The Role of Protein Tyrosine Kinase 6 in the Mammary Gland Epithelium and Breast Cancer

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THESIS

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This thesis is dedicated to my father Dechuan Peng and my mother Changhong Deng, who have been nothing but supportive during these years. Especially to my mom who is a brave breast cancer survivor and an inspiration to me, you made cancer research personal, and I will never forget that.

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LIST OF ABBREVIATIONS

AKT	Ak mouse strain, transforming
BRK	Breast tumor kinase
CSK	C-Src tyrosine kinase
DAB	3, 3'-Diaminobenzidine tetrahydrochloride
DCIS	Ductal carcinoma in situ
DMSO	Dimethyl sulfoxide
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FITC	Fluorescein isothiocyanate
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FRK	Fyn-related kinase
HIF	Hypoxia induced factor
HAN	Hyperplastic alveolar nodule
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
IGF	Insulin-like growth factor
IDC	Invasive ductal carcinoma
LCIS	Lobular carcinoma in situ
MAPK	Mitogen-activated protein kinase
MMTV	Murine mammary tumor virus
NT	Non-transgenic
PCR	Polymerase chain reaction
PI3K	Phosphoinositide-3 kinase
PR	Progesterone receptor
PTK6	Protein tyrosine kinase 6
PyMT	Polyoma middle T-antigen
shRNA	small hairpin RNA
Sik	Src-related intestinal kinase
siRNA	small interfering RNA
SNB	Sam68 nuclear body
STAT	Signal transducer and activator of transcription
STAP	Signal transducer and activator protein
STAR	Signal transduction and activation of RNA metabolism
SRMS	SRC-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristoylation sites
TDLU	Terminal ductal lobular unit
TMA	Tissue microarray

SUMMARY

Protein tyrosine kinase 6 (PTK6) is a soluble tyrosine kinase that is distantly related to SRC family kinases. In normal tissues, PTK6 is expressed in differentiated epithelia and involved in the maintenance of tissue homeostasis. In tumors, PTK6 expression is often de-regulated, as it could be down-regulated in oral, laryngeal and esophageal squamous cell carcinomas, but be overexpressed in the majority of breast cancer and ovarian cancer. Subcellular localization of PTK6 appears to be important as PTK6 is translocated from nucleus to cytoplasm during prostate tumorigenesis.

PTK6 overexpression has been widely observed in human breast tumors and breast cancer cell lines, and overexpression of PTK6 has been correlated with ERBB2 expression. PTK6 regulates pro-survival and proliferation molecules such as AKT, STAT3, ERK5 and ERK1/2, and knockdown of PTK6 inhibits proliferation in breast cancer cells. However, PTK6 expression was seldom detected in normal human mammary glands. This significant difference prompted us to ask: What role does PTK6 play in breast tumors? How is PTK6 induced during the tumorigenesis? To answer these questions, we analyzed human mammary gland tissue microarrays and discovered that PTK6 is expressed in the normal mammary epithelium in contrast to the common notion. Although active PTK6 was not detected in the normal mammary glands, high levels of activate PTK6 were detected in high grade carcinomas. This finding suggested that activation of PTK6 might be tightly controlled in normal breast and is de-regulated in breast tumors. Expression of PTK6 in a large portion of human breast cancer and pro-proliferation function of PTK6 in breast cancer cells led us to hypothesize that PTK6 promotes mammary gland tumorigenesis in vivo. To test this, we generated transgenic mice that express PTK6 in mammary glands under the control of Murine Mammary Tumor Virus Long Terminal Repeats (MMTV-LTR). Immunofluorescence

assay showed that the ectopic PTK6 was constitutively active in the mammary glands. Transgenic mice and non-transgenic control mice were monitored over 2 years and a 2.4-fold increase in breast tumor formation was detected in all the transgenic lines. Further analysis identified active STAT3 (Signal Transducer and Activator of Transcription 3) in the mammary glands from transgenic animals. STAT3 is a known PTK6 substrates that regulates pro-survival signaling, it is correlated with mammary gland involution and its expression is strictly regulated during the mammary gland development. Elevated levels of STAT3 expression have been detected in human carcinomas, and constitutively active STAT3 is correlated with poor prognosis. We detected activated STAT3 in mouse mammary glands as early as 12 weeks, and the activation persisted through the whole lifespan of the animals. Elevated levels of active STAT3 were also detected in the MMTV-PTK6 induced tumors comparing to the spontaneous tumors developed in the non-transgenic animals.

