anti-phospho-S6K Thr389 (#9205), anti-S6 (#2217), anti-phospho-S6 Ser240/244 (#5364), anti-4EBP1 (#9452), anti-phosho-4EBP1 Ser65 (#9451) were purchased from Cell Signaling Technologies. Laminin V (#Z0097) and Ki67 (#M7240) were purchased from Dako. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulin antibodies were purchased from Chemicon.

Chemical reagents

The IKKβ ATP competitive inhibitor, Compound A was a generous gift from the Baldwin Lab (Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill), and manufactured by Bayer Pharmaceuticals. Celecoxib (#S1261) was purchased from Selleckchem. BKM120 (#A-1108) and BYL719 (#A-1214) was purchased from Active Biochem. Aspirin (#A2093), Sodium salicylate (#A5376), Bafilomycin A (#B1793) and Chloroquine diphosphate (#C6628) was purchased from Sigma Aldrich. Aspirin/salicylate was prepared as previously described (Goel et al., 2003). Briefly,

aspirin was dissolved in 1M Tris-HCl (pH 7.5) to a stock concentration of 1M and final pH of 7.2. An equivalent volume of Tris-HCl (pH 7.2) was used as vehicle control.

Plasmids

JP1520-HA-PIK3CA-GFP, JP1520-HA-PIK3CA-WT (Addgene plasmid # 14570) and JP1520-HA-PIK3CA-HA-H1047R (Addgene plasmid # 14572) was a generous gift from Joan Brugge. pBABE-puro mCherry-EGFP-LC3B was a gift from Jayanta Debnath (Addgene plasmid # 22418).

Cell Culture and Immunoblotting

MCF10A cells were cultured in DMEM/Ham's F12 medium supplemented with 5% equine serum (Gibco-brl), 10µg/ml insulin, 500ng/ml hydrocortisone (Sigma-Aldrich), 20ng/ml EGF (R&D Systems) and 100ng/ml cholera toxin (List Biological Labs). MCF10A cells expressing PIK3CA WT and mutants were generated and grown as described previously (Debnath et al., 2003; Hutti et al., 2012). Stable pools were generated by selection in 2ug/ml puromycin. SUM159-PT cells were grown in Ham's F12 medium (Cellgro) supplemented with 5% FBS, 5µg/ml insulin (Sigma-Aldrich) and 1µg/ml hydrocortisone (Sigma-Aldrich). MCF7, MDA-MB-468 and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle medium (DMEM; Cellgro) supplemented with 10% Fetal Bovine Serum (FBS; Cyclone). For immunoblotting, cells were rinsed with PBS and lysed in RIPA buffer containing protease and phosphatase inhibitors. Lysates were resolved by SDS-PAGE and transferred by electrophoresis to nitrocellulose membrane (Bio-Rad), followed by immunoblotting.

PGE2α measurements

PGE2α from collected conditioned media were measured using a PGE2α ELISA kit according to manufacturers instructions (R&D Systems). Cells were stimulated with 10ug/ml arachidonic acid (Sigma-Aldrich) for 30 minutes prior to collection.

Morphogenesis Assay

MCF10A cells were grown in three-dimensional Matrigel cultures according to Debnath et al., 2003. Briefly, chambers slides were coated with growth factor-reduced Matrigel (BD Biosciences) and allowed to solidify for 30 minutes. $3x10^3$ cells suspended in assay media containing 2% Matrigel were overlayed on coated chamber slides. Assay medium contains DMEM/Ham's F12 (Cellgro) supplemented with 2% equine serum (Cellgro), 10ug/ml insulin, 500ng/ml hydrocortisone (Sigma-Aldrich), 5ng/ml EGF (R&D Systems) and 100ng/ml cholera toxin (List Biological Labs). For aspirin studies, acinars were allowed to grow for 4 days following treatment with aspirin every 2 days. Cells were then fixed and stained with Ki67 and laminin V on day 12 as previously described (Debnath et al., 2003). Images were acquired using the Nikon Eclipse Ti microscope.

Colony Formation in Soft Agar

For colony formation in soft agar, $5x10^4$ cells were suspended in growth media containing aspirin or vehicle and 0.4% noble agar. Cell suspension was plated on top a solidified layer of 0.8% noble agar also containing aspirin. Cells were fed with growth media containing aspirin, every 4 days. After 15 days, colonies were stained with 1mg/ml iodonitrotetrazolum chloride and quantified using Matlab software.

Quantitative real-time RT PCR

Total RNA was isolated using the RNeasy kit following the manufacturer's instructions (Qiagen). Reverse transcription was performed using Quantitect Reverse transcription kit according to the manufacture's instructions (Qiagen). Quantitative real-time RT-PCR was performed using SYBR Green PCR Master Mix (BioRad) and the ABI Prism 7900 sequence detector (Applied Biosystems). Quantification of COX-2 mRNA expression was calculated by the ΔΔCT method with GAPDH as reference. QRT-PCR primer sequences are listed below:

PTGS2 Forward 5'-TCAGCCATACAGCAAATCCTT-3'

PTGS2 Reverse 5'-GTGCACTGTGTTTGGAGTGG-3'

GAPDH Forward 5'-GCAAATTCCATGGCACCGT-3′

GAPDH Reverse 5'-TCGCCCCACTTGATTTTGGAGG-3′

Sulforhodamine B (SRB) Assay

Cell viability was assessed using sulforhodamine B assay as previously described (Brown et al., 2015). Briefly, adherent cells were fixed with 12.5% (w/v) trichloroacetic acid for 1 hour at 4°C. Cells were then rinsed three times with water and stained with a solution of 0.5% [w/v] SRB in 1% acetic acid for at least 30 minutes at room temperature. Cells were then washed three times with 1% acetic acid and allowed to dry. SRB was dissolved in 10 mmol/l Tris (pH 10.5). Absorbance of solubilized SRB was measured at 510 nm.

4. RESULTS

Aspirin inhibits growth of mutant *PIK3CA* breast cancer cells

To investigate the molecular basis for the epidemiological observations associating a beneficial therapeutic effect of aspirin in *PIK3CA*-mutant breast cancer, we first examined the effects of aspirin on immortalized non-tumorigenic breast epithelial cell line MCF10A, stably expressing the oncogenic mutant *PIK3CA H1047R*. When grown in 3 dimensional Matrigel culture, *PIK3CA H1047R* and *E545K*-expressing cells form large multi-acinar structures (**Figure 2-1A**) and display irregular deposition of the basement membrane marker laminin V (**Figure 2-1B**). Aspirin treatment of oncogenic mutant *PIK3CA* cells results in a spheroid-like acinar structure and a localization of laminin V to the periphery of the acini, similar to that observed with parental MCF10A cells (**Figure 2-1B**). The aspirin concentrations used here are consistent with measurements of aspirin in the serum of patients treated for chronic inflammatory diseases (Goodman et al., 2001; Juarez Olguin et al., 2004; Yin et al., 1998). Concomitantly, aspirin treatment also results in a decrease in the proliferation marker Ki67, and an overall reduction in average spheroid size (**Figure 2-1B**). A similar decrease in viability and disruption of stellate morphology and acinar formation is observed in aspirin-treated SUM159-PT breast cancer cells that harbor endogenous oncogenic *PIK3CA* (*H1047L*) (**Figure 2-1C**). Aspirin also robustly inhibits anchorage-independent growth of mutant *PIK3CA* breast cancer cell lines SUM159-PT (*H1047L*) cells and MCF-7 (*E545K*) breast cancer cells in soft agar (**Figure 2-1D**).

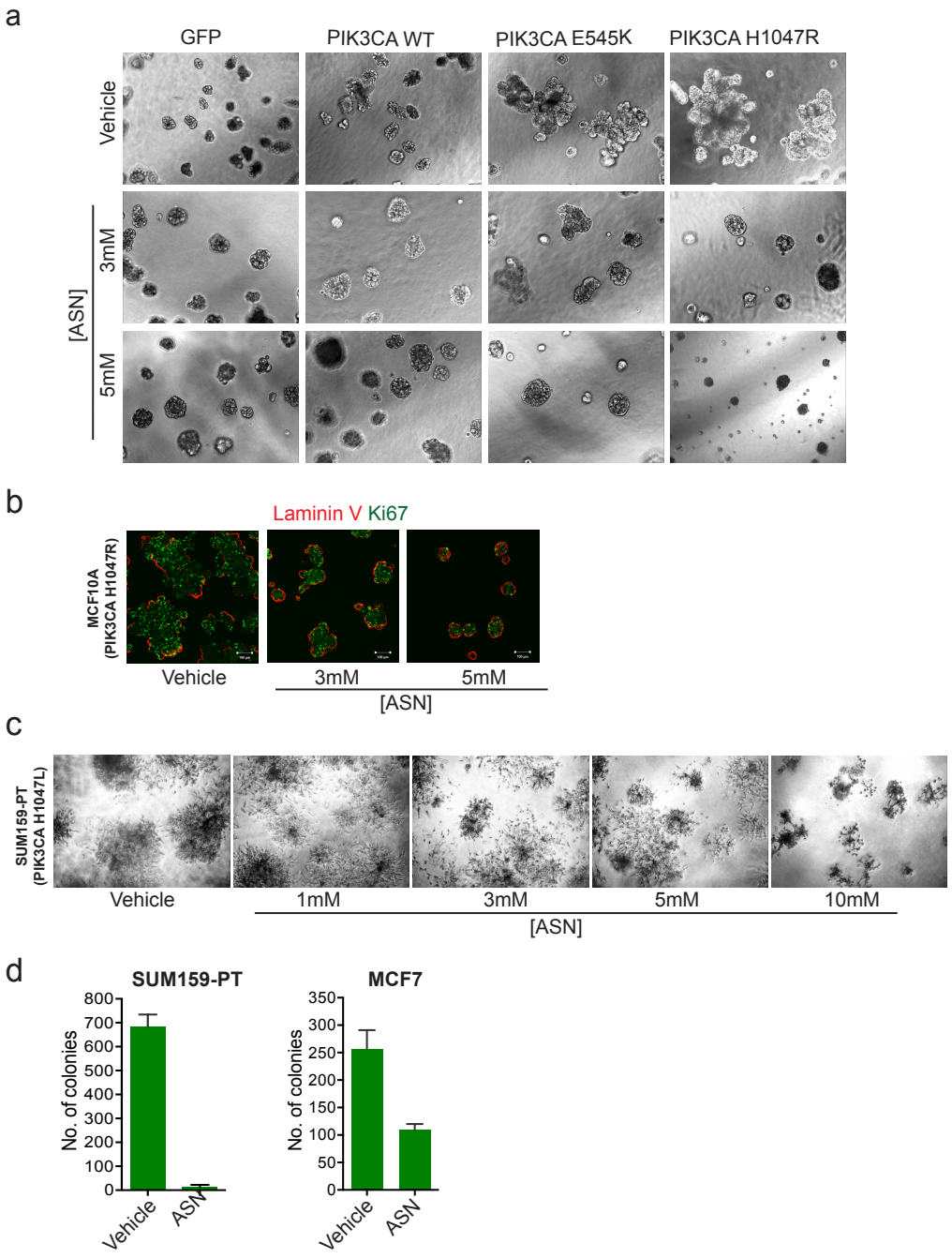


Figure 2-1: Aspirin inhibits growth of mutant *PIK3CA* breast cancer cells. (a) Representative images of MCF10A cells expressing JP1520-GFP, *PIK3CA* wild-type (WT) and *PIK3CA* mutants *E545K* and *H1047R*, grown in 3D Matrigel. (b) Representative images of MCF10A cells expressing mutant *PIK3CA H1047R*, grown in 3D Matrigel and stained for laminin V and Ki67. Cells were treated with 3mM and 5mM aspirin respectively, every two days, from day 4-12. Images were taken on day 12. (c) Disruption of stellate-like acinar structures of SUM159-PT grown in 3D Matrigel. Images were taken on day 10. Cells were treated with aspirin every 2 days from day 6 – 10. (d) Colony formation in soft agar, SUM159-PT and MCF7 cells were treated with 1.5mM aspirin every 4 days. On day 15, colonies were stained with iodonitrotetrazolum and quantified using Matlab.

Aspirin induces growth suppression of mutant *PIK3CA* breast cancer cells in an NF-κB and COX-2 independent manner

To explore the mechanistic basis for the growth inhibitory effects of aspirin in breast cancer cells expressing oncogenic *PIK3CA*, we tested the contribution of COX-2 and IKKβ/NF-κB, two well-characterized targets of aspirin. As revealed by RT-PCR, MCF10A cells expressing *PIK3CA H1047R* display elevated levels of *PTGS2*, the gene encoding COX-2 (**Figure 2-2A).** Similar induction of COX-2 mRNA is observed in MDA-MB-231 breast cancer cells expressing *PIK3CA H1047R* (**Figure 2-2A**). Expression of mutant *PIK3CA* also leads to elevated COX-2 protein (**Figure 2-2B**). Consistent with this observation, increased levels of PGE2α are observed in media from MCF10A cells expressing mutant *PIK3CA* (**Figure 2-2C**). Oncogenic *PIK3CA* also increases NF-κB activation as measured by IKKα/β, IκB and p65 phosphorylation (**Figure 2-2B**), similar to previous studies (Hutti et al., 2012). This observation coincides with the role for NF-κB as a transcriptional regulator of *PTGS2* (Kaltschmidt et al., 2002; Kim et al., 2009; Yamamoto et al., 1995). In light of this data, we investigated whether the growth inhibitory effects of aspirin in mutant *PIK3CA* breast cancer cells might be attributable to NF-κB/COX-2. We observed that treatment of mutant *PIK3CA* MCF10A cells or SUM159-PT breast cancer cells with Compound A (IKKβ ATP-competitive inhibitor) (Ziegelbauer et al., 2005), or Celecoxib (COX-2 specific inhibitor), has no effect on cell growth in 2D (**Figure 2-2D**). This suggests that the inhibitory effects of aspirin on the cell autonomous proliferation of breast cancer cells expressing oncogenic *PIK3CA* are unlikely to be mediated exclusively by induction of NF-κB and COX-2.

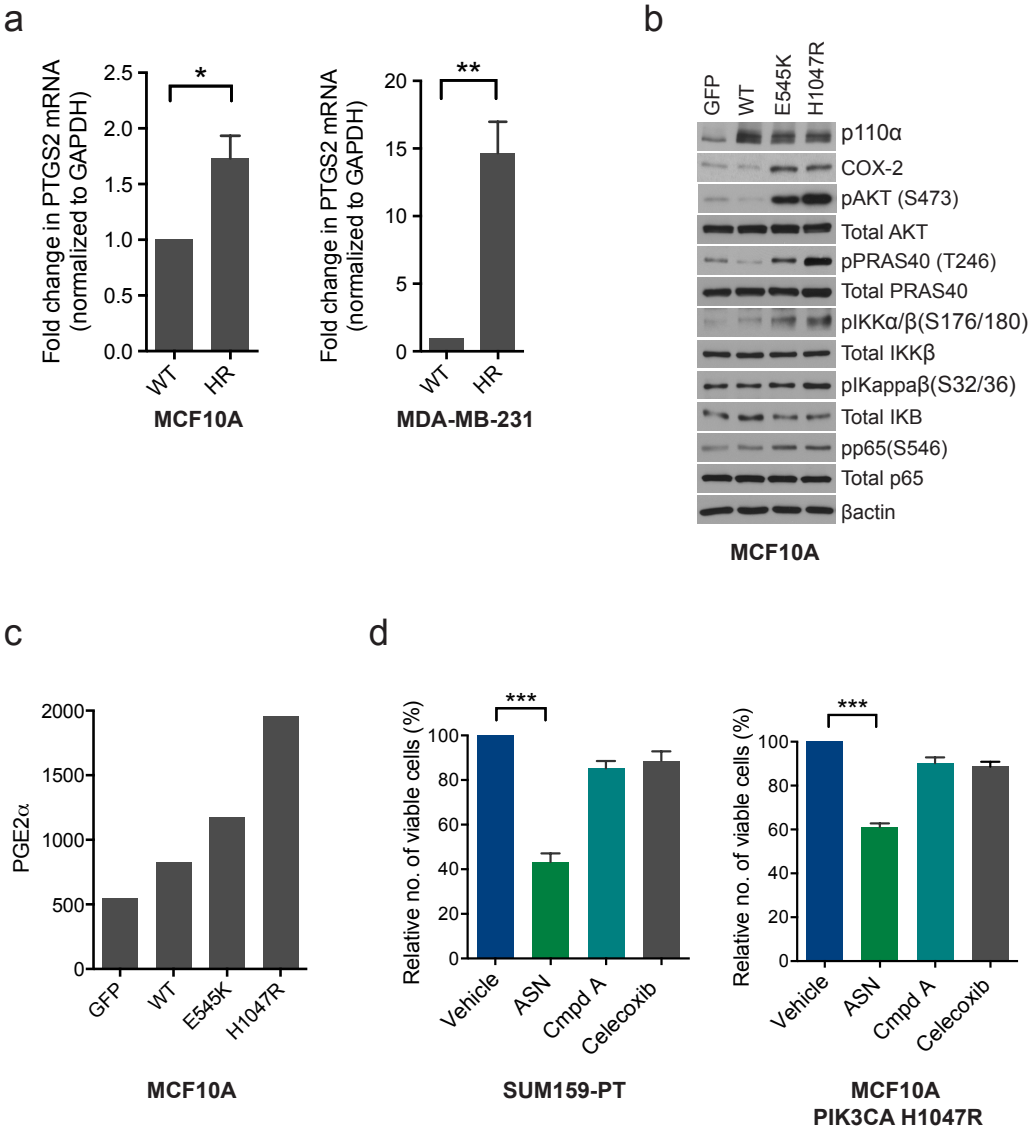


Figure 2-2: Aspirin induces growth suppression of mutant *PIK3CA* breast cancer cells in an NF-κB and COX-2 independent manner. (a) QRT-PCR, *PTGS2* mRNA expression in MCF10A and MDA-MB-231 cells expressing wild-type *PIK3CA* (WT) and mutant *PIK3CA H1047R*. Transcript levels were normalized to GAPDH. (b) Immunoblot analysis of MCF10A cells expressing *PIK3CA-WT* and *PIK3CA* mutants *E545K* and *H1047R* under EGF and insulin deprivation conditions. (c) PGE2α levels from conditioned media of MCF10A cells expressing *PIK3CA-WT* and *PIK3CA* mutants *E545K* and *H1047R*. Cells were stimulated with arachidonic acid for 30 minutes followed by ELISA. (d) Sulforhodamine cell viability, SUM159-PT cells and MCF10A expressing mutant *PIK3CA H1047R* were treated with 3mM aspirin (ASN), 5μM Compound A (Cmpd) and 10μM Celecoxib for 48hrs. Each bar represents three independent biological replicates ± the SEM. Statistical significance was determined using paired Student's t test, * p<0.05, ** p<0.01, ***p<0.001.