Previous studies showed that PTK6 and ERBB2 are co-overexpressed in many human breast carcinomas, and simultaneously overexpressing PTK6 and ERBB2 enhanced the tumorigenicity of MCF-10A cells in the immune-deficient mice. To study the possible synergistic effect of PTK6 and ERBB2 on breast tumorigenesis, we proposed that by introducing PTK6 into MMTV-ERBB2 transgenic animals, we will observe a change in kinetics of breast tumor formation, may it be accelerated tumor formation or increased tumor size. We crossed the MMTV-PTK6 mice with the MMTV-ERBB2 (B2) mice and compared tumorigenesis between B2/PTK6 double transgenic and B2 single transgenic animals. We did not detect a statistically significant difference in tumor size or lung metastasis between single and double transgenic animals. Although a higher proliferation rate was observed in B2/PTK6 tumors, it was counteracted by increased apoptosis. In addition, although endogenous mouse PTK6 is not expressed in normal mouse mammary glands, it was

induced in both B2 and B2/PTK6 tumors and may have partially masked the effect of ectopic PTK6. We also detected endogenous PTK6 induction in several breast cancer models including MMTV-H-Ras, MMTV-Polyoma middle T and our MMTV-PTK6 mice.

To evaluate the importance of PTK6 induction in breast tumor formation, we hypothesized that PTK6 ablation will impair ERBB2-induced breast tumor formation. We crossed MMTV-ERBB2 mice with *Ptk6* null (*Ptk6* $^{-/-}$) mice to generate B2, *Ptk6* $^{-/-}$ mice, and observed a significant delay in breast tumor initiation. BrdU staining showed that proliferation rates in mammary glands were much lower in B2, *Ptk6*$^{-/-}$ mice than in B2, *Ptk6*$^{+/+}$ animals. Immunoblotting and immunofluorescence staining showed that although the total ERBB2 level doesn't seem to be altered by the absence of endogenous PTK6 in the breast tumors, activation of focal adhesion kinase (FAK) and p130Cas was decreased in *Ptk6* $^{-/-}$ tumors, suggesting that in addition of delayed tumor formation, disrupting PTK6 expression may also decrease the metastasis potential of ERBB2 induced breast tumors. Then we examined the outcome of targeting PTK6 in breast cancer treatment. Knockdown of PTK6 increased trastuzumab-induced growth inhibition in ERBB2 overexpressing cell line SK-BR-3, and sensitized breast cancer cells like SK-BR-3, BT-474 and MCF-7 to chemotherapy and irradiation treatment.

These findings suggest that PTK6 plays an important role in regulating breast epithelial cells. Overexpressing PTK6 or de-regulated PTK6 activation could lead to breast tumor formation. Disrupting *Ptk6* gene expression delays tumor formation and possibly decreases metastasis potential in mouse. Down-regulating PTK6 increases the effect of anti-cancer therapies in breast cancer cells.

1. INTRODUCTION

1.1 Background

Protein Tyrosine Kinase 6 (PTK6) is a non-receptor tyrosine kinase that is expressed in the epithelial cells of various tissues across multiple species. A partial cDNA of PTK6 was originally identified in the cultured normal human melanocytes by reverse transcriptase-PCR. Almost at the same time, PTK6 cDNA was cloned from metastatic human breast tumor with primers targeting the highly conserved catalytic domain of protein tyrosine kinases, and thus given the name BRK (Breast tumor kinase) (Mitchell et al. 1994). The mouse orthologue of BRK was identified in the intestinal epithelium in a screen for tyrosine kinases that regulate regeneration, and was named Sik (Src-related intestinal kinase). Mouse PTK6 shares 80% amino acid identity with human PTK6 (Siyanova et al. 1994, Vasioukhin et al. 1995). PTK6 was identified in normal tissues and tumors, as well as in non-transformed and cancer cell lines. A growing number of publications have reported PTK6 as an active player that regulates various signaling pathways involved in differentiation, apoptosis, cell to cell contact, epithelial to mesenchymal transition, cell survival, proliferation and migration. The functions of PTK6 are tissue specific and context dependent: PTK6 has been suggested to be a tumor suppressor in laryngeal and esophageal squamous cell carcinoma (Ma et al. 2012, Liu et al. 2013), but it also promotes cell survival and migration in prostate tumors (Brauer et al. 2010, Zheng et al. 2011, Zheng et al. 2012, Zheng et al. 2013), and its overexpression could be oncogenic in lymphocytes (Kasprzycka et al. 2006). PTK6 is overexpressed in the majority of breast tumors and breast cancer cell lines, but its functions in the mammary gland are still not fully understood and sometimes controversial. Our study focused on both normal mammary glands and breast tumors and provided new understanding of PTK6 signaling functions.

1.2 PTK6 protein structure and regulatory residues

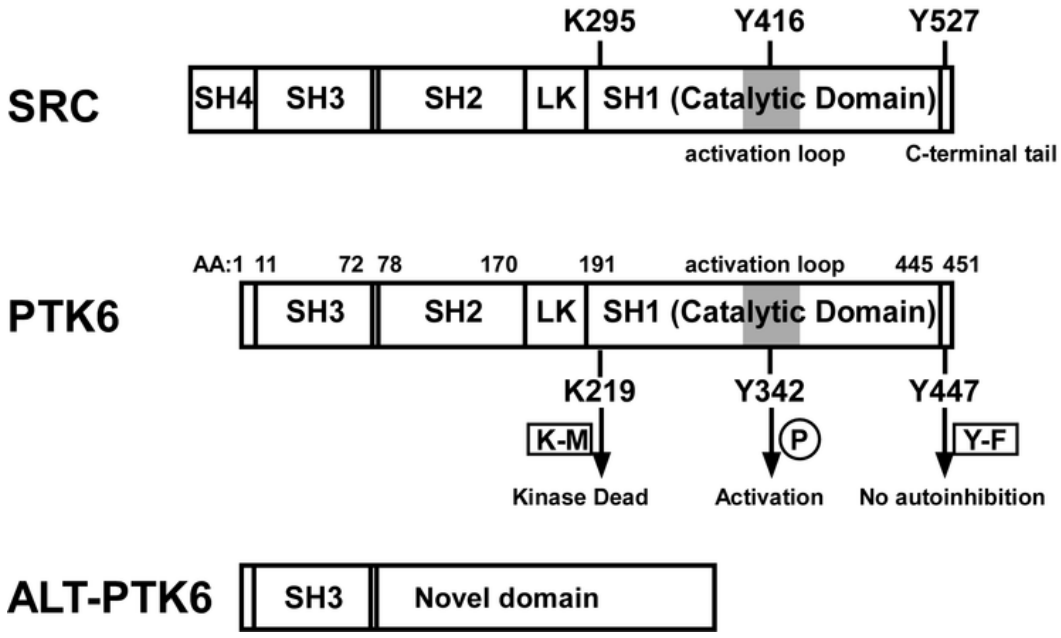
Human *PTK6* gene localizes to chromosome 20q13.3 and encodes a 451 amino acid protein (Park et al. 1997). The overall amino acid homology between human PTK6 and human SRC is 46% and the kinase catalytic domain homology is 56% (Mitchell et al. 1994). However, both human and mouse PTK6 does not contain the RDLRAAN defining motif of SRC family, suggesting PTK6 belongs to a novel kinase family (Lee et al. 1993, Mitchell et al. 1994). Later study revealed that *PTK6* gene contains 8 exons and has distinct intron/exon boundaries (Mitchell et al. 1997, Lee et al. 1998, Llor et al. 1999, Serfas et al. 2003) and forms distinct protein kinase family with SRMS, FRK (also known as RAK) (Reviewed in Brauer, et al. 2009) and drosophila Src42A/Dsrc41. *PTK6* is considered to share a common ancestor of the *SRC* family but branched out early during evolution (D'aniello et al. 2008). Although no mutated form of PTK6 has been found in any tissue to date (Easty et al. 1997, Lee et al. 1998), an alternative spliced product of the *PTK6* gene(ALT-PTK6) has been identified in breast cancer cells T-47D (Mitchell et al. 1997) and later in a number of prostate and intestinal cancer cell lines (Brauer et al. 2011). The alternative splicing of the PTK6 transcript results in excising out exon 2 and producing a truncated, catalytically inactive 134 amino acid protein that contains the N-terminal SH3 domain and a novel C-terminus. ALT-PTK6 often co-exists with full length PTK6 and plays a role in PTK6 signaling, possibly as a competitive inhibitor (Brauer et al. 2011)