Aspirin activates AMPK and decreases mTORC1 signaling in mutant *PIK3CA* cells

AMPK and mTORC1 are major sensors of nutrient and growth factor signaling that regulate cell proliferation and size (Hardie, 2011; Laplante and Sabatini, 2012). Since aspirin directly binds and activates AMPK through an allosteric mechanism (Hawley et al., 2012), we investigated the contribution of AMPK and mTORC1 signaling in mediating the growth defects induced by aspirin in mutant *PIK3CA* breast cancer. Firstly, expression of mutant *PIK3CA E545K* and *H1047R* in MCF10A cells results in downregulation of AMPK activity as reported by phosphorylation of AMPK (S172) and its substrate Acetyl-CoA carboxylase (ACC) (S79). This is concomitant with increased mTORC1 signaling, as read out by phosphorylation of S6K1 (T389), 4EBP1 (S65) and S6 (S240/244), all of which are blocked by the *PIK3CA* specific inhibitor BYL719 (**Figure 2-3A**). As expected, oncogenic *PIK3CA* also increases phosphorylation of Akt (S473) and downstream substrates PRAS40 (T246) and GSK-3β (S9). Interestingly, in breast cancer cells harboring oncogenic *PIK3CA*, aspirin treatment leads to activation of AMPK coincident with inhibition of mTORC1 activity in a dose-dependent manner (**Figure 2-3B**). As expected, re-activation of Akt signaling is observed under conditions of attenuated mTORC1 activity and loss of feedback inhibition, similar to chronic rapamycin treatment (**Figure 2-3B**) (Shi et al., 2005; Wan et al., 2007). Importantly, inhibition of COX-2 and IKKβ/NF-κB does not significantly attenuate mTORC1 activity (**Figure 2-3C**). Therefore, aspirin-induced inhibition of mTORC1 is independent of COX-2 and IKKβ/NF-κB signaling. Interestingly, both Celecoxib and Compound A modestly increase phosphorylation of AMPK (S172), however this does not translate

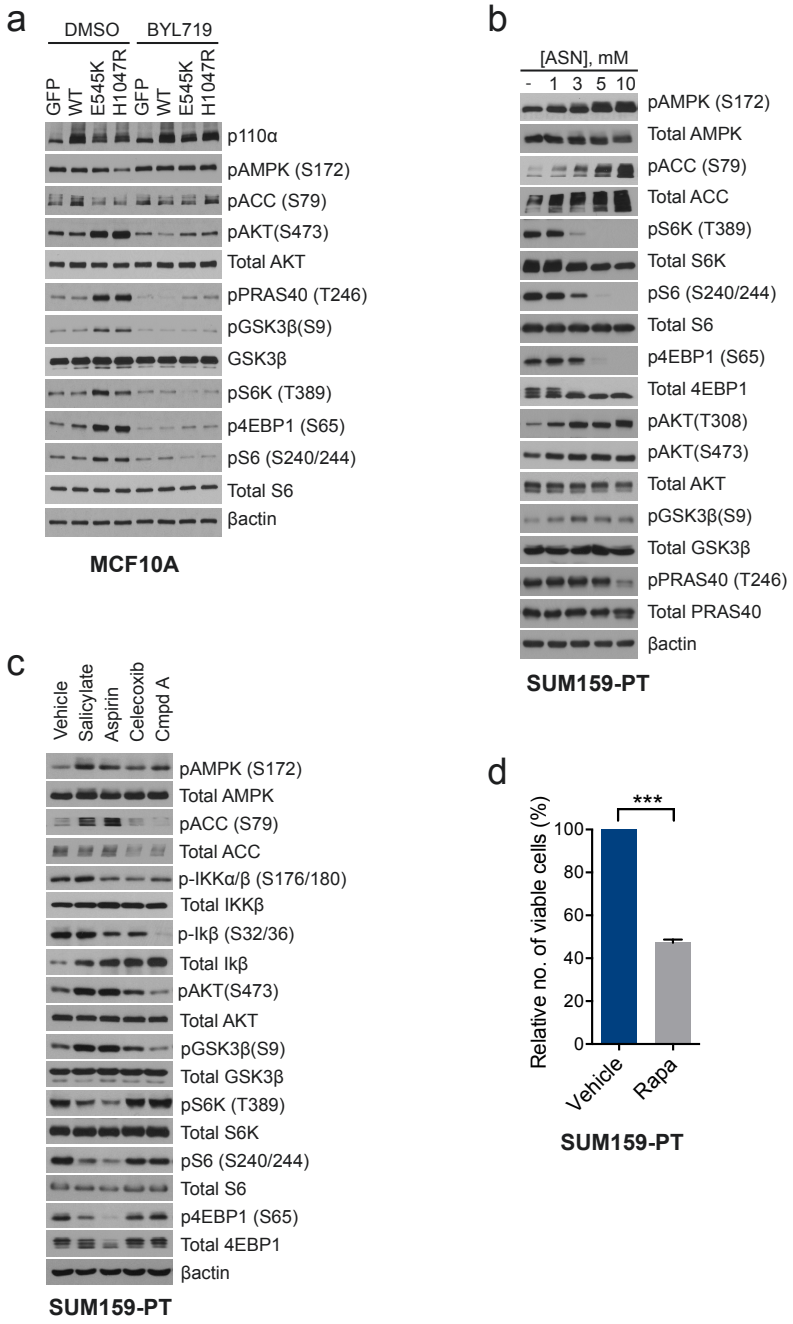


Figure 2-3: Aspirin activates AMPK and decreases mTORC1 signaling in mutant *PIK3CA* cells. (a) Immunoblot analysis of MCF10A cells expressing vector control JP1520-GFP, *PIK3CA-WT*, and mutant *PIK3CA E545K* and *H1047R* respectively. Cells were treated with 0.5μM BYL719 for 16hrs in growth media lacking EGF and insulin. Immunoblot analysis of (b) SUM159-PT cells treated with increasing concentrations of aspirin for 16hrs and (c) SUM159-PT cells treated with 3mM salicylate, 3mM aspirin, 10μM Celecoxib and 5μM Compound A for 16hrs. (d) Sulforhodamine cell viability, SUM159-PT cells treated with 100nM rapamycin for 48 hrs. Each bar represents three independent biological replicates ± the SEM. Statistical significance was determined using paired Student's t test, ***p<0.001.

into inhibition of mTORC1 signaling (**Figure 2-3C**). The mechanism that accounts for this increase remains unclear. In support of a role for mTORC1 inhibition in growth suppression, we observed that rapamycin, an mTORC1 inhibitor, decreases growth of SUM159-PT cells (**Figure 2-3D**).

Aspirin sensitizes mutant *PIK3CA* breast cancer cells to PI3K inhibitors to augment growth suppression

We next investigated the ability of aspirin in combination with PI3K inhibitors to attenuate the growth of *PIK3CA*-mutant breast cancer cell lines. In MCF10A cells expressing oncogenic *PIK3CA* (*H1047R*), co-treatment of aspirin sensitizes cells to BYL719, when compared to control GFP or wild-type p110α-expressing cells (**Figure 2-4A**). Similarly, in SUM159-PT cells (*PIK3CA H1047L*) enhanced growth inhibition is observed upon co-treatment with aspirin and BYL719, compared to each drug alone (**Figure 2-4B**). However, this combination shows little additive effect in MDA-MB-468, a *PTEN*-mutant breast cancer line (**Figure 2-4C**). In these cells, co-treatment of aspirin with BKM120, a pan class I PI3K inhibitor, shows an additive effect (**Figure 2-4C**), consistent with the notion that PTEN-deficient tumors signal primarily through p110β (*PIK3CB*), and would therefore be insensitive to p110α-specific inhibitors (Wee et al., 2008). By stark contrast, MDA-MB-231 cells that do not harbor PI3K pathway mutations are insensitive to the combination of aspirin with either BYL719 or BKM120 (**Figure 2-4D**).

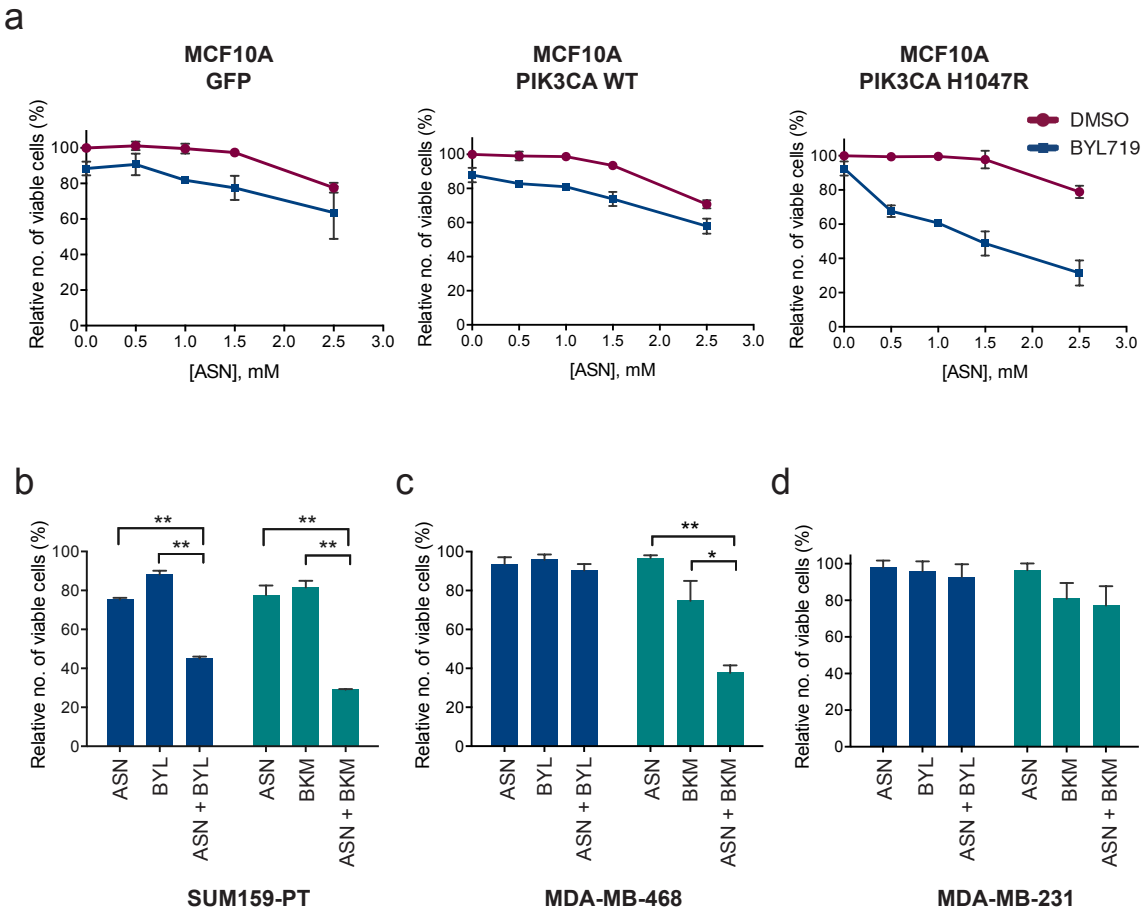


Figure 2-4: Aspirin sensitizes mutant *PIK3CA* breast cancer cells to PI3K inhibitors to augment growth suppression. (a) Sulforhodamine assay, MCF10A cells expressing JP1520-GFP, *PIK3CA-WT* and *PIK3CA H1047R* respectively, treated with increasing concentration of aspirin and 1μM BYL719 for 48 hrs. (b) Sulforhamine assay, SUM159-PT, MDA-MB-468 and MDA-MB-231 cells treated with 1.5mM aspirin (ASN) and 1μM BYL719 (BYL) or 1μM BKM120 (BKM) for 48hrs. Cell viability was determined relative to vehicle control. Each bar represents three independent biological replicates ± the SEM. Statistical significance was determined using paired Student's t test, * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

Co-treatment of aspirin and PI3K inhibitors leads to enhanced activation of AMPK, inhibition of mTORC1 and induction of autophagy

In terms of signaling responses, the combination of aspirin with either PI3K inhibitors leads to an increase in phosphorylation of AMPK (S172) and ACC (S79) along with enhanced suppression of phosphorylation of S6K1 (T389), S6 (S240/244) and 4EBP1 (S65) (**Figure 2-5A**). Similar to aspirin, co-treatment of SUM159-PT cells with rapamycin and PI3K inhibitors - BYL719 and BKM120, also leads to a significant decrease in cell viability relative to each drug alone (**Figure 2-5B**). This trend also holds true for the AMPK activator A769662 (**Figure 2-5C**).

AMPK and mTORC1 signaling are important regulators of autophagy. Given that co-treatment of aspirin and BYL719 leads to activation of AMPK and suppression of mTORC1 signaling, we investigated the effect of this drug combination on autophagy. We observed that treatment of SUM159-PT cells with a combination of aspirin and BYL719, results in an increase in LC3I/II protein expression by western blot analysis (**Figure 2-5D**) as well as an increase in the number of fluorescently labeled mcherry-LC3 puncta formed, relative to each drug alone (**Figure 2-5E**). In light of these findings, we then investigated whether activation of autophagy could be responsible for the decrease in cell viability observed upon co-treatment with aspirin and BYL719. To address this, we treated SUM159-PT cell with two independent autophagy inhibitors Chloroquine and Bafilomycin A, respectively. Our findings indicate that both autophagy inhibitors were able to modestly rescue the decrease in cell viability induced by co-treatment of aspirin and BYL719 (**Figure 2-5F,G**).

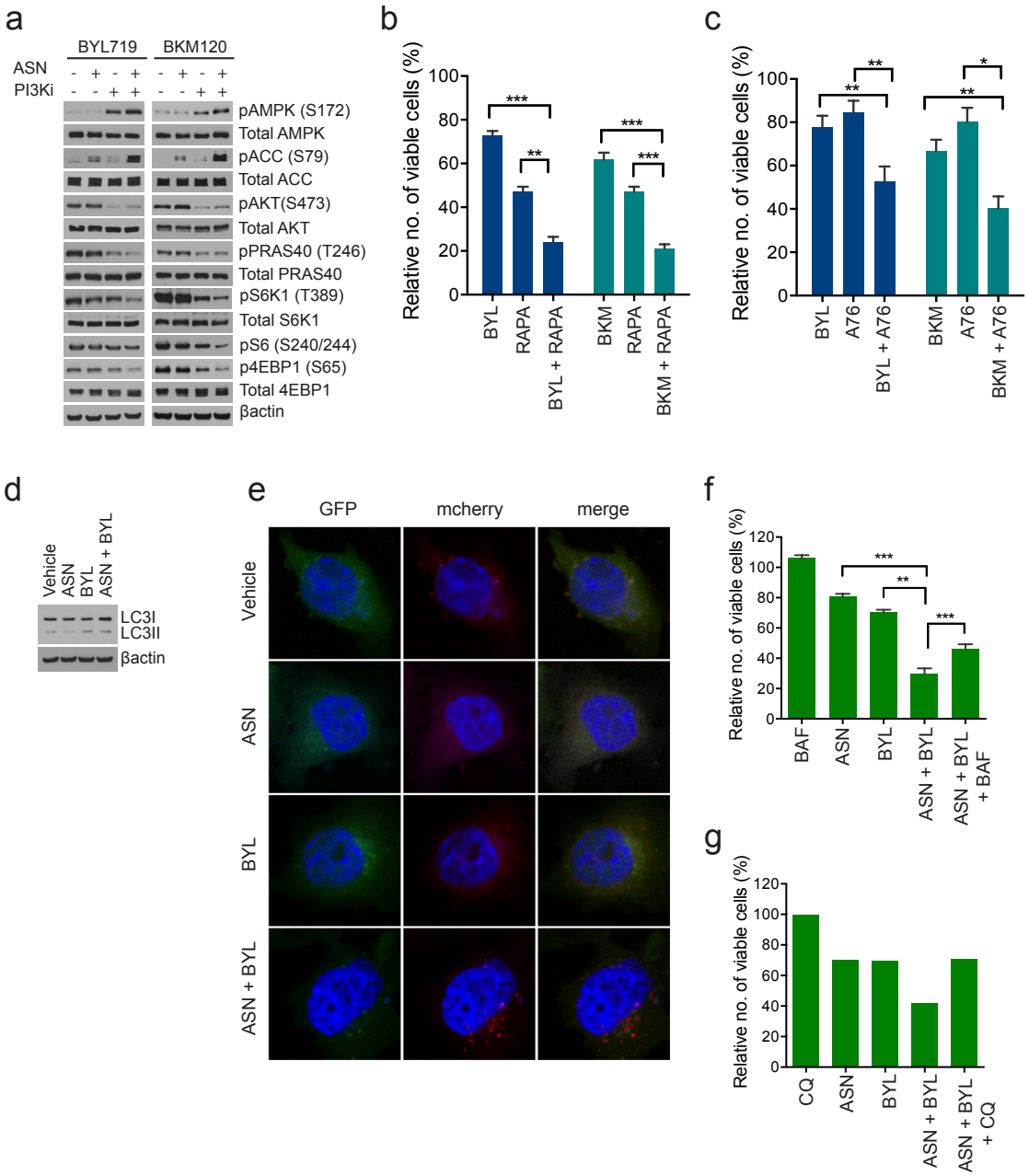


Figure 2-5: Co-treatment of aspirin and PI3K inhibitors leads to enhanced activation of AMPK, inhibition of mTORC1 and induction of autophagy. (a) Immunoblot, SUM159-PT cells treated with 1.5mM aspirin and 1μM BYL719 for 24hrs or 1μM BKM120 for 16hrs. Sulforhodamine assay: (b) SUM159-PT cells treated with 50nM Rapamycin (RAPA) and 1μM BYL719 (BYL) or 1μM BKM120 (BKM) for 48hrs; (c) SUM159-PT cells treated with 100μM A769662 (A76) and 1μM BYL719 or 1μM BKM120 for 48hrs. (d) Immunoblot of LC3I/II in SUM159-PT cells treated with 1.5mM aspirin (ASN) and 1μM BYL719 for 48hrs. (e) Immunofluorescent images of SUM159-PT cells expressing a mcherry-EGFP-LC3B reporter, treated with 1.5mM aspirin and 1μM BYL719 for 48hrs. (f,g) Sulforhodamine assay, SUM159-PT cells treated with 1.5mM aspirin, 1μM BYL719, 10nM Bafilomycin A (BAF) and 10μM Chloroquine (CQ) for 48hrs. Each bar represents three independent biological replicates ± the SEM. Cell viability was determined relative to vehicle control. Statistical significance was determined using paired Student's t test, * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

5. DISCUSSION (SEE CONCLUSION CHAPTER FOR FURTHER DISCUSSION)

Over the last few decades, a growing body of literature has suggested that aspirin might be helpful in the treatment of cancer. This is apparent in the case of colorectal cancer (CRC) where the strongest evidence for aspirin use has been accrued. In contrast, there are few well-characterized *in vitro* and animal studies on aspirin's benefit in breast cancer. Furthermore, the relationship between aspirin use in PI3K-dependent breast cancers has not been thoroughly explored. In this study, we investigate whether adjuvant aspirin treatment could improve the efficacy of specific *PIK3CA* and PI3K inhibitors currently under clinical trial evaluation for the treatment of breast cancer. To date, many of these inhibitors display limited clinical efficacy (Klempner et al., 2013). Our study indicates that aspirin decreases the growth and viability of *PIK3CA* mutant cells. While epidemiology studies associating aspirin use with *PIK3CA* mutation status were performed in colorectal cancer, our *in vitro* data suggest that a similar phenomenon might occur in breast cancer, and should be investigated. This is supported by our finding that MCF10A cells expressing mutant *PIK3CA* appear to be more sensitive to aspirin in comparison to cells expressing wild-type *PIK3CA* when grown in 3D Matrigel culture. It is also noteworthy, that lower concentrations of aspirin robustly affects the growth of *PIK3CA* mutant colonies in soft agar.

Additionally, we demonstrate that aspirin in combination with PI3K inhibitors results in enhanced growth suppression of *PIK3CA/PTEN* mutant cells. Our data suggests that this growth suppression phenotype is due to enhanced AMPK activation and inhibition of mTORC1 signaling. In support of the observed sensitivity of PI3K-activated cells to this drug combination, we show that mutant *PIK3CA* inhibits AMPK

activity but activates mTORC1 signaling. This is most likely due to mutant *PIK3CA*-induced activation of Akt. Akt phosphorylation of AMPK-α1 at S487 in the serine/threonine-rich loop has been shown to inhibit AMPK-α1 activation and phosphorylation at T172 (Hawley et al., 2014). In the case of mTORC1 signaling, Akt activation has been shown to activate mTORC1 signaling through several mechanisms including phosphorylation of TSC2 and PRAS40 (Inoki et al., 2002; Manning et al., 2002; Vander Haar et al., 2007). Although activation of AMPK has been shown to inhibit mTORC1 signaling, future experiments aimed at deciphering whether aspirin-induced inhibition of mTORC1 signaling is AMPK dependent, are required. While our studies indicate that the contribution of aspirin in cell autonomous growth suppression is not solely due to inhibition of COX-2 and IKKβ/NF-κB, the importance of these latter targets might be more relevant in an *in vivo* setting. This is due to the fact that decreasing COX-2 activity and NF-κB signaling impedes cancer growth and metastasis via several mechanisms that target the immune system, platelet activation/angiogenesis and other components of the tumor microenvironment (Markosyan et al., 2013; Wang et al., 2014; Williams et al., 2000; Xu et al., 2014). It is also worth noting that the proposed combination of aspirin and PI3K pathway inhibitors does not result in overt cell death but rather growth suppression. Thus, it is imperative that we evaluate the efficacy of this drug combination *in vivo*. While our *in vitro* studies show a cytostatic effect, it is possible that our *in vivo* studies may reveal a cytotoxic effect. This is supported by a study, which demonstrated that aspirin treatment in a xenograft model of TSC2 deficient ELT3 cells (which display constitutive mTORC1 signaling), results in tumor regression characterized by cleaved caspase-3 and cleaved-PARP (Li et al., 2014).

6. CONCLUSION

Taken together, our findings provide a mechanistic rationale for the use of aspirin in combination with PI3K inhibitors for the treatment of breast cancers that show PI3K-pathway dependency. It also highlights the importance of performing future studies to assess whether *PIK3CA* mutation status would be a reliable biomarker for identifying breast cancer patients who are most likely to benefit from adjuvant aspirin therapy. There has been great interest in performing human clinical trials to evaluate the potential chemotherapeutic effects of aspirin in breast cancer. It will be interesting to see whether the beneficial outcome outweighs the side effects due to aspirin usage, and how it compares to other chemotherapeutic agents currently under clinical evaluation.

CHAPTER III

A NOVEL ROLE FOR PI3K-REGULATED PENTRAXIN 3 IN BASAL-LIKE BREAST CANCER

The data presented in this chapter is part of a collaborative project with the Karnoub Lab at Beth Israel Deaconess Medical Center, Harvard Medical School. Whitney Henry designed and performed all the experiments outlined in this chapter unless stated otherwise. Her contributions will be an integral part of a peer-reviewed manuscript.

1. ABSTRACT

The phosphoinositide 3-kinase (PI3K) pathway regulates all aspects of breast cancer development, yet the mechanism(s) by which the PI3K pathway promotes tumorigenesis is still not fully elucidated. In this study, we employ a well-characterized cell line model consisting of immortalized mammary epithelial MCF10A cells expressing wild-type *PIK3CA* and oncogenic *PIK3CA H1047R* respectively. Microarray analysis revealed that constitutive PI3K pathway activation preferentially affects the gene expression levels of cytokines, chemokines and secreted factors. Here we identify a novel PI3K-regulated gene called long Pentraxin 3 (*PTX3*). PTX3 is a soluble pattern recognition receptor that plays a fundamental role in the immune system. However, the function of this protein in breast cancer malignancy is largely unknown. In this study, we demonstrate that the PI3K pathway regulates the expression of *PTX3* in a NF Kappa B dependent manner. We also show that PTX3 plays a functional role in mediating PI3K-driven growth factor independent proliferation in two-dimensional (2D) monolayer as well as growth in three-dimensional (3D) Matrigel. Together, these findings highlight a relevance for PTX3 in PI3K-activated basal-like breast cancer.

2. INTRODUCTION

Pentraxin 3 (PTX3) is a highly conserved humoral pattern recognition receptor (Breviario et al., 1992; Lee et al., 1993). It belongs to the pentraxin superfamily which is classified into two main subtypes based on length and structure. Short pentraxin protein members consists of acute-phase C-reactive proteins (CRPs) and serum amyloid P component (SAP) proteins (Bottazzi et al., 2010). They are synthesized in the liver and released in response to inflammatory mediators. In contrast, PTX3 is a glycosylated protein composed of an extended asymmetric octamer attached to a conserved C-terminal pentraxin domain (Bottazzi et al., 1997; Breviario et al., 1992; Inforzato et al., 2010). It contains a distinct N-terminal domain not shared by any known protein and a hydrophobic signal peptide sequence indicative of a secretory protein (Breviario et al., 1992). PTX3 is locally produced and released by several cell types including dendritic cells, mononuclear phagocytes, endothelial cells and epithelial cells (Deban et al., 2011). It interacts with many different hosts ligands including complement factors, extracellular matrix components, P-selectin and apoptotic cells (Bottazzi et al., 2010).

PTX3-null (PTX3-/-) mouse studies have shown that PTX3 plays a critical role in resistance against selected pathogens including fungi, bacteria and viruses (Garlanda et al., 2002). As a result, PTX3 is currently being investigated as an anti-microbial agent. PTX3 also promotes the organization of hyaluronic acid-rich extracellular matrices and loss of PTX3 is associated with female infertility (Salustri et al., 2004). Similar to short pentraxins, PTX3 functions in the phagocytosis of specific cargos via Fc receptors (Lu et al., 2008). It also facilitates opsonization of various targets through its association with the complement system (Ma et al., 2009; Nauta et al., 2003). The

complement system is a fundamental innate effector system that identifies and orchestrates the clearance of microorganisms and apoptotic cells.

Although PTX3 has been widely studied in the context of immunology, very little is known about the role of this protein in cancer. As a result, one of the research projects in the Karnoub lab involves investigating the function of PTX3 in breast cancer. They first identified PTX3 among the top ten genes whose mRNA expression was significantly increased in MDA-MB-231 breast cancers cells when cocultured with mesenchymal stem cells (MSCs). Previous work from their lab showed that co-existance of tumor cells with mesenchymal stem cells (MSCs)s induces transcriptional changes in the tumor cells that are significant for their growth and metastasis (Cuiffo et al., 2014; El-Haibi et al., 2012; Karnoub et al., 2007). Consequently, it was hypothesized that PTX3 may play an important role in breast cancer progression.

Further studies performed by the Karnoub lab has also revealed that PTX3 expression is clinically relevant to breast cancer, and is preferentially associated with basal-like breast tumors. Specifically, PTX3 expression seems to correlate best with the claudin low subtype, which is characterized by poor differentiation and a mesenchymal state enriched with stem cell capacity. Consistent with this finding, PTX3 expression was increased in HMLE cells expressing embryonic transcription factors Twist and Snail respectively (Elenbaas et al., 2001). Additionally, the Karnoub lab has gathered evidence that implicates PTX3 in promoting various cancer stem cell related phenotypes.

The phosphoinositide 3-kinase (PI3K) pathway is one of the most frequently mutated pathways in breast cancer. Moreover, somatic mutations in *PIK3CA*, the gene

encoding the catalytic subunit of PI3K, are highly prevalent in breast cancers. These mutations increase PI3K activity and induce transformation of mammary epithelial cells as well as the formation of heterogeneous mammary tumors *in vivo* (Zhao and Vogt, 2008). Interestingly, recent studies have shown that the gene expression profile of mutant *PIK3CA* tumors is most closely associated with the claudin-low subtype of human breast cancers, using both mouse and human patient datasets respectively (Hanker et al., 2013; Hutti et al., 2012). It was also shown that mutant *PIK3CA* confers features of stemness as indicated by its regulation of discrete epithelial-mesenchymal transition (EMT) and stem cell markers, and induction of mammosphere formation (Hanker et al., 2013). In light of these findings, we investigated the relationship between PI3K signaling, PTX3 and breast cancer progression.

3. MATERIALS AND METHODS

Antibodies and Reagents

Anti-p110α (#4249), anti-phospho-Akt Ser473 (#4060), anti-phospho-Akt Thr308 (#2965), anti-Akt (#4691), anti-phospho-Pras40 Thr246 (#2997), anti-Pras40 (#2691), anti-phospho-GSK3β Ser9 (#9336), anti-GSK3β (#9315S), anti-βactin (#4970), anti-phospho-IKKα/β Ser176/180 (#2697), anti-phospho-IκBα Ser32/36 (#9246), anti-IκBα (#9247), anti-phospho NF-Kappa-B p65 Ser536 (#3033), anti-NF-Kappa-B p65 (#8242), were purchased from Cell Signaling Technologies. Anti-PTX3 (#LS-C138995) was purchased from LifeSpan BioSciences Inc. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulin antibodies were from Chemicon. The IKKβ ATP competitive inhibitor, Compound A was a generous gift from the Baldwin Lab (Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill), and manufactured by Bayer Pharmaceuticals. The pan-class I PI3K inhibitor BKM120 was purchased from Active Biochem (#A-1108).

Plasmids

JP1520-HA-PIK3CA-GFP, JP1520-HA-PIK3CA-WT (Addgene plasmid # 14570) and JP1520-HA-PIK3CA-HA-H1047R (Addgene plasmid # 14572) was a generous gift from Joan Brugge. pLP-LNCX-PIK3CA-WT (Addgene plasmid # 25633) and pLP-LNCX-PIK3CA-H1047R (Addgene plasmid # 25635) was a gift from Todd Waldman. Human PTX3 was cloned into pBABE-puro vector by Anthony Collmann, Karnoub lab. pBABE-puro was a gift from Hartmut Land & Jay Morgenstern & Bob Weinberg (Addgene plasmid # 1764).

Cell Culture and Immunoblotting

SUM159-PT cells were grown in Ham's F12 medium (Cellgro) supplemented with 5% FBS, 5μg/ml insulin (Sigma-Aldrich) and 1μg/ml hydrocortisone (Sigma-Aldrich). MCF10A cells were cultured in DMEM/Ham's F12 medium supplemented with 5% equine serum (Gibco-brl), 10μg/ml insulin, 500ng/ml hydrocortisone (Sigma-Aldrich), 20ng/ml EGF (R&D Systems) and 100ng/ml cholera toxin (List Biological Labs). Cells used were tested for mycoplasma contamination. For immunoblotting, cells were rinsed with PBS and lysed in RIPA buffer containing protease and phosphatase inhibitors. Lysates were resolved by SDS-PAGE and transferred by electrophoresis to nitrocellulose membrane (Bio-Rad), followed by immunoblotting.

RNA interference

For shRNA-mediated knockdown of PIK3CA, single-stranded oligonucleotides sense and antisense pairs, encoding specific PIK3CA target sequences were synthesized and cloned into PLKO.1 vector. Lentiviral supernantant was collected after 48 hours from 293T cells co-transfected with the respective PLKO-shRNA constructs, VSVG and psPAX2. Cells stably expressing PIK3CA shRNAs were selected and cultured in growth media containing 2ug/ml puromycin. The hairpin sequences were generated as follows: PIK3CA shRNA#1, sense, 5'-CCGGGCACAATCCATGAACAGCATTCTCGAGAATGCTGTTCATGGATTGTGCTTTTTTG-3';

PIK3CA shRNA#1, antisense, 5'-
AATTCAAAAACACAATCCATGAACAGCATTCTCGAGAATGCTGTTCATGGATTGTG-3'
PIK3CA shRNA#2, sense, 5'-
CCGGGCATTAGAATTTACAGCAAGACTCGAGTCTTGCTGTAAATTCTAATGCTTTTTTG-3';
PIK3CA shRNA#2, antisense, 5'-
AATTCAAAAAGCATTAGAATTTACAGCAAGACTCGAGTCTTGCTGTAAATTCTAATGC-3'
PIK3CA shRNA constructs were generated by Emilie Collman, PhD, Toker Lab.
Cells stably expressing PLKO-PTX3 shRNA (puromycin) constructs were provided by the Karnoub lab. PTX3 shRNA constructs were originally obtained from the DFCI RNAi core facility.

3D Culture Assay

MCF10A cells were grown in 3D Matrigel cultures as previously described (Isakoff et al., 2005). Briefly, chambers slides were coated with growth factor-reduced Matrigel (BD Biosciences) and allowed to solidify for about 30 minutes. $3x10^3$ cells suspended in assay media containing 2% Matrigel were overlayed on coated chamber slides. Assay medium contains DMEM/Ham's F12 supplemented with 2% equine serum, 10ug/ml insulin, 500ng/ml hydrocortisone, 5ng/ml EGF and 100ng/ml cholera toxin. Assay media was replenished every 3-4 days. Images were acquired using the Nikon Eclipse Ti microscope.

Quantitative RT PCR analysis

Total RNA was isolated using RNeasy RNA extraction kit (Qiagen). Quantitect Reverse Transcription kit (Qiagen) was used for reverse transcription. Quantitative RT-PCR was performed using SYBR Green PCR master mix (BioRad) in an ABI Prism 7900 sequence detector (Applied Biosystems). Relative mRNA expression was determined using theΔΔCT method, and normalized to GAPDH.

Real-time quantitative PCR primer sequences were as follows:

GAPDH Forward 5'-GCAAATTCCATGGCACCGT-3′

GAPDH Reverse 5'-TCGCCCCACTTGATTTTGGAGG-3′

PTX3 Forward 5'-GCTCTCTGGTCTGCAGTGTT-3'

PTX3 Reverse 5'-CTTGTCCCATTCCGAGTGCT-3'

Sulforhodamine B (SRB) Assay

Cell viability was assessed using sulforhodamine B assay as previously described (Brown et al., 2015). Briefly, adherent cells were fixed with 12.5% (w/v) trichloroacetic acid for 1 hour at 4°C. Cells were then rinsed three times with water and stained with a solution of 0.5% [w/v] SRB in 1% acetic acid for at least 30 minutes at room temperature. Cells were then washed three times with 1% acetic acid and allowed to dry. SRB was dissolved in 10 mmol/l Tris (pH 10.5). Absorbance of solubilized SRB was measured at 510 nm. For proliferation assay under serum free conditions – SUM159-PT cells were first seeded in complete growth media. After approximately 16 hours, adherent cells were rinsed twice with PBS and changed to serum free F12 media. Serum-free media was replaced every two days.

Anoikis Assay

Cells were harvested after three days of growth in suspension and stained with Annexin V and 7-AAD using the PE Annexin V apoptosis kit (BD Pharmingen) according to manufacturer's instruction. Cells were then assessed by flow cytometry (FACSCalibur; Becton-Dickinson) and analyzed using FlowJo (Tree Star Inc) software.

Colony Formation in Soft Agar

Colony formation in soft agar was performed as described previously (Isakoff et al., 2005). Briefly, 5 x 10^4 cells suspended in MCF10A growth media containing 0.4% noble agar was plated on top a solidified layer of 0.8% noble agar. Cells were fed with growth media every 4 days. After 28 days, colonies were stained with 1mg/ml iodonitrotetrazolum chloride and quantified.

4. RESULTS

The PI3K pathway regulates PTX3 expression in breast cancer

Our first indication that the PI3K pathway regulates PTX3 expression came from a microarray analysis of genes differentially modulated by mutant *PIK3CA H1047R* compared to wild-type (WT) *PIK3CA*, in immortalized mammary epithelial MCF10A cells. Similar to previous studies, mutant *PIK3CA H1047R* cells show increased Akt activation and substrate phosphorylation (**Figure 3-1A**) and display features of cellular transformation including EGF-independent proliferation (**Figure 3-1B**), colony growth in soft agar (**Figure 3-1C**) and evasion of anoikis (**Figure 3-1D**). Gene ontology analysis revealed a significant enrichment of genes involved in the inflammatory response, cytokine and chemokine signaling followed by other important cellular processes including cell differentiation, angiogenesis, cell migration and proliferation (**Figure 3-2**). Notably, one of the genes found to be transcriptionally upregulated by mutant *PIK3CA* is *PTX3*. Transcriptional upregulation of PTX3 mRNA was validated by qRT-PCR (**Figure 3-3A**). Expression of mutant *PIK3CA H1047R* also leads to an increase in PTX3 protein level and this is reversed upon treatment with the pan PI3K inhibitor BKM120 (**Figure 3-3B**). Similarly, knockdown of mutant *PIK3CA* in SUM159-PT breast cancer cells which harbor an endogenous mutant *PIK3CA* (*H1047L*), results in downregulation of PTX3 mRNA (**Figure 3-3C)** and protein level (**Figure 3-3D**). Together, this data indicates that the PI3K pathway regulates PTX3 expression.