Like many protein tyrosine kinases, PTK6 contains multiple SRC Homology (SH) domains including a kinase domain (SH1) that performs catalytic function, a linker region between SH1 and SH2 domain, a SH2 domain that binds to phosphorylated tyrosine, and a SH3 domain that interact with proline-rich regions (Mitchell et al. 1994, Mitchell et al. 1997, Kim et al. 2005). However, PTK6 has a unique amino-terminal sequence and lacks the SH4 domain which contains

the myristoylation/palmitoylation signals that target SRC to the plasma membrane (Figure 1). Consequently, PTK6 can localize at membrane (Zheng et al. 2013), in the cytoplasm (Derry et al. 2003) or nucleus (Derry et al. 2000) depending on the environmental context, and is capable of interacting with substrates at different subcellular localizations.

The SH3 domain of PTK6 is involved in intramolecular interactions and governs substrate recognition and phosphorylation (Derry et al. 2000, Qiu et al. 2004, Qiu et al. 2005, Lukong et al. 2008). Interaction of SH3 domain and linker region is also involved in auto-inhibition (Qiu et al. 2002, Kim et al. 2007, Ko et al. 2009). The SH2 domain of PTK6 contains a consensus alpha/beta-fold and peptide binding surface which can bind to phosphorylated tyrosine in a sequence-specific context (Mitchell et al. 2000, Born et al. 2005). However, the binding affinity of phosphorylated tyrosine peptide by PTK6 SH2 domain is weaker than SRC family members, shown by the dissociation constant measured by surface plasmon resonance analysis (Hong et al. 2001, Hong et al. 2004). PTK6 can auto-phosphorylate multiple tyrosine residues including the Y-342 which is in the activation loop of catalytic domain, and the phosphorylation of Y-342 greatly increases catalytic activity (Mitchell et al. 1994, Qiu et al. 2002). The activation of PTK6 will be inhibited when the C-terminal Y-447 is phosphorylated and binds to the SH2 domain, changing the structure of PTK6 to a more "closed" conformation (Derry et al. 2000, Qiu et al. 2002). Mutating tyrosine 447 to phenylalanine which is identical to tyrosine but lacks the phosphorylatable -OH group will prevent SH2 binding and auto-inhibition, and renders PTK6 constitutively active in the appropriate context (Zheng et al. 2013). It is still unclear what regulates Y447 phosphorylation, since the SRC regulator CSK (c-SRC tyrosine Kinase) that phosphorylates the carboxyl terminal tyrosine on SRC does not phosphorylate PTK6 (Qiu et al. 2002). The catalytic activity of PTK6 can be abolished by mutating lysine 219 in the ATP binding site to methionine (K219M) (Kamalati et al. 1996).

Figure 1: PTK6 protein structure and regulatory amino acid residues (Not drawn to scale) Both SRC and full-length PTK6 contain SH3, SH2 and SH1 domains, as well as a linker (LK) region between SH2 and SH1 domains. However, PTK6 lacks the N-terminal SH4 domain that contains myristoylation signal which is important for membrane association. Lysine 295 in SRC and 219 in PTK6 are in the ATP-binding sites and mutate these residues to methionine will abolish the kinase activity. Tyrosine 527 in SRC and 447 in PTK6 can be phosphorylated and interact with SH2 domain thus auto-inhibit the catalytic activity, and mutating these residues to phenylalanine will prevent auto-inhibition. Tyrosine 416 in SRC and 342 in PTK6 are in the activation loops and phosphorylation of these residues indicates the activation of these kinases. Alternative spliced variant of PTK6 (ALT-PTK6) is a 15kDa protein and contains SH3 domain and a novel truncated SH2 domain.



These regulatory residues are highly conserved across species (Figure 2), making it possible to study the function of human PTK6 in mouse models.

1.3 PTK6 substrates, regulators and binding partners

PTK6 has been shown to interact with a wide range of signaling molecules involved in transcriptional regulation, differentiation, cell survival, proliferation and migration. PTK6 functions as a kinase to phosphorylate substrates or as an adaptor protein independent of catalytic activity.

1.3.1 RNA binding proteins

The nuclear RNA binding protein Sam68 (Src-associated in mitosis, 68kDa) is a known substrate for SRC-family tyrosine kinases during mitosis (Fumagalli et al. 1994, Taylor et al. 1994). It is a member of RNA-binding proteins family called STAR (Signal Transduction and Activation of RNA metabolism) which mediates RNA splicing in response to extracellular signals (Lukong et al. 2003). In response to EGF treatment, PTK6 directly phosphorylates Sam68 in nucleus and inhibits its RNA-binding activities (Derry et al. 2000, Coyle et al. 2003, Haegebarth et al. 2004, Lukong et al. 2005), PTK6 phosphorylation also cause Sam68 to re-localize to SNBs (Sam68 Nuclear Bodies) (Lukong et al. 2005, Sellier et al. 2010). Recent study in breast cancer cell lines MDA-MB 231 and MDA-MB-453 showed phosphorylation of Sam68 is required for HGF-induced cell migration, suggesting the pro-migration role of PTK6 in breast cancer cells (Locatelli et al. 2011). Similar RNA-binding inhibition is observed when PTK6 co-localizes and phosphorylates SLM-1 and SLM-2(Sam68-like mammalian proteins) (Haegebarth et al. 2004).

PSF is a polypyrimidine tract-binding (PTB) protein-associated splicing factor. In breast tumor cell line BT-20, PTK6 phosphorylates PSF upon EGF stimulation, results in inhibition of its RNA-binding ability and leads to cell cycle arrest (Lukong et al. 2009).

Figure 2. Human and mouse PTK6 are highly homologous

Full length protein sequences of human and mouse PTK6 are aligned for comparison. They both contain 451 amino acids and share 80% sequence identity (identical amino acids are labelled with asterisks). Important regulatory amino acid residues (K219, Y342 and Y447, circled) are conserved between species. Antibodies distinguishing human (SC-1188, C-18, Santa Cruz Biotechnology) and mouse (Sc-916, C-17, Santa Cruz Biotechnology) PTK6 are raised against epitopes mapping at the C-terminus of PTK6 of different origins.

1.3.2 Transcriptional regulators

STAT (Signal Transducer and Activator of Transcription) family members were originally identified as cytokine signal mediators (Darnell et al. 1994, Zhong et al. 1994, Decker et al. 1999) that can be activated by growth factors such as EGF and PDGF (Zhong et al. 1994, Duncan et al. 1997, Henriksen et al. 2002). Different STATs play different roles during mammary gland developments (reviewed in Watson et al. 2008). Although STAT3 expression in normal mammary gland is transient and only occurs during involution stage, elevated STAT3 activity was detected in breast tumors compared with adjacent non-neoplastic tissues (Diaz et al. 2006). Phosphorylation on Y705 activates STAT3. Constitutively activated STAT3 has been reported in breast tumors and breast cancer cell lines (Garcia et al. 1997, Sartor et al. 1997, Diaz et al. 2006, Watson et al. 2008, Barbieri et al. 2010). Increased levels of activated STAT3 have been linked with breast cancer cell proliferation, survival and migration, and indicated poor prognosis (Burke et al. 2001, Li et al. 2002, Cheng et al. 2008, Ranger et al. 2009), on the other hand, down regulating STAT3 results in inhibition of cell proliferation and migration, as well as increased apoptosis in the breast cancer cells (Burke et al. 2001, Ling et al. 2005, Song et al. 2005, Lin et al. 2010, Zhao et al. 2010), suggesting STAT3 may be a valuable target in breast cancer treatment.

STATs can be activated by SRC family kinases, particularly by c-SRC (Smith et al. 1998, and reviewed in Silva et al. 2004). This finding shed light on the relationship between PTK6 and STAT3. Expressing PTK6 in HeLa cells induces phosphorylation of STAT3, and this effect is dependent on the kinase activity of PTK6. *In vitro* kinase assay showed that PTK6 directly phosphorylates STAT3 at tyrosine residue 705 and activates STAT3 (Liu et al. 2006). Activated STAT3 will undergo dimerization and be translocated to the nucleus where it performs its transcriptional regulator functions (Reviewed in Bowman et al. 2000). Expressing PTK6 and

STAT3 simultaneously stimulates cell proliferation and this effect surpasses the growth advantages conferred by expressing PTK6 or STAT3 alone (Liu et al. 2006). PTK6 also promotes STAT3 activation in colon. Disrupting *Ptk6* gene expression in mouse impaired AOM induced STAT3 activation and colon tumorigenesis (Gierut et al. 2011).