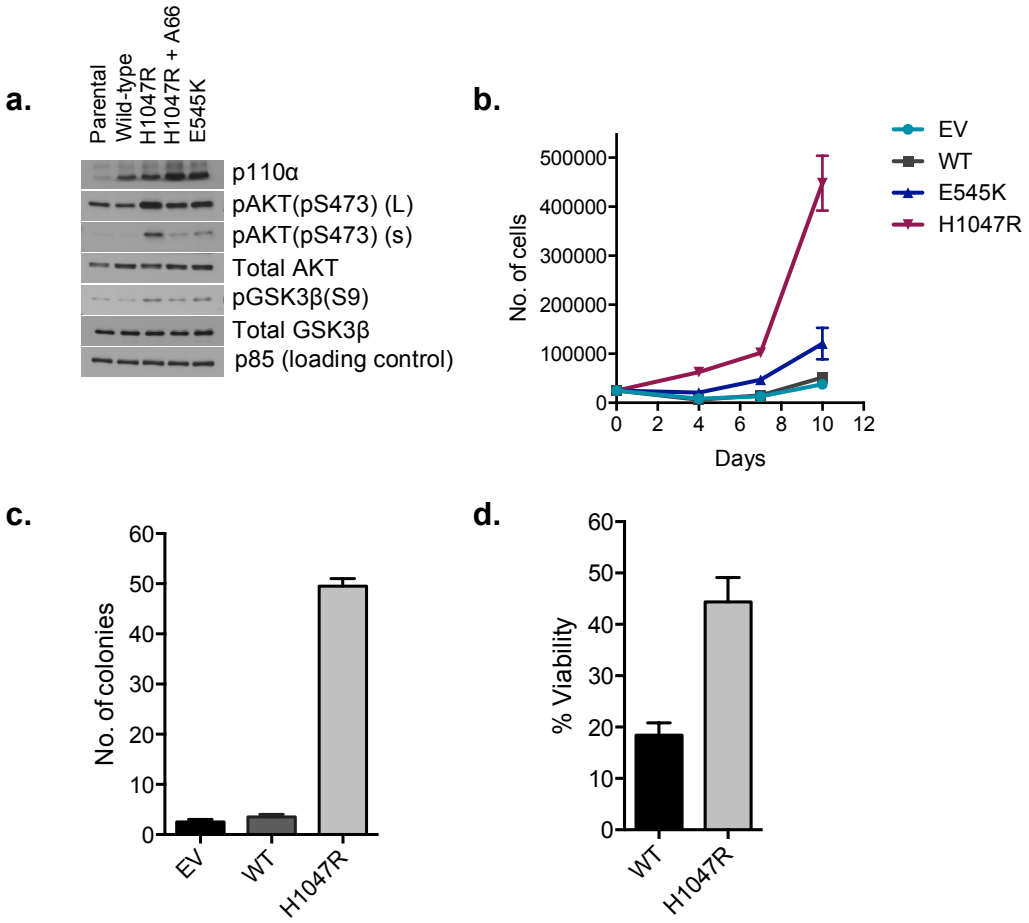
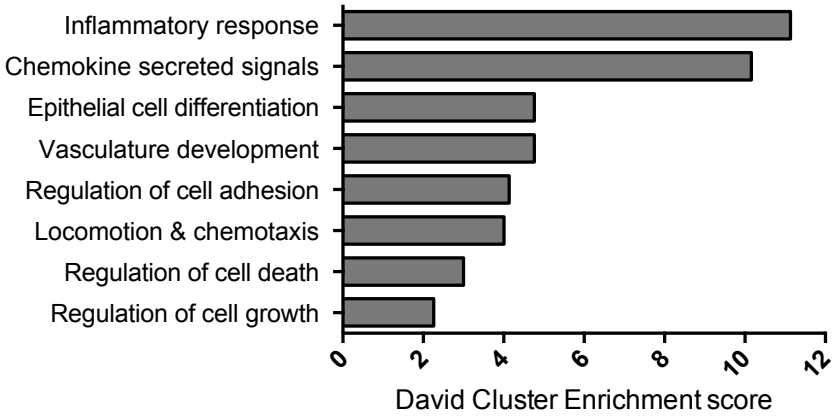


Figure 3-1: Mutant *PIK3CA H1047R* induces cellular transformation phenotypes. (a) Immunoblot analysis using antibodies specific to the proteins indicated for lysates of MCF10A cells stably expressing *PIK3CA-WT* and *PIK3CA* mutant *E545K* and *H1047R* respectively. Mutant *PIK3CA H1047R* cells were treated 0.77μM A66 (*PIK3CA* specific inhibitor) for 30mins. (b) EGF-independent proliferation, (c) Colony formation in soft agar, and (d) anoikis assay. Details for each phenotypic assay described above are provided in the Materials and Methods section.

a.



b.

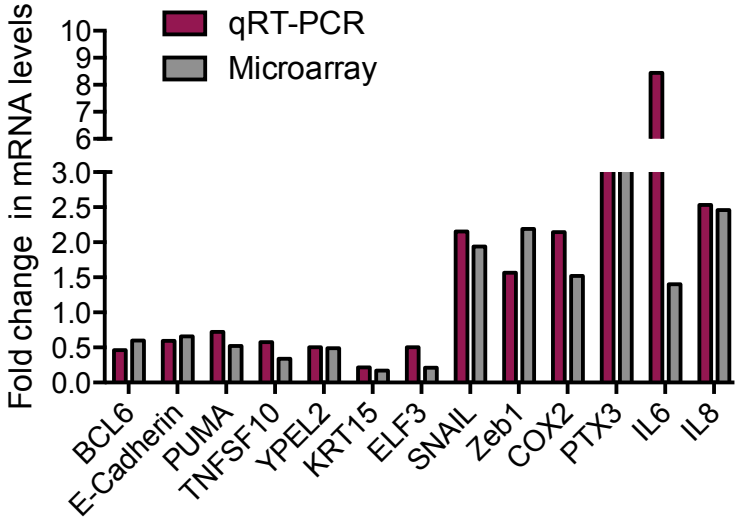


Figure 3-2: PI3K pathway activation results in differential expression of genes involved in inflammation, cytokine signaling and extracellular matrix remodeling. Gene expression profiling was performed on MCF10A cells expressing mutant *PIK3CA H1047R* compared to wildtype *PIK3CA*. (a) Gene ontology analysis performed on genes with a fold change of >1.5 or <0.6, and a FDR<5%.
(b) Quantitative RT PCR validation of PI3K regulated genes obtained from microarray.

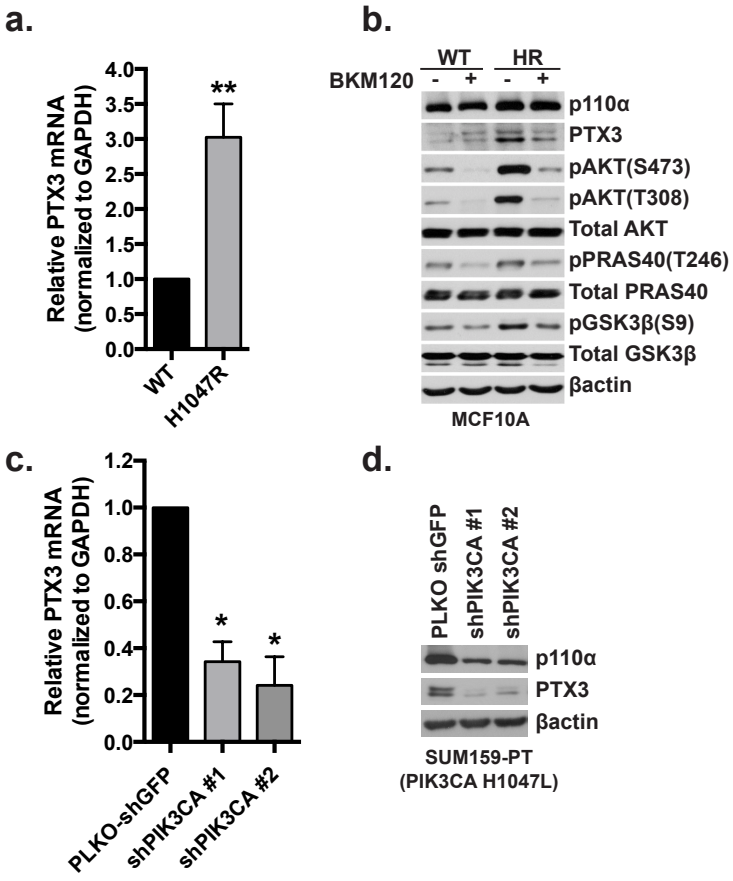


Figure 3-3: PTX3 expression is regulated by the PI3K pathway. (a) Quantitative RT PCR measurements of PTX3 mRNA levels and (b) immunoblot analysis of PTX3 protein levels, in MCF10A cells stably expressing wild-type *PIK3CA* (WT) and mutant *PIK3CA H1047R* (HR). Cells were treated for 24hrs with 1μM of BKM120, a pan PI3K inhibitor under EGF and insulin deprived conditions.
(c) Quantitative RT PCR measurements of PTX3 mRNA levels and (d) PTX3 protein levels, upon shRNA-mediated knockdown of *PIK3CA* in SUM159-PT breast cancer cells. Each bar represents three independent biological replicates ± the SEM. Transcript levels were normalized to GAPDH. Statistical significance was determined using paired Student's t test, * $p<0.05$, ** $p<0.01$.

Modulation of PTX3 expression by the PI3K pathway is NF-κB dependent

To investigate the mechanism by which PI3K signaling regulates PTX3 expression, we first performed a promoter analysis where we identified a putative NF-κB binding site at the PTX3 promoter (**Figure 3-4A**). Given that mutant *PIK3CA* increases NF-κB signaling as noted by the increase in phosphorylation of IKKα/β (S176/180), IκB (S32/36) and p65 (S536), as well as downregulation of total IκB (**Figure 3-4B**), we examined the contribution of NF-κB signaling in PI3K-mediated regulation of PTX3. Indeed, treatment of MCF10A cells expressing mutant *PIK3CA H1047R* with the IKKβ ATP-competitive inhibitor, Compound A (Ziegelbauer et al., 2005), attentuates PI3K-induced PTX3 levels. Furthermore, treatment of SUM159-PT breast cancer cells with Compound A also decreases PTX3 mRNA (**Figure 3-4D**) and protein expression (**Figure 3-4C**). This indicates that NF-κB signaling mediates regulation of PTX3 by the PI3K pathway.

PTX3 contributes to PI3K-driven growth factor independent proliferation and survival

One fundamental feature of a cancer cell is its capacity to sustain proliferative signaling in the absence of crtical growth factors. Previous work has shown that constitutively active mutant *PIK3CA E545K* and *H1047R*, but not wild-type *PIK3CA*, can permit the growth of immortalized mammary epithelial MCF10A cells in the absence of the epidermal growth factor (EGF) (Isakoff et al., 2005). Thus, we investigated the contribution of PTX3 in facilitating mutant *PIK3CA* growth factor independent cell proliferation. We observed that RNAi-mediated silencing of PTX3 decreases monolayer

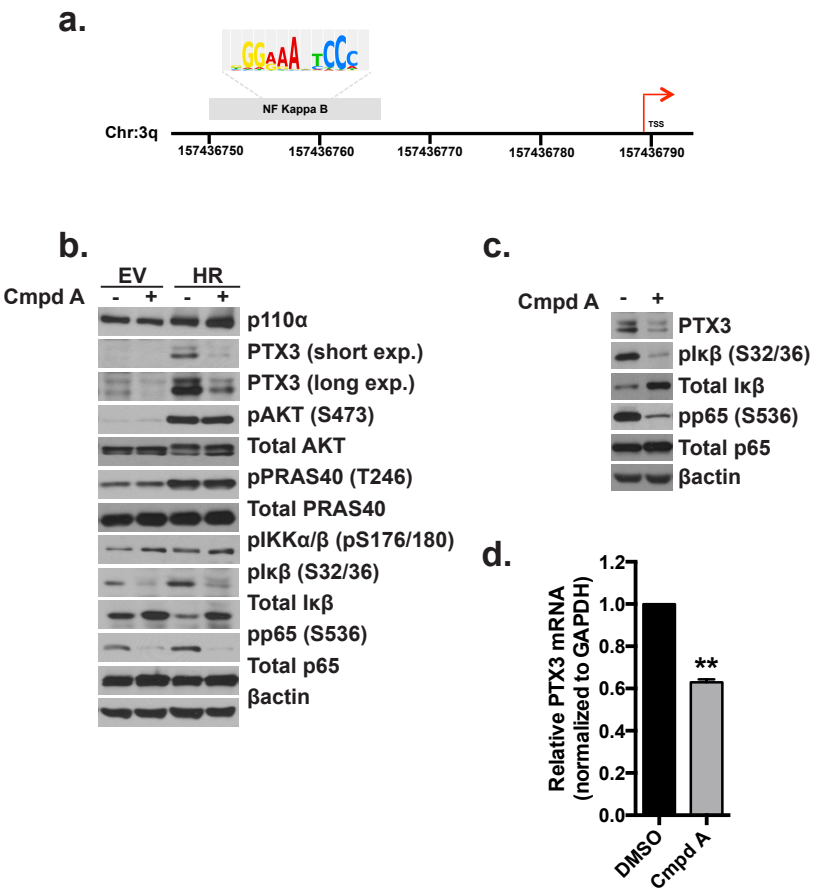


Figure 3-4: The PI3K pathway regulates PTX3 in a NF-κB dependent manner. (a) Promoter analysis - presence of a putative NF-κB binding site at the PTX3 promoter; Matrix logo depicts graphical representation of the predicted NF-κB family consensus motif. Chr: chromosome, TSS: transcription start site. (b) Immunoblot analysis of MCF10A cells stably expressing empty vector (EV) and mutant *PIK3CA H1047R* (HR) respectively. Cells were cultured in growth media lacking EGF and insulin for 24hrs followed by treatment with 5μM Compound A (Cmpd) for 24hrs. (c) Immunoblot analysis and (d) quantitative RT PCR measurements, of SUM159-PT breast cancer cells treated with 5μM Compound A for 16hrs. Each bar represents three independent biological replicates ± the SEM. Transcript levels were normalized to GAPDH and fold change was determined relative to DMSO treated cells. Statistical significance was determined using paired Student's t test, ** $p<0.01$.

growth of MCF10A cells expressing mutant *PIK3CA H1047R* under EGF and insulin deprived conditions (**Figure 3-5A, 3-5B**). Conversely, over-expression of PTX3 in SUM159-PT breast cancer cells increases proliferation under serum-starved conditions (**Figure 3-6**).

Although conventional monolayer cultures are powerful tools for studying cell proliferation, and growth responses to various cellular stresses, many studies have shown that growth of cells in a 3D culture system is a valuable model for understanding biological processes with *in vivo* relevance. Thus, we next examined the effect of shRNA-mediated silencing of PTX3 on the growth of *PIK3CA* mutant cells in 3D Matrigel. We observed that MCF10A cells expressing mutant *PIK3CA H1047R* develop large multi-acinar structures in comparison to control wild-type *PIK3CA* cells when grown in matrigel. Moreover, downregulation of PTX3 decreases the size of mutant *PIK3CA H1047R* acinar structures (**Figure 3-7**). Together, these data implicates PTX3 in PI3K-dependent proliferation and survival in both 2D and 3D Matrigel culture.

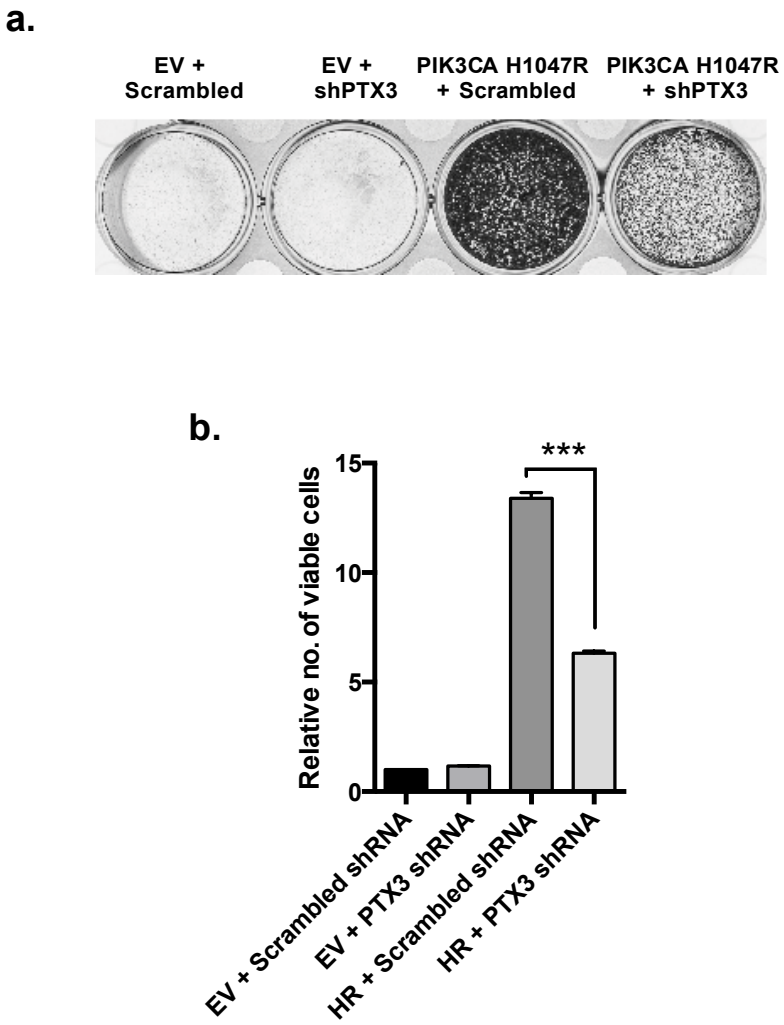


Figure 3-5: Knockdown of PTX3 decreases the viability of mutant *PIK3CA* cells under EGF and insulin deprived conditions. (a) Representative images of sulforhodamine (srb) stained MCF10A cells stably expressing empty vector (EV) and mutant *PIK3CA H1047R* (HR) co-infected with scrambled or PTX3 shRNA lentiviral constructs respectively. Cells were cultured in growth media lacking EGF and insulin. Images were taken on day 5. (b) Relative number of viable cells was determine by comparison to empty vector + scrambled (control) cells, using srb assay. Each bar represents three independent replicates ± the SEM. Statistical significance was determined using paired Student's t test, *** $p<0.001$.

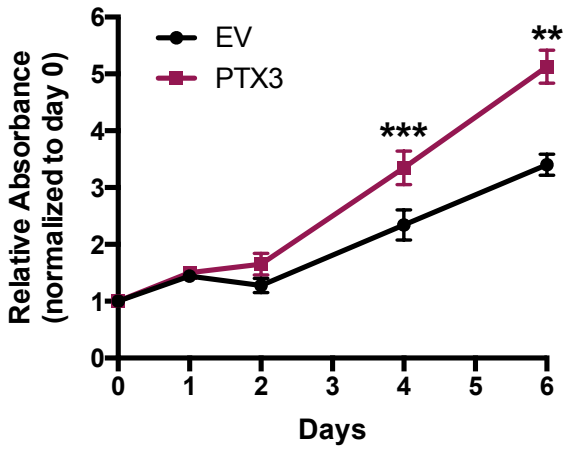


Figure 3-6: Overexpression of PTX3 increases proliferation of SUM159-PT cells under serum-starved conditions. Proliferation assay - relative absorbance values were compared to day 0. Each point represents four independent replicates ± the SEM. Statistical significance was determined using paired Student's t test, ** $p<0.01$, *** $p<0.001$.

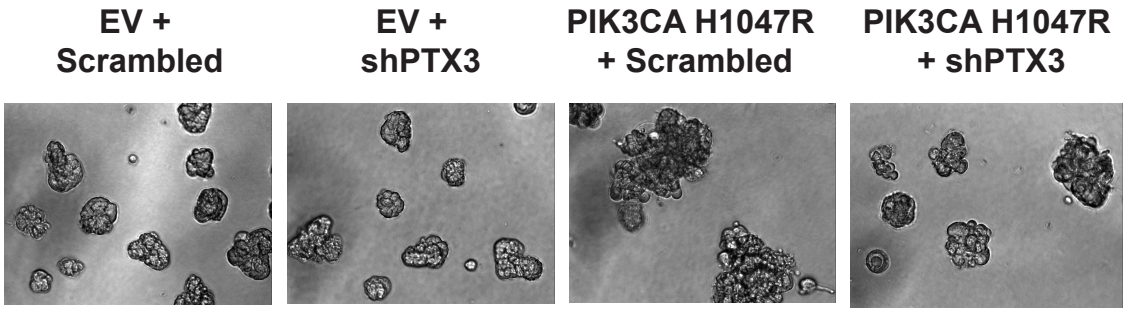


Figure 3-7: Knockdown of PTX3 decreases growth of mutant *PIK3CA* cells in 3D Matrigel. Representative images of MCF10A cells stably expressing empty vector (EV) and mutant *PIK3CA H1047R* (HR) co-infected with scrambled or PTX3 shRNA lentiviral constructs respectively. Cells were grown in 3D matrigel and images shown were taken on day 6.

5. DISCUSSION

PTX3 is a protein that is typically known for its role in immune surveillance where it facilitates pathogen recognition by immune cells, and for protection against autoimmunity (Garlanda et al., 2005). In contrast, less is known about the role of this protein in cancer. Here we show that oncogenic PI3K signaling increases PTX3 gene expression via the NF-κB pathway. We identify a cell autonomous role for PTX3 in mediating PI3K-dependent monolayer growth under nutrient and growth factor deprived conditions. The effect of PTX3 on cell proliferation is not limited to growth in 2D but also 3D Matrigel. This cell autonomous role for PTX3 is of particular interest since many of the functional studies on PTX3 involve its paracrine signaling with immune cells and extracellular matrix proteins. However, the exact mechanism by which PTX3 functions to elicit this cell autonomous effect requires further investigation.

On the other hand, much of the work done on the PI3K pathway focuses on its autonomous impact on the tumor cell and less is known about how PI3K-activation in tumor cells affect the tumour microenvironment. Given that PTX3 influences chemotaxic migration of macrophages and immune cells, angiogenesis and inflammation (Garlanda et al., 2005), future *in vivo* studies are required to examine the full complement of PTX3 function in PI3K-driven breast cancer.

It has been shown that MCF10A cells transformed by mutant *PIK3CA* most closely resemble triple negative, basal like breast cancer (Hutti et al., 2012). This subtype of breast cancer shows constitutive PI3K activation typically due to loss of either PTEN or INPP4B tumor suppressors and to a lesser extent *PIK3CA* mutations. Interestingly, our collaborators have shown that PTX3 expression is also specifically

enriched in the claudin-low subtype of basal-like breast cancer. Still, it is unclear why elevated PTX3 is enriched in this subtype given than *PIK3CA* mutations that render the PI3K pathway constitutively active, are highly prevalent in luminal A and HER2 positive breast cancers. We hypothesize that this preferential association of PTX3 in basal-like breast cancers may be due to the differential expression and activity of PTX3 co-regulators. It is also possible that changes in the chromatin landscape at the PTX3 promoter varies among each subtype. This would influence the accessibility of transcriptional regulators at the PTX3 locus and consequently affect its expression levels. Further studies will be required to test these hypotheses. Given that basal-like breast cancers typically show poor outcomes, it would also be useful to determine whether PTX3 is a reliable prognostic biomarker for breast cancer survival.

While our findings are consistent with previous studies which indicate an oncogenic role for PTX3, other recent studies have demonstrated that PTX3 may function as a tumor suppressor. This suggest that the role of PTX3 may be context dependent and tissue specific.

6. CONCLUSION

Overall, our findings provide evidence for the relevance of PTX3 in breast cancer. We show that PTX3 is a novel PI3K-regulated gene whose transcription is regulated by the NF-κB pathway. We also demonstrate that PTX3 is an important mediator of PI3K-dependent proliferation under growth factor deprived conditions and in 3D Matrigel. This study may have future diagnostic and therapeutic implications for PI3K-driven basal-like breast cancer.

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CHAPTER IV

IDENTIFICATION AND CHARACTERIZATION OF PI3K-REGULATED LINCRNA C14ORF34 IN BREAST CANCER

This project was done in collaboration with Postdoctoral Fellow David Hendrickson, Rinn Lab, Department of Stem Cell and Regenerative Biology, Harvard University. Whitney Henry independently discovered c14orf34 in a model of PI3K-induced cellular transformation, provided clinically relevant c14orf34 gene expression profiling data, and compiled preliminary evidence for a role for c14orf34 in breast cancer malignancy.

1. ABSTRACT

Although lincRNAs have recently been implicated in tumorigenesis, the characterization of this nascent class of noncoding RNAs in cancer biology requires further investigation. Here we discover a novel lincRNA called c14orf34 in breast cancer. Our studies indicate that c14orf34 is upregulated in mammary epithelial cells transformed by oncogenic Src, in a manner that is dependent on the transcription factor, STAT3. Similarly, c14orf34 is also increased in mutant *PIK3CA* transformed mammary epithelial cells and is regulated by the PI3K pathway. Gene expression profiling revealed that c14orf34 is preferentially associated with basal-like breast cancer cells and is elevated in a subset of invasive breast carcinomas from human patients. RNAi-mediated depletion of c14orf34 results in a decrease in breast cancer cell proliferation, cell migration and loss of invasive structures when grown in 3D Matrigel. Using RNA-Seq, we identified several genes that are differentially expressed upon overexpression of c14orf34 in MCF10A cells. Many of these genes are also induced in MCF10A transformed cells and play important roles in cancer progression. Together, our findings highlight the importance of lincRNA c14orf34 in the molecular etiology of breast cancer.

2. INTRODUCTION

Over the last few decades, intense efforts have been made on understanding the role of protein-coding genes in breast cancer malignancy. However, advancements in RNA-sequencing technologies have revealed that protein-coding genes represent <2% of the human genome. In contrast, a large proportion of the human transcriptome is composed of noncoding RNA transcripts including long noncoding RNAs (lncRNAs) (Consortium et al., 2007). Large intergenic noncoding RNAs (lincRNAs) are a new subclass of lncRNAs. They are located between two genes and range from 200 nucleotides to >10kb (Ponting et al., 2009). Similar to protein-coding mRNA, lincRNAs may be 5' capped, spliced and polyadenylated, despite their lack of protein-coding capability (Affymetrix and Cold Spring Harbor Laboratory, 2009; Carninci et al., 2005; Mercer et al., 2010). It is estimated that there are >1000 lincRNAs encoded in the mammalian genome (Guttman et al., 2009). Gene expression analysis and more recently, functional studies have supported a role for lincRNAs in a vast array of biological processes including cell cycle regulation, apoptosis, cellular trafficking, immune surveillance, and embryonic stem cell pluripotency (Khaitan et al., 2011; Loewer et al., 2010; Wang et al., 2008; Wapinski and Chang, 2011).

While the mechanism(s) by which lincRNAs function is still largely unknown, it has been demonstrated that lincRNAs can modulate the expression of specific target genes. It has been postulated that this can be achieved if the lincRNA (1) acts as a decoy to inhibit the binding of transcription factors or other DNA binding proteins, (2) as molecular scaffolds to facilitate formation of ribonucleoproteins and (3) as guides to direct various chromatin–modifying complexes to their sites of action (Wang and Chang,

2011). Many lincRNAs involved in x-inactivation including the well-studied lincRNA Xist, are cis-acting chromatin-associated lincRNAs (Augui et al., 2011; Lee, 2009). Another possibility, albeit with less supportive evidence, is that the mere act of transcription of the lincRNA at a specific genomic site may influence the expression of its neighboring genes (Ulitsky and Bartel, 2013).

Emerging studies have implicated lincRNAs in a diverse array of cancers including breast cancer (Calin et al., 2007; Gupta et al., 2010; Lin et al., 2007; Prensner et al., 2011). Functional studies indicate that these noncoding RNAs may modulate many cellular processes involved in tumorigenesis. For example, overexpression of the antisense noncoding RNA ANRIL, in prostate cancer cells, results in the transcriptional repression of the INK4n/ARF/INK4a tumor suppressor genes, which regulate cell cycle progression and senescence (Yap et al., 2010). In melanoma cells, RNAi-mediated knockdown of the highly expressed lincRNA SPRY4-IT1 results in defects in cell growth and increases rates of apoptosis (Khaitan et al., 2011). LincRNAs can also function in a tumor suppressive capacity. Studies show that lincRNAp21 mediates global repression of genes to facilitate p53-dependent cellular apoptosis after induction of the p53 transcriptional response to DNA damage and cellular insults (Huarte et al., 2010). Additionally, lincRNAs may also serve as prognostic biomarkers as exemplified with lincRNA Hotair. Elevated expression of Hotair was shown to promote breast cancer metastasis and was associated with poor survival (Gupta et al., 2010).

Despite the growing body of literature on lincRNAs, less than 1% of the identified human lincRNAs are characterized (Chisholm et al., 2012). Clearly, our understanding of lincRNA biology is far from complete. Furthermore, the identification, regulation and

functional characterization of lincRNAs involved in breast cancer pathogenesis require further investigation. Here we identify a novel lincRNA called c14orf34 in breast cancer using two independent systems of cellular transformation. We show that c14orf34 expression is clinically relevant in human breast cancer and is elevated in basal-like breast cancer cells. In addition, we investigate the transcriptional regulation of this lincRNA and provide evidence for its role in breast cancer development. Given that many lincRNAs function as fine-tune regulators of gene expression, we assess the role of c14orf34 in this capacity.

3. MATERIALS AND METHODS

Antibodies, plasmids and reagents

pBabe puro HA PIK3CA WT (Addgene plasmid #12522) and pBabe puro HA PIK3CA H1047R (Addgene plasmid # 12524) was a gift from Jean Zhao. pBABE-puro was a gift from Hartmut Land & Jay Morgenstern & Bob Weinberg (Addgene plasmid # 1764). Isogenic MDA-MB-231 cells expressing PIK3CA WT, PIK3CA E545K and PIK3CA H1047R were a kind gift from J. Backer lab. MCF10A-Src inducible cell lines were generated by the Struhl's lab (Iliopoulos et al., 2009).

Retroviral expression of c14orf34

C14orf34 was cloned by David Hendrickson as previously described (Hacisuleyman et al., 2014).

RNA interference

For c14orf34 and PIK3CA shRNAs, single-stranded oligonucleotides sense and antisense pairs, encoding the indicated target sequences were synthesized and cloned into PLKO.1 vector. Stable cell lines were maintained in 2μg/ml puromycin.

PIK3CA shRNA#1, sense, 5'-
CCGGGCACAATCCATGAACAGCATTCTCGAGAATGCTGTTCATGGATTGTGCTTTTTTG-3';

PIK3CA shRNA#1, antisense, 5'-
AATTCAAAAACACAATCCATGAACAGCATTCTCGAGAATGCTGTTCATGGATTGTG-3'

PIK3CA shRNA#2, sense, 5'-

CCGGGCATTAGAATTTACAGCAAGACTCGAGTCTTGCTGTAAATTCTAATGCTTTTT

TG-3';

PIK3CA shRNA#2, antisense, 5'-

AATTCAAAAAGCATTAGAATTTACAGCAAGACTCGAGTCTTGCTGTAAATTCTAATG

C-3'

C14orf34 shRNA#1, sense, 5'-

CCGGAAGAGAAAAGCTGAAGGACACCTCGAGGTGTCCTTCAGCTTTTCTCTTTTTT

TG

C14orf34 shRNA#1, antisense, 5'-

AATTCAAAAAAAGAGAAAAGCTGAAGGACACCTCGAGGTGTCCTTCAGCTTTTCTC

TT

C14orf34 shRNA#2, sense, 5' –

CCGGACCTCAAATCTTTCGAGAACACTCGAGTGTTCTCGAAAGATTTGAGGTTTTTT

G

C14orf34 shRNA#2, antisense, 5'-

AATTCAAAAAACCTCAAATCTTTCGAGAACACTCGAGTGTTCTCGAAAGATTTGAGG

T

Cell Culture and Immunoblotting

MCF10A and DCIS cells were cultured in Ham's F12/DMEM (Cellgro) supplemented with 5% equine serum (Cellgro), 500ng/ml hydrocortisone (Sigma-Aldrich), 100ng/ml cholera Toxin (List Biological Laboratories), 20ng/ml EGF (R&D Systems) and 10ug/ml

insulin. SKBR3 cells were maintained in 10% FBS/McCoy's (Cellgro). MCF7, and MDA-MB-468, MDA-MB-453, MDA-MB-231 cells were maintained in Dulbecco's modified Eagle medium (DMEM; Cellgro) supplemented with 10% Fetal Bovine Serum (FBS; Cyclone). T47D and BT549 cells were cultured in 10% FBS/DMEM, supplemented with 1mg/ml insulin (Sigma-Aldrich). SUM159-PT cells were grown in Ham's F12 medium (Cellgro) supplemented with 5% FBS, 1ug/ml hydrocortisone (Sigma-Aldrich) and 5ug/ml insulin (Sigma-Aldrich). ZR75-1 and HCC1806 were maintained in RPMI 1640 supplemented with 10% FBS (Cellgro). For all western blotting, cells were lysed in RIPA buffer with protease and phosphatase inhibitors.

3D Morphogenesis Assay

MCF10A cells were grown in three dimensional Matrigel cultures as described previously (Debnath et al., 2003). Briefly, $3x10^3$ cells were suspended in modified growth medium containing 2% growth factor-reduced Matrigel, 2% Horse serum (Cellgro) and 5ng/ml EGF (R&D systems). Cell mixture was plated on top of a solidified layer of growth factor-reduced Matrigel. Cells were fed every 4 days.

Quantitative real-time RT PCR

Total RNA was isolated using the RNeasy kit following the manufacturer's instructions (Qiagen). Reverse transcription was performed using Quantitect Reverse transcription kit according to the manufacture's instructions (Qiagen). Quantitative real-time RT-PCR was performed using SYBR Green PCR Master Mix (BioRad) and the ABI Prism 7900

sequence detector (Applied Biosystems). Relative mRNA expression was calculated by the ΔΔCT method with GAPDH as reference. Primer sequences:

C14orf34 sense: 5'- GTGTACATTTCTGGGTAGCTT

C14orf34 antisense: 5' - AAAGGAAAACAATACAGGCTTG

GAPDH sense: 5' - GCAAATTCCATGGCACCGT

GAPDH antisense: 5'- TCGCCCCACTTGATTTTGG

Transwell Migration Assay

Transwell migration assay was performed as previously described (Chin and Toker, 2010). Cells were allowed to migrate through an 8μm-pore transwells (Corning) for 16 hours. NIH3T3 conditioned media was used as a chemoattractant in the lower chamber.

RNA-Seq Analysis and ChIP-Seq Analysis

RNA-Seq and ChIP-Seq analysis was done by David Hendrickson. RNA-Seq analysis was performed as previously described (Hacisuleyman et al., 2014). Raw ChIP-Seq data was retrieved from (Fleming et al., 2015).

4. RESULTS

Identification and gene expression profiling of a novel lincRNA called c14orf34 in breast cancer

As a first attempt to identify novel lincRNAs that are involved in breast cancer malignancy, we employed a well-established immortalized mammary epithelial MCF10A cell line model containing a tamoxifen-inducible Src kinase oncoprotein (Src). After 36 hours of treatment with tamoxifen (TAM), these cells show multiple features of cellular transformation including colony formation in soft agar, increased migration and invasion and tumor formation capability in immunocompromised mice (Iliopoulos et al., 2009). By performing RNA-sequencing analysis of these MCF10A cells, we discovered a novel lincRNA called c14orf34 that is strongly upregulated upon Src induction (**Figure 4-1A, 4-1B**). LincRNA c14orf34 resides ~112kb from the kinesin receptor KTN1 and ~ 321kb from the E3 ubiquitin ligase family member 2, PEL12. In support of c14orf34 being an independent transcript, we noted that c14orf34 is transcribed in the opposite direction to either flanking gene. More detailed analysis of this lincRNA revealed that it contains 3-4 exons depending on isoform type. Next, we examined the expression of c14orf34 in a panel of breast cancer cell lines with various genetic aberrations. Strikingly, we identified a preferential upregulation of c14orf34 in basal-like, triple-negative breast cancer cell lines, many of which display a high metastatic potential and poor prognosis (**Figure 4-1C**). Furthermore, analysis of RNA-sequencing data of invasive breast carcinomas obtained from the TCGA database**,** revealed that c14orf34 is genetically modified in ~4% of breast cancer patients. Most alterations resulted from mRNA upregulation and genomic amplification, although deletion of the c14orf34 locus was

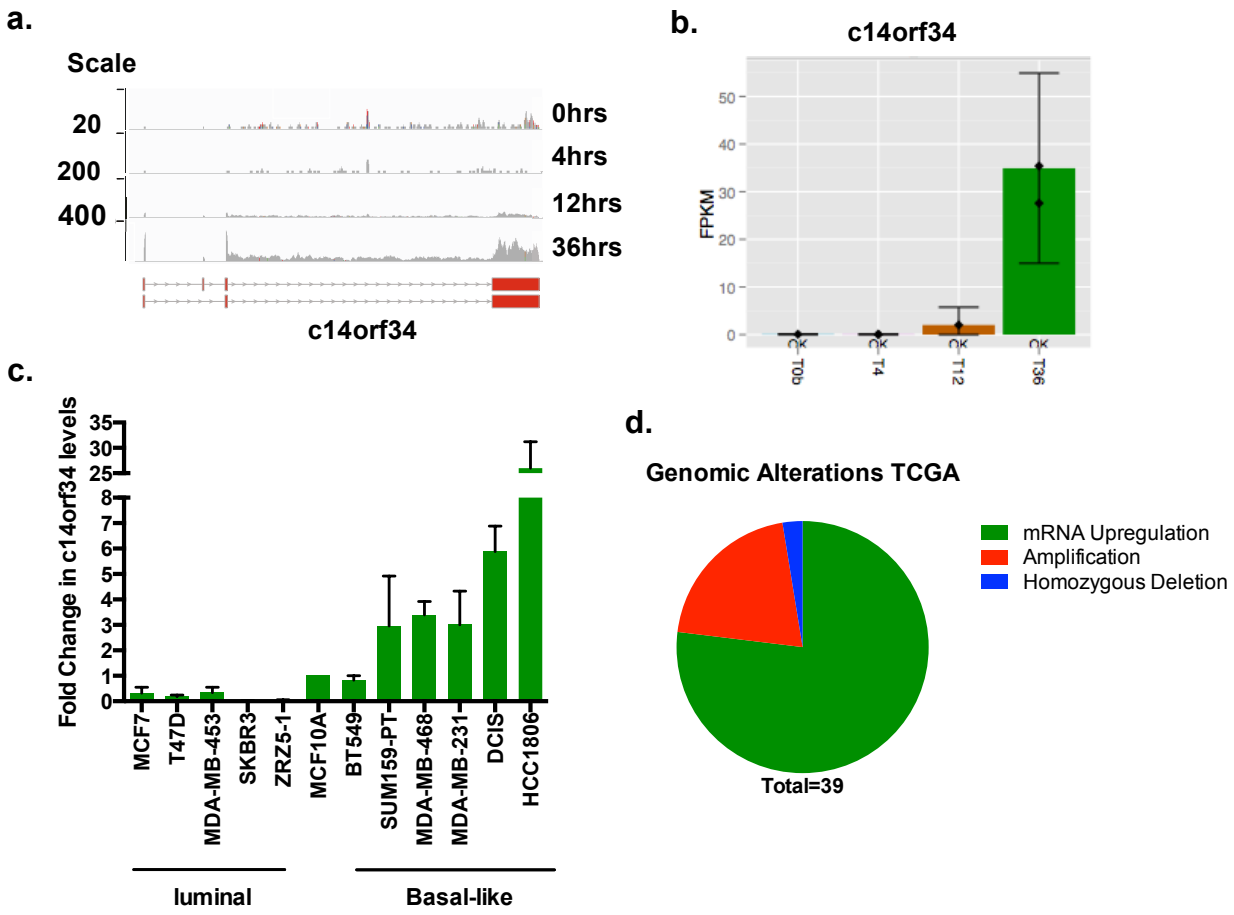

Figure 4-1: Identification and structural characterization of a novel lincRNA, c14orf34. (a-b) RNA Sequencing, relative expression of c14orf34 at various time-points post Src induction in immortalized mammary epithelial cells MCF10A. FPKM, fragments per kilobase of transcript per million mapped reads. (c) Relative expression of c14orf34 in a panel of breast cancer cell lines, compared to MCF10A cells. Expression was determined by qRT-PCR and normalized to GAPDH. Values are representative from three independent experiments. (d) Genomic alterations of c14orf34 in a subset of human breast cancer patients with invasive breast carcinomas. Data was generated from TCGA database and represents 4% of 965 tumor samples analyzed.

observed in one patient (**Figure 4-1D**).