PTK6-mediated STAT3 activation can be regulated by STAP-2 (Signal-transducing adaptor protein-2, also known as BKS, Brk Substrate) (Ikeda et al. 2009). STAP-2 is an adaptor protein that binds to STAT3 and regulates downstream signaling pathways. STAP-2 can be phosphorylated by PTK6 on tyrosine 250 and enhance STAT3 activation (Ikeda et al. 2009). Knocking down STAP-2 with RNAi decreases PTK6-mediated STAT3 activation in T-47D cells (Ikeda et al. 2010). Also, PTK6-mediated STAT3 activation can be inhibited by SOCS3 (Suppressor of Cytokine Signaling 3). SOCS3 is the only known inhibitor of PTK6, it interacts with PTK6 via its SH2 domain and binds to the kinase domain of PTK6 and inhibits the catalytic activity. Increased SOCS3 expression inhibits breast cancer cell proliferation (Gao et al. 2012).

Another STAT family member STAT-5b is also a substrate of PTK6 (Weaver et al. 2007). PTK6 phosphorylates STAT5b on tyrosine 699 and promotes its transcriptional activity which results in proliferation boost (Weaver et al. 2007). PTK6-mediated STAT5b activation could also be modulated by STAP-2 (Ikeda et al. 2011).

Study of the PTK6 promoter region identified a few cis-acting elements including those for Sp1, AP2, SIE and NF-kB (Mitchell et al. 1994). Both Sp1 and NF-kB have been shown to bind to these cis-acting elements, suggesting PTK6 may be regulated by these transcriptional regulators (Kang et al. 2002). PTK6 has also been indicated to be regulated by a zinc finger DNA-binding protein KLF9 (Krüppel-Like Factor-9) (Simmen et al. 2007).

1.3.3 Survival, proliferation and migration regulators

AKT is a serine-threonine kinase that plays an important role in cell survival and proliferation, elevated levels of activated AKT in tumors is often linked with poor outcome (Lin et al. 2005, Soderlund et al. 2005). Activation of AKT requires phosphorylation on threonine residue 308 and serine residue 473. Our lab identified AKT as a direct substrate of PTK6: PTK6 phosphorylates AKT on tyrosine residues 315 and 326 in a SRC family kinase independent manner, and knocking down PTK6 in the prostate cell line BPH1 decreases EGF induced AKT activation (Zheng, Peng, et al. 2010).

Paxillin is a scaffold protein that is recruited to the leading edge of cells upon the initiation of migration to provide docking sites for multiple signaling, adaptor, and structural proteins (reviewed in Turner 2000). PTK6 phosphorylates Paxillin on Y31 and Y118 upon EGF stimulation, and phosphorylation on Paxillin promotes the activation of small GTPase Rac1 via the function of CrkII, resulting in increased cell motility and invasion (Turner 2000).

p130 CRK-associated substrate (p130Cas) was originally identified as a highly phosphorylated protein in cells transformed by v-Src and v-Crk oncogenes, later it turned out to be an adaptor molecule that regulates cell motility and invasion (reviewed in Bouton et al. 2001, Miranti et al. 2002), it has been identified as an important regulator of mammary epithelial cell proliferation and survival: Transgenic mice expressing p130Cas displayed extensive mammary epithelial hyperplasia and delayed involution(Cabodi et al. 2006). A double transgenic line expressing p130Cas and activated ERBB2 simutaneously in the mammary glands developed multifocal breast tumors with a significantly shorter latency than the strain expressing ERBB2 alone, suggesting that p130Cas promotes ERBB2 dependent tumorigenesis (Cabodi et al. 2006). It was shown that p130Cas was an essential transducer element for ERBB2-dependent foci formation and anchorage-

independent growth, and down-regulating p130Cas in the mammary glands decreased the growth of ERBB2 induced breast tumors (Cabodi et al. 2010). Recently, our lab discovered that PTK6 phosphorylates p130Cas and promotes peripheral adhesion complex formation and cell migration, and increased cell migration conveyed by expression of membrane-targeted PTK6 can be impaired by knockdown of p130Cas or ERK5 (Zheng et al. 2011).