C14orf34 is regulated by the transcription factor STAT3 during Src-induced transformation

Given that the transcription factor STAT3 has been shown to play an important role in Src-induced transcriptional responses during cellular transformation, we analyzed published chromatin immunoprecipitation (ChIP) data performed in the MCF10A Src-inducible model, to determine whether STAT3 directly binds to the c14orf34 promoter. An enrichment of STAT3 binding to the c14orf34 promoter region was observed as early as 4 hours post Src induction, with a significant increase at 36 hours; coinciding with the surge in c14orf34 transcript levels at this time-point (**Figure 4-2A**). To further corroborate this finding, we evaluated c14orf34 expression by qRT-PCR upon siRNA-mediated knockdown of STAT3 in the MCF10A-Src system. Our data show that depletion of STAT3 abolishes Src-induced upregulation of c14orf34 (**Figure 4-2B**).

C14orf34 is regulated by the PI3K pathway

Next, we established a cell line model of MCF10A cells expressing wild-type *PIK3CA* or oncogenic *PIK3CA H1047R* (**Figure 4-3A**). Oncogenic *PIK3CA H1047R* induces cellular transformation as indicated by the increase in colony formation in soft agar compared to wild-type *PIK3CA* (**Figure 4-3B**). Given the similarities in the transcriptional signature of MCF10A cells transformed by mutant *PIK3CA H1047R* and MCF10A-Src transformed cells (**Figure 4-3C**) we investigated whether the PI3K pathway regulates the expression of c14orf34. An increase in c14orf34 expression was

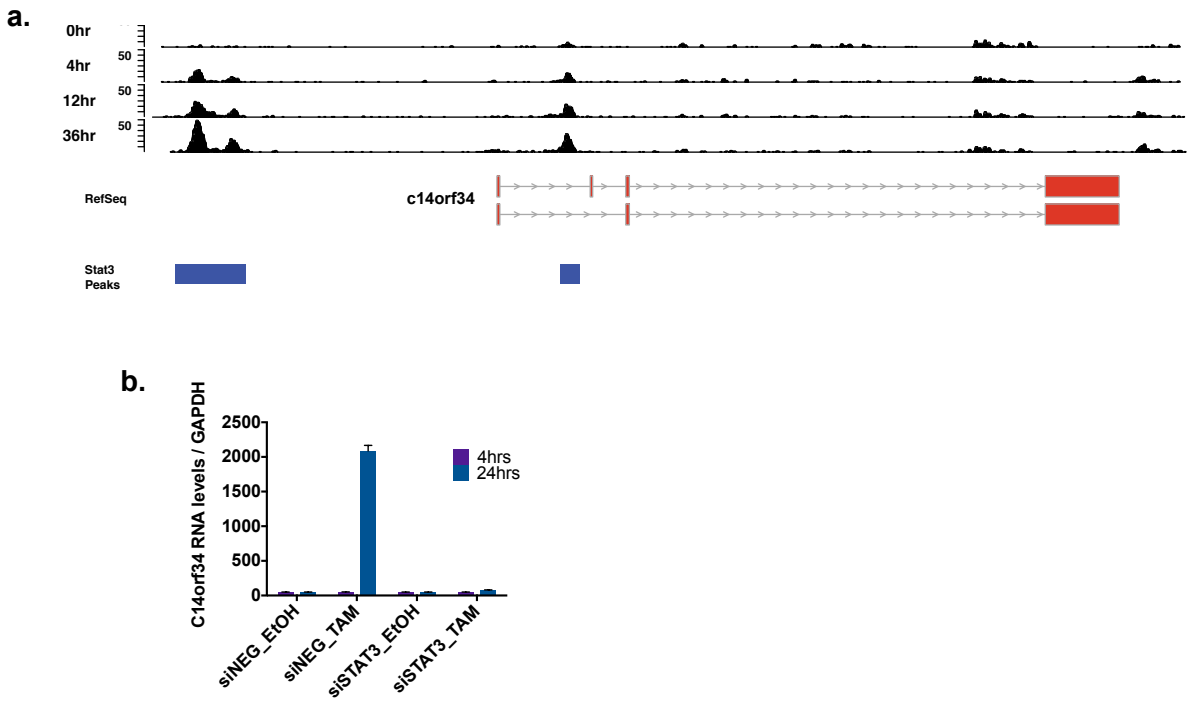

Figure 4-2: Regulation of c14orf34 by the transcription factor, STAT3. (a) STAT3 Ch-IP enrichment in MCF10A cells post Src induction, at c14orf34 locus. (b) Expression of c14orf34 in siRNA-mediated knockdown of STAT3 in MCF10A-Src transformed cells. Transcript levels were determined by qRT-PCR and normalized to GAPDH. Values represent the average of three technical triplicates.

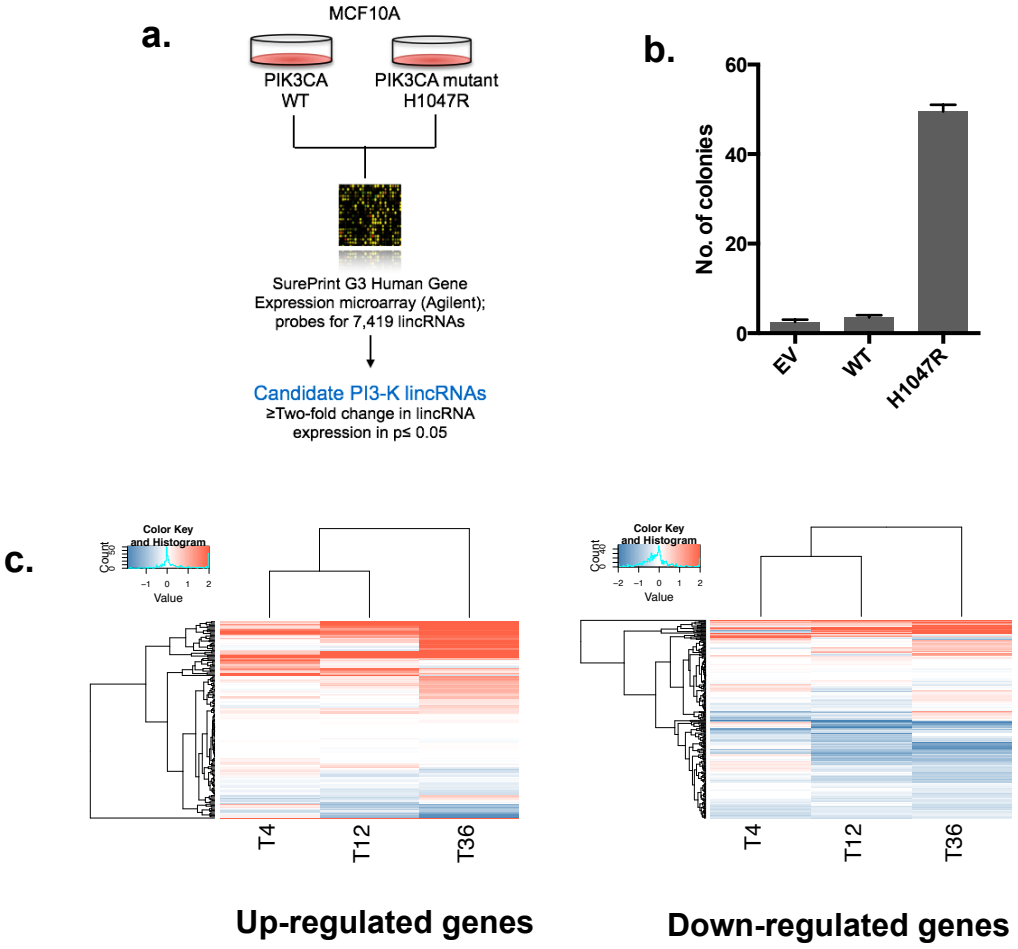

Figure 4-3: Similarity in transcriptional profile in Src-transformed and mutant *PIK3CA*-transformed MCF10A cells. (a) Schematic of MCF10A system used to identify PI3K regulated genes involved in transformation. (b) Colony growth in soft agar, MCF10A cells stably expressing *PIK3CA* wild-type, mutant *PIK3CA E545K* and *H1047R* were compared relative to empty vector control (EV). (c) Heat map showing the expression pattern of genes in Src-transformed system that are increased or decreased by two folds in mutant *PIK3CA* transformed MCF10As.

observed in MCF10A cells expressing mutant *PIK3CA H1047R* relative to wild-type *PIK3CA* cells (**Figure 4-4A**). Likewise, a similar upregulation of this lincRNA was observed in isogenic MDA-MB-231 cells expressing mutant *PIK3CA E545K* and *H1047R* respectively (**Figure 4-4B**). In contrast, c14orf34 was downregulated upon knockdown of *PIK3CA* in SUM159-PT (mutant *PIK3CA H1047L*) breast cancer cells (**Figure 4-4C**). Together this data indicates that c14orf34 is regulated downstream of the PI3K pathway.

Loss of c14orf34 decreases breast cancer cell proliferation, migration and integrity of invasive cellular structures

In order to assess the biological role of c14orf34 in breast cancer, we performed shRNA-mediated loss-of-function studies. Two distinct shRNA constructs, each targeting a different exon of c14orf34, was cloned (**Figure 4-5A**). More than 80-90% knockdown efficiency was observed by qRT-PCR, in SUM159-PT cells (**Figure 4-5B**) and DCIS cells (**Figure 4-6B**). We first investigated the effects of loss of c14orf34 on cell proliferation. We observed a modest decrease in the proliferation of SUM159-PT breast cancer cells upon knockdown of c14orf34 (**Figure 4-5C**). Next, we explored the effects of c14orf34 on cell motility. Our findings indicate that knockdown of c14orf34 leads to a decrease in transwell migration of SUM159-PT breast cancer cells. We also observed a loss in the invasive protrusions from DCIS spheroids grown in Matrigel upon depletion of c14orf34 (**Figure 4-6B-C**). This is suggestive of a role for c14orf34 in cell invasion.

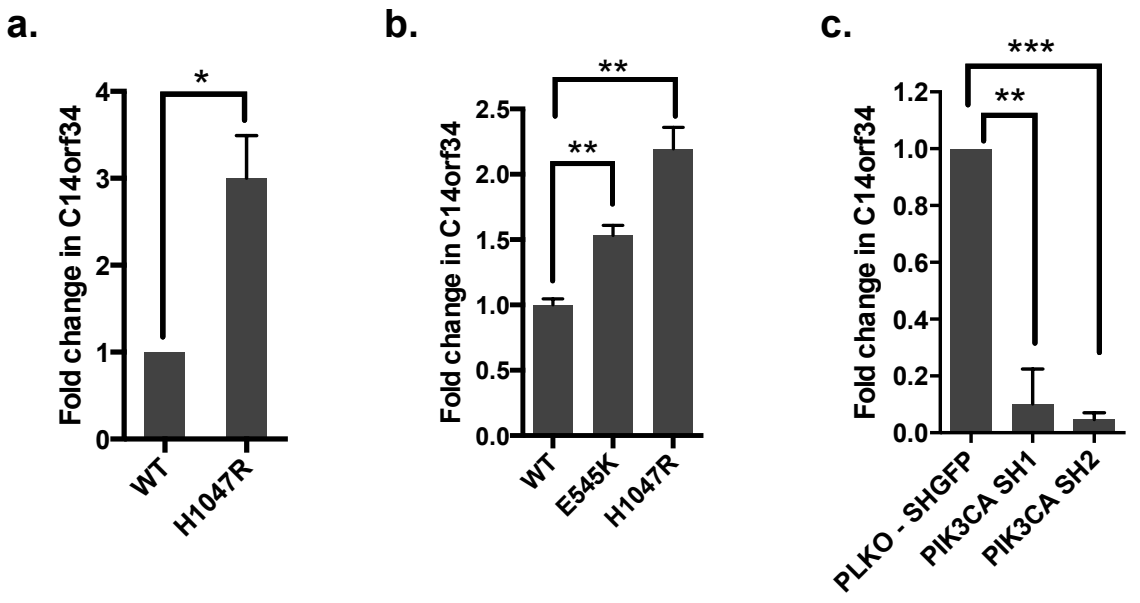

Figure 4-4: C14orf34 is regulated by the PI3K pathway. QRT PCR, relative expression of c14orf34 in: (a) MCF10A cells expressing *PIK3CA-WT* (WT) compared to mutant *PIK3CA H1047R*, (b) Isogenic MDA-MB-231 cells expressing *PIK3CA-WT*, mutant *PIK3CA E545K* and *H1047R* respectively and (c) shRNA-mediated knockdown of *PIK3CA* in SUM159-PT cells. Transcript levels were normalized to GAPDH. Data represents average of three independent experiments ± the SEM. Statistical significance was determined using paired Student's t test, * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

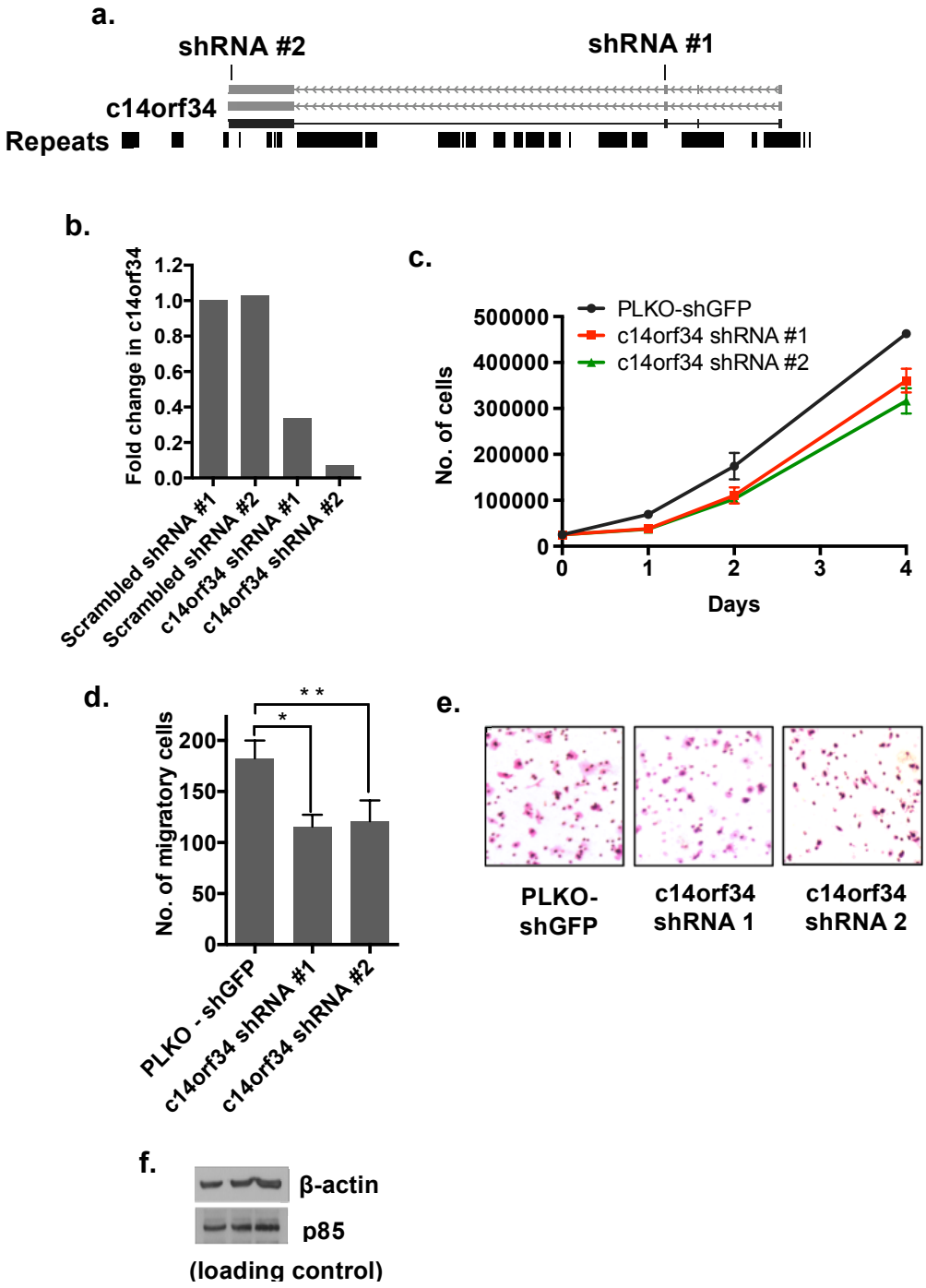

Figure 4-5: Loss of c14orf34 decreases breast cancer cell proliferation and migration.
(a) Schematic showing regions targeted by c14orf34 shRNA#1 and shRNA#2. (b) Validation of knockdown of c14orf34 in SUM159-PT cells used for migration and proliferation assays. (c) Proliferation assay and (d-f) Transwell migration assay of SUM159-PT cells expressing two different c14orf34 shRNA constructs.

Overexpression of c14orf34 results in a gene expression profile that resembles that of a transformed cell

Given previous studies have suggested a role for lincRNAs as regulators of gene expression, we performed RNA-sequencing to investigate the effect of overexpression of c14orf34 in untransformed MCF10A cells. We observed that a significant number of genes that are upregulated in the Src-transformed cells are also upregulated in the c14orf34 overexpressing cells (**Figure 4-7**). Notably, overexpression of a pool of lincRNAs that do not affect transformation closely resembles the gene expression profile of untransformed MCF10A cells. Interestingly, some of these c14orf34-regulated genes are known to play a role in cell motility and invasiveness. This may provide some insight into mechanism by which c14orf34 affects these cellular processes.

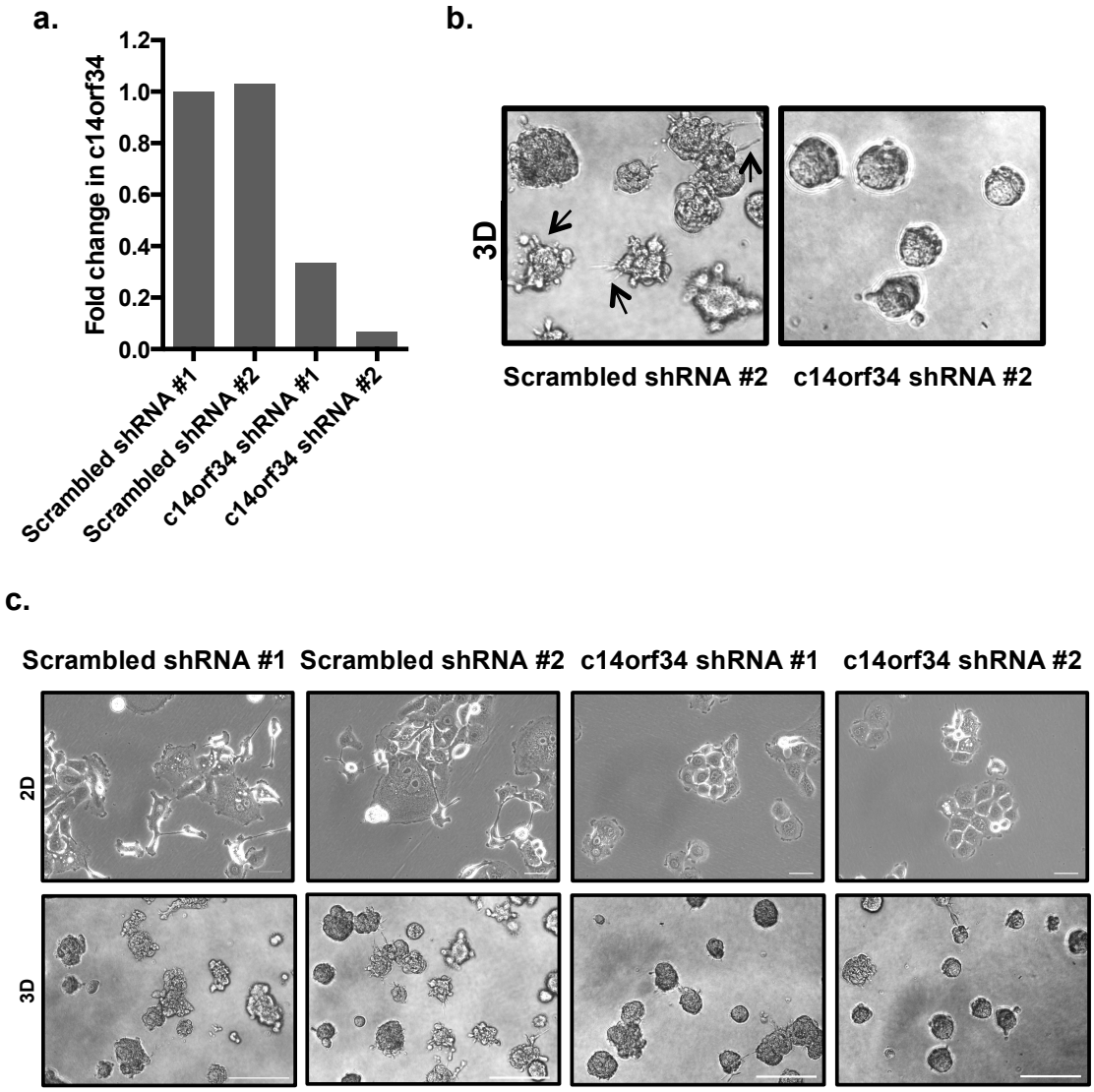

Figure 4-6: Depletion of c14orf34 affects the integrity of invasive protrusions. (a) QRT PCR validation of knockdown of c14orf34 in DCIS cells. Transcript levels were normalized to GAPDH and fold change was calculated relative to scrambled shRNA #1. (b) Representative phase-contrast images of DCIS acinars infected with lentiviral scrambled or c14orf34 shRNA constructs, grown in 3D Matrigel. Images displayed were taken on day 5. (c) (Top) Morphological effects of knockdown of c14orf34 in DCIS cells grown in 2D, scale bar = 100μm and (Bottom) in 3D Matrigel cultures. For 3D, images shown were taken at day 5, scale bar = 500μm.

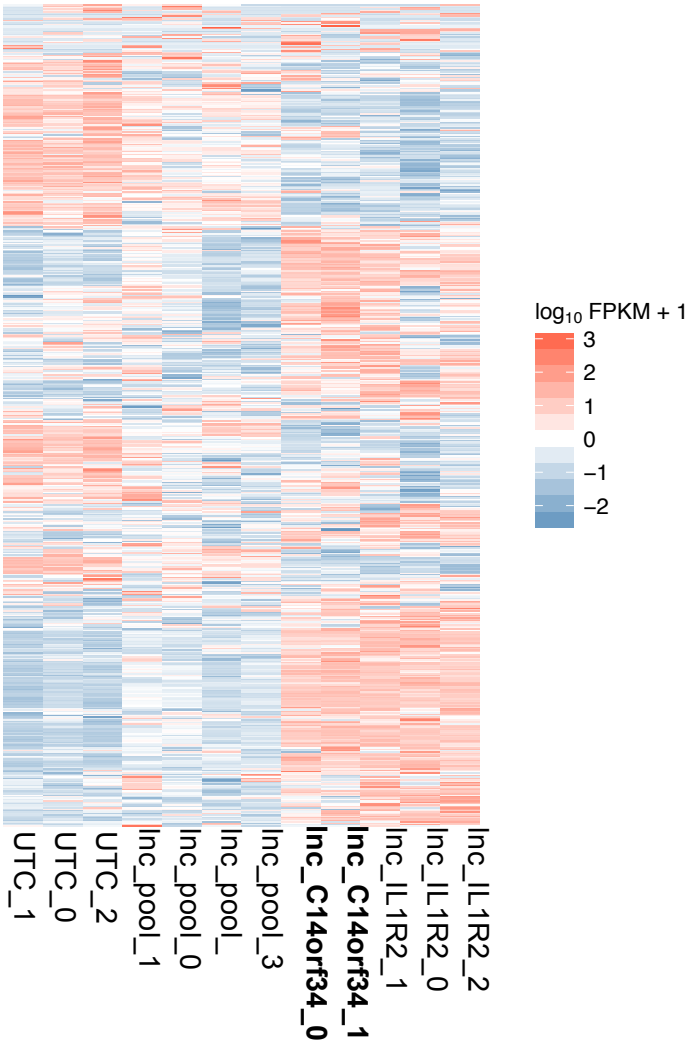

Figure 4-7: Heat map showing expression profile of MCF10A cells overexpressing c14orf34. All the genes depicted in the heat map were upregulated in MCF10A-Src transformed cells. UTC: Untransformed MCF10A cells; lnc_pool are set of lincRNAs that are not involved in transformation; lnc_ILR2 is a lincRNA potentially involved in cell transformation.

5. DISCUSSION

In this study, we identified a novel lincRNA called c14orf34 and provide evidence for a potential role in breast cancer migration and invasion. Our studies indicate that c14orf34 is transcriptionally upregulated in immortalized mammary epithelial cells that have undergone transformation by oncogenic PI3K or oncogenic Src, respectively. This is mediated by the transcription factor, STAT3. Notably, expression of c14orf34 is increased in basal-like breast cancer cells which coincidently also show a preferentially increase in STAT3 activity. While tissue specificity and orchestrated transcriptional regulation might suggest functionality, this must be interpreted with caution. Studies have shown that the chromatin landscape can vary with lineage specificity and cell fate, which may inadvertently lead to accessibility of transcription factor binding sites at cryptic promoters (Ulitsky and Bartel, 2013).

Thousands of lincRNAs have been annotated to date. However, many appear to offer little fitness advantage to the cell and thus the number of possible functional lincRNAs is heavily debated. Indeed, there are clear examples of well-characterized lincRNAs like Xist, which play a critical role in dosage compensation (Penny et al., 1996). Here we provide evidence for a role for c14orf34 in breast cancer. Loss of c14orf34 leads to a decrease in cell proliferation, motility and possibly - cell invasiveness, refuting the idea that it might be a trivial promiscuous transcript.

Thus far, no mammalian ortholog of c14orf34 has been found. This is not completely surprising, as many lincRNAs appear to be poorly conserved and tend to undergo rapid evolution. For instance, only ~ <12% of human and mouse lincRNAs have homologs found in other species (Cabili et al., 2011; Church et al., 2009) and <6%

of zebrafish lincRNAs share homologous sequences in human or mouse (Penny et al., 1996). While lack of conservation may bolster the argument that lincRNAs are merely cryptic noise, knowledge of Xist challenges this notion. Although Xist is critical for dosage compensation, most of its sequence is poorly conserved and deletion of exon 4, which shows the most obvious conservation pattern, is inconsequential (Caparros et al., 2002). Moreover, analysis of exon one which is thought to be important for Xist function, and which contains most of the conserved repetitive sequences yields low PhastCons scores (Beletskii et al., 2001; Wutz et al., 2002). A more comprehensive catalog of lincRNAs from several vertebrate species along with advancements in computational assessment tools, may improve comparative lincRNA studies. Only then will we be able to gain a more accurate picture of lincRNA conservation.

Our study also indicates that c14orf34 affects global gene expression. Importantly, the gene expression profile due to overexpression of c14orf34 shows great similarity to that of Src-transformed cells. This finding, together with the fact that overexpression of c14orf34 does not affect its neighboring genes; suggest that c14orf34 might be a trans-acting lincRNA. The first example of a trans-acting lincRNA was demonstrated with Hotair (Gupta et al., 2010). Since then, other trans-acting lincRNAs have been documented. C14orf34 might mediate its function by interacting with various chromatin modifiers and/or transcriptional regulators such as PRC1, PRC2, LDS1 among other factors, as observed for other lincRNAs such as Hotair, Hottip, Mistral and Xist (Bertani et al., 2011; Penny et al., 1996; Tsai et al., 2010; Wang et al., 2011). Despite these examples, it is still debatable whether these RNA-protein interactions are direct and the factors that dictate the specificity of these interactions are still not clearly

defined. Another possibility is that the lincRNA can interact with other RNA and DNA molecules to facilitate its task (Ulitsky and Bartel, 2013).

Future studies will require further investigation to elucidate the mechanistic action of c14orf34. In addition to lincRNA-interaction studies, localization studies could also be employed. While there are well-studied examples of lincRNAs that are enriched in the nucleus (Brown et al., 1992; Hutchinson et al., 2007; Sone et al., 2007), many lincRNAs appear to cytoplasmic (Coccia et al., 1992; Kino et al., 2010; Yoon et al., 2012). It would be interesting to determine how lincRNAs are exported from the nucleus; how they evade RNA decay and remain stable, and whether other modes of functionality apart from the ones described above exist.

6. CONCLUSION

In summation, this study supports the relevance of lincRNAs in human cancer pathogenesis. To our knowledge, it is the first study that investigates the regulation and biological function of c14orf34. It also implicates for the first time, a lincRNA as a novel downstream effector of PI3K signaling. Future studies will be required to understand the entire complement of c14orf34 biology and its significance in other processes critical for breast cancer initiation and progression.

CHAPTER V

CONCLUSION

1. SUMMARY OF THESIS

The overall objective of this thesis work was twofold: (1) to investigate the chemotherapeutic benefit of aspirin in PI3K-dependent breast cancers and (2) to explore novel downstream effectors of PI3K signaling that may contribute to breast cancer pathogenesis.

In chapter 2, we demonstrate that aspirin preferentially sensitizes mutant PI3K breast cancer cells to PI3K inhibitors to augment growth suppression. By performing mechanistic studies, we show that the growth inhibitory effect of aspirin in combination with PI3K inhibitors may be due to enhanced activation of AMPK, inhibition of mTORC1 signaling and subsequent induction of autophagy. Importantly, we also demonstrate that pharmacologic inhibition of COX-2 and IKKβ, which are also regulated by the PI3K pathway, and well-known targets of aspirin, does not decrease the viability of *PIK3CA* mutant cells in our cell-based models. Given the growing interest in personalized medicine, this pre-clinical study may provide insight for the stratification of patients who are most likely to benefit from adjuvant aspirin therapy.

Additionally, we discovered novel protein-coding genes and long noncoding RNAs that are regulated by the PI3K pathway using a combination of biochemical and genetic approaches. In chapter 3, we show that the PI3K pathway regulates the *PTX3* gene in a NF-κB dependent manner. Furthermore, we highlight a role for PTX3 in PI3K-driven proliferation and survival in 2D and 3D Matrigel cultures. In chapter 4, we also identify a novel lincRNA transcript called c14orf34, which is upregulated in two independent models of cellular transformation, one of which is driven by mutant *PIK3CA*. We provide evidence supporting transcriptional regulation of c14orf34 by the

transcription factor STAT3. By performing gene expression profiling, we show that c14orf34 is associated with basal-like breast cancer cell lines and elevated in a subset of invasive breast carcinomas from human patients. In addition, we provide preliminary evidence that suggest a role for c14orf34 in cell proliferation, motility and invasion, and in the regulation of global gene expression.

In summation, this thesis work suggests that aspirin in combination with PI3K pathway inhibitors may be an efficacious therapeutic strategy in PI3K-addicted breast cancers. It reinforces the complexity of PI3K signaling in breast cancer development and offers potentially new avenues for diagnostic and therapeutic purposes. The implications of these findings are further discussed below.

2. DISCUSSION

Although aspirin is primarily administered as an antipyretic and analgesic, numerous observational and randomized clinical trials have suggested a potential chemotherapeutic use (Baron et al., 2003; Rothwell et al., 2011; Rothwell et al., 2012). Recently, two independent studies demonstrated that aspirin use is associated with superior cancer-specific survival in colorectal cancer patients with mutant-*PIK3CA* but not among those with the wild-type gene (Domingo et al., 2013; Liao et al., 2012). Despite these seminal observations, the molecular basis for this phenomenon remains poorly understood. Furthermore, whether this observation occurs in breast cancer is unknown. Given that (1) mutation of *PIK3CA* is highly prevalent in breast cancer and that (2) aspirin use is associated with a decrease in breast cancer mortality and distant recurrence (Holmes et al., 2010), the relationship between PI3K pathway activation and the protective benefit of aspirin in breast cancer was investigated.

Here we show that aspirin affects the viability and growth of *PIK3CA* mutant cells in 2D and 3D cultures. While our findings suggest that the cell autonomous growth inhibitory effects of aspirin maybe ascribed to activation of AMPK and inhibition of mTORC1 signaling, we cannot exclude the contribution of COX-2 and NF-κB signaling – two well-known targets of aspirin. The relevance of these latter targets maybe more significant in an *in vivo* setting. Several studies have shown that inhibition of COX-2 and NF-κB signaling decreases cancer growth and metastasis via several mechanisms that target the immune system, platelet activation/angiogenesis and other components of the tumor microenvironment (Markosyan et al., 2013; Wang et al., 2014; Williams et al., 2000; Xu et al., 2014). In fact, studies have indicated a preferential benefit for aspirin in

colorectal cancer patients whose tumors express high COX-2 expression (Chan et al., 2007; Chan et al., 2009). In one epidemiology study, the strongest antitumor effects of aspirin were observed in colorectal cancer patients whose tumors harbored both *PIK3CA* mutation and COX-2 expression, although this was not statistically significant due to small sample size (Liao et al., 2012). Furthermore, our study has also revealed that mutant *PIK3CA* affects the expression of many inflammatory-related genes. We have shown that mutant *PIK3CA* can also activate IKKβ/NF-κB signaling which plays a critical role in the regulation of inflammatory genes, including the *PTGS2* gene, which encodes for cyclooxygenase-2 (COX-2). Therefore, these other attributes of aspirin may help explain the sensitivity of mutant *PIK3CA* cancers; however, our cell culture models would not be amenable for such evaluation. On the other hand, while COX-2 expression strongly associates with the benefit of aspirin in colorectal cancer, an epidemiology study conducted by Holmes *et al.* has shown that the improved breast cancer survival associated with aspirin use is independent of COX-2 status (Holmes et al., 2011). This is consistent with laboratory studies, which also show that aspirin's antitumor effects may be COX-2 independent and may be due to other modes of action including aspirin's effect on the Wnt/β-catenin pathway (Bos et al., 2006; Dovizio et al., 2013; Hanif et al., 1996; Thun et al., 2012).

Our finding also highlights the importance of careful consideration of the timing of administration of aspirin and the dosage used, in order to obtain a beneficial outcome. This has been echoed by many epidemiology studies on aspirin use in cancer. Here we show that lower doses of aspirin were sufficient to inhibit colony formation in soft agar in mutant *PIK3CA* breast cancer cells. In contrast, a higher concentration was required to

affect growth of established breast cancer acinar in 3D Matrigel. Furthermore, *PIK3CA* mutant cells appeared more sensitive to aspirin compared to wild-type *PIK3CA* cells in this latter model. One possible explanation for this differential association between aspirin and *PIK3CA* mutation status could be due to a change in the dependency of tumor cells on mutant *PIK3CA*-regulated activities, at various stages of tumor evolution.

Our work indicates that aspirin in combination with PI3K inhibitors results in enhanced growth suppression of *PIK3CA* mutant SUM159-PT or *PTEN* mutant MDA-MB-468 breast cancer cells, but not *KRAS* mutant MDA-MB-231 breast cancer cells. This finding may be due to the increased sensitivity of PI3K pathway mutant cells to PI3K inhibitors and possibly, aspirin as well. In our model, we propose that PI3K-dependent breast cancer cells show greater growth suppression upon co-treatment with aspirin and PI3K inhibitor BYL719 or BKM120, due to enhanced AMPK activation and inhibition of mTORC1 signaling. This is consistent with recent studies, which show that *PIK3CA* mutation status correlates with increased sensitivity to mTORC1 inhibitors (Meric-Bernstam et al., 2012; Weigelt et al., 2011). Our study also indicates that mutant *PIK3CA* inhibits AMPK signaling and activates mTORC1. In support of our hypothesis that aspirin may be effective in mutant *PIK3CA*/mTORC1 dependent cancers, a previous study has shown that that aspirin shows potent antitumor effects in a xenograft model of TSC2 deficient ELT3 cells, which display constitutive mTORC1 activity (Li et al., 2014). It is worth noting that the proposed combination of aspirin and PI3K pathway inhibitors does not result in overt cell death but rather growth suppression. Thus, it is imperative that we evaluate the efficacy of this drug combination *in vivo*, given the critical role of aspirin on the immune system, platelet activation and the tumor

microenvironment. It is possible that while our *in vitro* studies show a cytostatic effect, *in vivo* studies may reveal a cytotoxic effect.

Overall, our work supports the initiation of pre-clinical and clinical trials, to evaluate the efficacy of adjuvant aspirin therapy in breast cancer patients with PI3K-pathway dependency. Aspirin is a simple and inexpensive drug already approved by the FDA with many potential chemotherapeutic properties. In contrast to other drugs being tested for cancer treatment, it has fewer severe side effects and due to its pleiotropic effects, may be effective at targeting both the primary tumor and distant metastases. **Figure 5-1** summarizes the mechanism(s) described above, which may explain why aspirin is beneficial in mutant *PIK3CA* or PI3K-activated breast cancer.

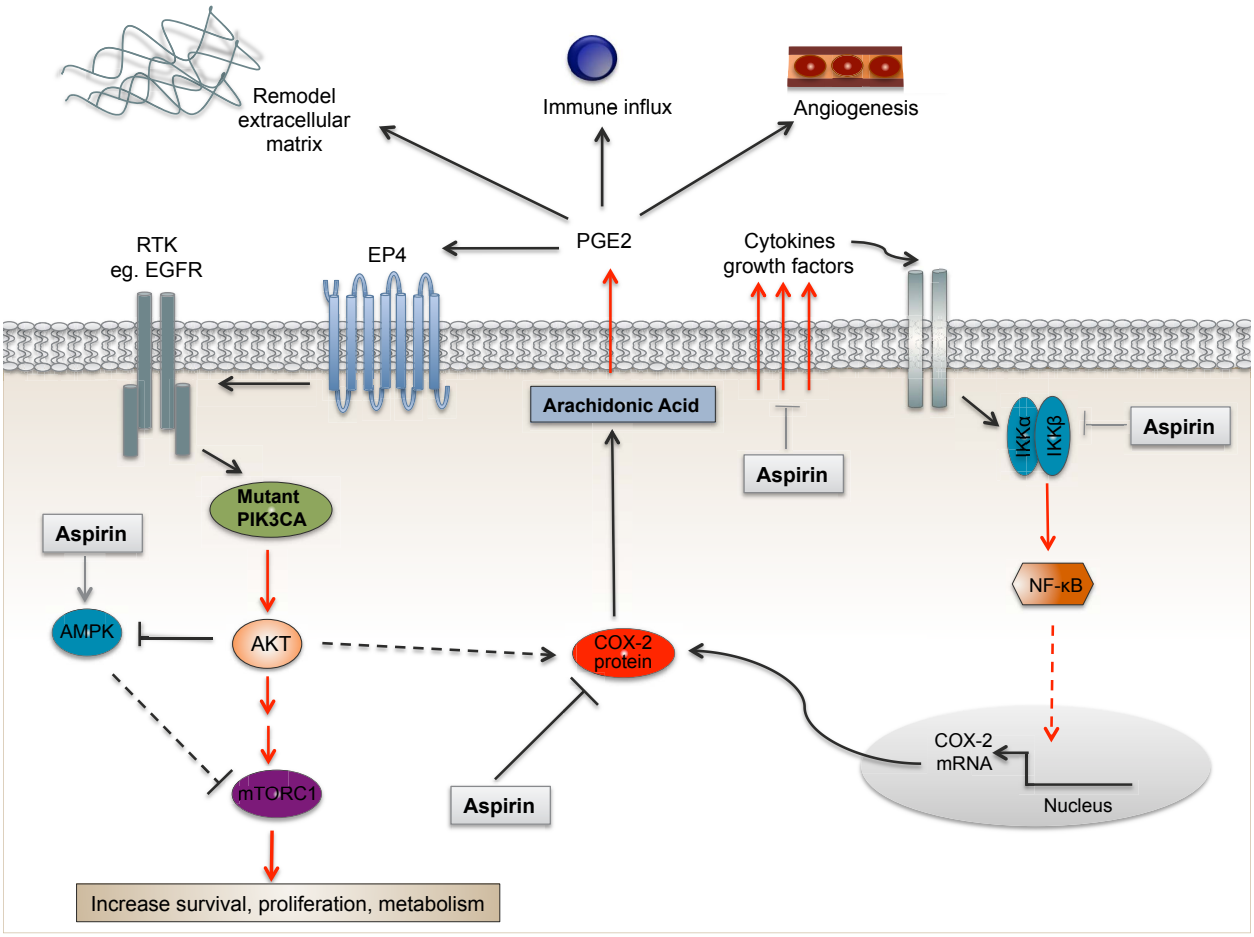

Figure 5-1: Aspirin's mechanism(s) of action in mutant *PIK3CA*-dependent cancers. Mutant *PIK3CA* regulates AMPK/mTORC1 signaling as well as the NF-κB/COX-2/PGE signaling axis - all of which are important targets of aspirin. Aspirin's anti-tumor effect may be due to a combination of cell-autonomous inhibition of growth and survival signaling pathways, in addition to modulation of the immune system, angiogenesis and extracellular matrix remodeling.

This thesis work also focuses on understanding the mechanism(s) by which PI3K pathway activation contributes to breast cancer development and progression. Until recently, very little was known about the biological significance of PTX3 in breast cancer pathogenesis. Furthermore, its relationship to the PI3K signaling pathway was mostly speculative. Our study illustrates that activation of PI3K signaling increases *PTX3* gene expression via the NF-κB pathway. We also demonstrate that PTX3 contributes to PI3K-dependent monolayer growth under nutrient and growth factor deprived conditions. This finding is noteworthy as it ascribes a cell autonomous role for PTX3 in breast cancer development in contrast to previous studies which attribute the biological functions of PTX3 to its role in paracrine signaling with immune cells and extracellular matrix proteins (Bottazzi et al., 2010). Conversely, the fact that PTX3 affects chemotaxic migration of macrophages and immune cells, angiogenesis and inflammation, may help to explain how the PI3K pathway may manipulate the tumor microenvironment and immune system to faciliate tumorigenesis.

It is important to keep in mind that while our findings suggest a pro-tumorigenic role for PTX3 in cancer, other studies have proposed the opposite and advocate for harnessing PTX3 activity for cancer treatment (Bonavita et al., 2015; Leali et al., 2011). This warrants more careful evaluation of the role of PTX3 in cancer as its functional significance might be context dependent, tissue specific and more complex that initially conceived.

This thesis work also explored the relationship between the PI3K pathway and lincRNA expression. Specifically, we identified a novel lincRNA called c14orf34 and provided evidence for a potential role in breast cancer. It is noteworthy that c14orf34

was identified in two independent models of cellular transformation – due to overexpression of oncogenic *c-Src* and mutant *PIK3CA H1047R* respectively. While this can be viewed as a consequence of promiscuous transcriptional activity at a genomic locus, it can also be interpreted as a result of functional significance. In support of the latter, we also provide preliminary functional data indicative of a role for c14orf34 in breast cancer migration and invasion. Furthermore, we show that overexpression of c14orf34 results in a gene expression profile that closely resembles that of a transformed cell. While this suggests that c14orf34 might be a trans-acting lincRNA, further investigation is required to elucidate the precise mechanism by which c14orf34 mediates its function. This is further outlined in the future directions section.

It is interesting that c14orf34 shows increased expression in basal-like/triple negative breast cancer cell lines. This subtype of breast cancer is typically associated with poor differentiation and high metastatic potential. Thus, the notion that c14orf34 may play a role in cell invasion and motility, would be consistent with its association with this subtype of breast cancer. Given that basal-like/triple negative breast cancers are associated with poor prognosis, it would be valuable to determine whether expression of c14orf34 would be a suitable prognostic biomarker. The lincRNA Hotair has already set a precedent for lincRNAs in breast cancer metastasis and the role for c14orf34 in this process is a rational hypothesis. **Figure 5-2** summarizes the transcriptional responses to PI3K activation in breast cancer development, described above.

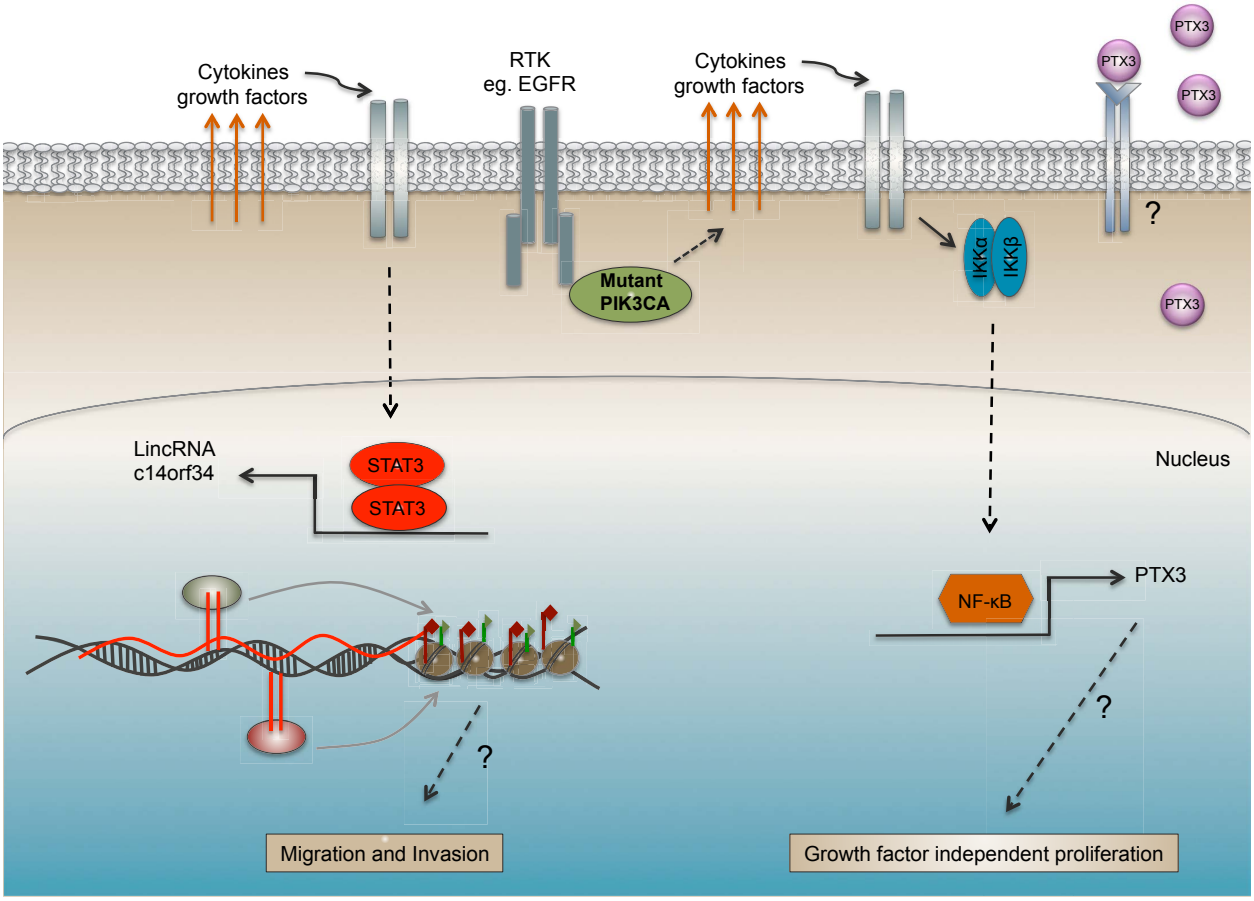

Figure 5-2. Transcriptional responses to PI3K pathway activation in breast cancer. Activation of mutant *PIK3CA* leads to differential expression of cytokines and inflammatory factors that instigate a variety of receptors whose signaling cascade leads to activation of transcription factors such as STAT3 and NF-κB. In this model, lincRNA c14orf34 is activated by STAT3, and functions in cell invasion and migration. Pentraxin 3 (PTX3) is regulated by NF-κB and plays a role in growth factor independent proliferation and survival. The mechanism(s) by which these downstream PI3K-regulated effectors mediate their function requires further investigation.

3. FUTURE DIRECTIONS

The importance of activation of PI3K signaling in breast cancer pathogenesis is indisputable. However, it is becoming well accepted that effective treatment of breast cancer will entail a combinatorial approach rather than single agent targeting of PI3K-pathway components. In chapter 2, we provide evidence that imply that the co-administration of aspirin with PI3K inhibitors would be an effective treatment strategy in *PIK3CA*/*PTEN* mutant breast cancer.

Firstly, we show that 3D acinar structures of MCF10A cells expressing mutant *PIK3CA H1047R* show increased sensitivity to aspirin compared to cells expressing wild-type *PIK3CA*. This observation suggests that tumors harboring *PIK3CA* mutations or PI3K-pathway dependency are more likely to become susceptible to aspirin induced growth defects. To distinguish whether the presence of *PIK3CA* mutation *per se* associates with increased aspirin sensitivity, it will be important to test this hypothesis using isogenic pairs of wild-type and mutant *PIK3CA* breast cancer cell lines. This can be accomplished using the CRISPR/CAS9 technology. Alternatively, a larger panel of breast cancer cell lines with various genetic aberrations could be assessed for differential sensitivity to aspirin. Additionally, it would be worthwhile to perform an epidemiology study to validate the association between aspirin use, *PIK3CA* mutation status and improved survival among breast cancer patients.

It is also noteworthy that a lower concentration of aspirin was sufficient to robustly inhibit colony formation in soft agar. In contrast, higher concentrations of aspirin were required to block acinar growth in Matrigel. One key difference between these two experiments is that the first evaluates the effects of aspirin on the initiation

stages while the later assesses its effect on acinar maintenance and growth. It would be interesting to test whether aspirin treatment affects tumor incidence and growth in a genetically engineered mouse model of mutant *PIK3CA*-dependent tumorigenesis or other PI3K-addicted breast cancer models.

Similarly, this growth suppression attribute of aspirin could have important implications for colonization of metastatic lesions. Our findings also suggest that aspirin can affect the integrity of protrusive structures associated with highly motile breast cancer cells. Together, this supports the notion that aspirin may play an important role in reducing metastasis and this should be carefully investigated using a combination of cell-based migration and invasion assays as well as *in vivo* assays amenable for studying metastasis. This idea is further supported by epidemiology studies, which indicate that aspirin use is associated with increased survival and reduced distant recurrences in breast cancer patients (Holmes et al., 2010).

Next, we show that aspirin improves the efficacy of PI3K inhibitors to enhance growth suppression specifically in breast cancer cell lines harboring *PIK3CA* or *PTEN* mutations. While encouraging, it is imperative that we test this drug combination using a genetically engineered mouse model of PI3K-driven breast cancer. The use of this type of mouse model is particularly important because it will provide a physiological environment that will allow one to assess the additional contributions of aspirin on the immune system, angiogenesis and the tumor microenvironment.

In chapter 3, we reveal a new relationship between the PI3K pathway and PTX3, in the context for breast cancer. While our studies demonstrate that PTX3 plays a role in

PI3K-dependent proliferation and growth in cell line models, it is important that we evaluate the function of PTX3 in mouse models of PI3K-driven breast cancers. Given that PTX3 null -/- female mice are subfertile, it may be worthwhile to generate a mammary gland specific PTX3 conditional knockout mouse. This can be crossed with a genetically engineered mouse model of mutant *PIK3CA*, *PTEN* loss or other PI3K pathway lesion. This new strain will allow one to investigate the importance of PTX3 in various aspects of PI3K-driven breast cancer development. This model would also be advantageous in examining the effect of loss of tumor-derived PTX3 on tumor-stromal interactions. This is especially important given the interplay between PTX3 and immune cells as well as extracellular matrix components.

Another important question that should be addressed is the mechanism by which PTX3 mediates its function. Given that PTX3 is a soluble factor, it will important to identify the upstream receptor that mediates PTX3 signaling. This can be achieved by employing a ligand based-receptor capture technology. Proteomic and gene expression profiling may also provide some insight into the major signaling nodes that are affected upon differential expression of PTX3. PTX3 has been shown to undergo many posttranslational modifications although the significance of these remains unknown. Future studies directed at addressing this unexplored area may also reveal novel insight about the biology of PTX3.

While there are many studies in which the PI3K pathway mediates cancer phenotypes through protein-coding genes and miRNAs, no study has explored a role for lincRNAs in this pathway. In chapter 4 we identified a novel PI3K-regulated lincRNA called c14orf34 that is upregulated during malignant transformation. This finding spawns

a number of questions regarding the mechanism and functional characterization of c14orf34 in cancer. Firstly, our preliminary findings are suggestive of a role for c14orf34 in cell invasion and motility and more quantitative cell migration and invasion assays should be performed in other breast cancer cell lines in order to ascribe this role as a general phenomenon. Other functional growth assays including colony formation in soft agar should also be employed as it correlates best with *in vivo* tumorigenesis. To this end, cloning of the other c14orf34 isoforms using 5' and 3' RACE will be very useful. It would be interesting to determine whether these isoforms share functional redundancy and similar gene expression patterns.

More importantly, the mechanism by which c14orf34 mediates these functions should be explored. Knowledge of its cellular localization by RNA FISH or cellular fractionation could provide some insight into this question. Additionally, performing protein or RNA/DNA interaction screens using RNA pulldowns followed by mass spectrometry (protein interactors) or sequencing technology (DNA or RNA interactors), would be informative. Deletion mapping experiments could then be performed to examine the region of c14orf34 that facilitates this interaction.

Other uncharacterized PI3K-regulated lincRNA transcripts have also been curated (unpublished) during the course of this study. Given that less than 1% of lincRNAs have been characterized in detail, future experiments aimed at characterizing these lincRNAs would be a significant contribution to the lincRNA field. Furthermore, an understanding of the functions of PI3K-regulated lincRNAs may facilitate the design and implementation of new diagnostic and therapeutic approaches for breast cancer.

4. FINAL NOTE

The goal of this thesis work was to gain an understanding of PI3K signaling that would inform the development of rational therapeutic approaches for breast cancer treatment. Here we discovered that aspirin sensitizes mutant *PIK3CA* breast cancer cells to PI3K inhibitors to enhance growth suppression. We also performed mechanistic studies, which suggest that this growth inhibitory effect may be due to activation of AMPK and inhibition of mTORC1 signaling. Given the pleiotropic effects of aspirin, it is important that we evaluate this drug combination in an *in vivo* model of breast cancer. Our study also reinforces the need to carefully consider patient stratification, dosage concentration and methodical scheduling, as these may affect the overall efficacy of this drug combination.

Our work also focused on the transcriptional responses to PI3K activation in breast cancer. We demonstrated that the PI3K pathway regulates novel protein-coding genes and long noncoding RNAs (lncRNAs) such as *PTX3* and lincRNA *c14orf34*. We uncovered a role for these uncharacterized transcripts in mediating various PI3K-driven phenotypes using a variety of functional cell-based assays. This body of work is the first to describe a role for any of these aforementioned transcripts in the development of breast cancer that show PI3K pathway dependency.

Overall, this thesis work underlines the complexity of PI3K signaling in breast cancer and may potentially offer innovative opportunities for diagnostic and therapeutic purposes.

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