Focal adhesion kinase (FAK) is regulated by growth factors and G-protein linked receptor signaling and is highly phosphorylated in focal adhesion contacts of normal cells (Reviewed in (Parsons 2003, Schaller 2001). FAK can activate AKT to promote survival (Xia et al. 2004) and is also able to form complexes with Paxillin or p130Cas to transmit survival signals (Zouq et al. 2009). Elevated levels of FAK are often detected in human breast tumors and in the invasive ductal carcinomas, FAK overexpression is positively correlated with histological grade and proliferation (Theocharis et al. 2009), disrupting FAK expression in mammary glands impairs tumor formation and metastasis in transgenic mouse model of human breast cancer (Provenzano et al. 2008). Our lab discovered that PTK6 directly phosphorylates FAK and activates AKT. In human prostate cancer cell line PC3, knockdown of PTK6 disrupts FAK and AKT activation and promotes anoikis, which can be rescued by exogenous expression of FAK (Zheng et al. 2012).

1.4 PTK6 expression profile

PTK6 has been detected in the epithelial cells of normal tissues and in tumors. In normal tissue, PTK6 is involved in epithelial differentiation and contributes to maintenance of tissue homeostasis, while in cancer PTK6 is linked with pro-survival, proliferation and migration pathways.

1.4.1 PTK6 expression in normal tissues

PTK6 expression was detected at mouse embryonic day 15.5 in the differentiating granular layer of the skin (Vasioukhin et al. 1995) and at mouse embryonic day 18.5 in the differentiating intestine

(Siyanova et al. 1994). In GI tract, PTK6 is expressed in the non-dividing, differentiated cells of the villus epithelium, and can be induced in the crypts by irradiation. Disrupting *Ptk6* expression in mouse results in longer villi, delayed differentiation and increased proliferation in the crypts possibly due to compensating effect (Vasioukhin et al. 1995, Llor et al. 1999, Haegebarth et al. 2006). PTK6 has also been detected in normal oral epithelium (Petroa et al. 2004) and normal luminal prostate epithelial cells (Derry et al. 2003). In skin, PTK6 expresses in the differentiating layers in the suprabasal keratinocytes (Wang et al. 2005) and involves in calcium-induced keratinocyte differentiation (Vasioukhin et al. 1997, Tupper et al. 2011).

Previous study suggested PTK6 expression is low or non-detectable at any developmental stage in normal breast tissues (Llor et al. 1999, Mitchell et al. 1994). While this theory still holds in mouse mammary glands, our finding suggested that PTK6 is actually expressed in the normal human mammary glands and merits more study to explore its physiological functions in normal breast.

1.4.2 PTK6 expression in cancer

PTK6 has been detected in many cancers, and expression and localization of PTK6 varied greatly from their normal counterparts. In normal prostate and low grade benign prostate hyperplasia, PTK6 is localized in the nucleus, while in more advanced prostate tumors, PTK6 is translocated to cytoplasm (Derry et al. 2003). Alteration of PTK6 subcellular localization was also detected in human oral squamous cell carcinomas (OSCC): In normal oral epithelium and moderately differentiated OSCC cells, PTK6 was localized in the nucleus and cytoplasm, while in poorly differentiated OSCC cells, PTK6 was localized in perinuclear regions. In addition to the localization change(Petroa et al. 2004), PTK6 was also found to be down-regulated in OSCC and laryngeal squamous cell carcinoma and esophageal squamous cell carcinoma (Liu et al. 2013, Ma et al. 2012, Petroa et al. 2004), suggesting PTK6 may play a role as tumor suppressor. However,

not all head and neck cancer exhibited PTK6 down-regulation: immunohistochemistry revealed that PTK6 was expressed at higher levels in 38% of head and neck squamous cell carcinomas compared with normal tissue (Lin et al. 2004)

PTK6 is expressed in 70% of high grade ovarian carcinomas but not detected in normal ovarian epithelium (Schmandt et al, 2006). Similar to the result in tumors, PTK6 is expressed in 9 out of 19 human ovarian cancer cell lines but not detected in immortalized ovarian epithelium (Schmandt et al, 2006). Knocking down PTK6 in human ovarian cancer cell line DOV13 inhibits IGF-1 induced anchorage independent growth (Irie et al. 2010).

In colon cancers, PTK6 mRNA is higher in tumor than in adjacent normal tissue (Llor et al, 1999). *Ptk6*$^{-/-}$ mice were resistant to AOM induced formation of aberrant crypt foci (ACF) and tumorigenesis. Disruption of *Ptk6* impaired STAT3 activation following AOM injection. PTK6 also promoted activation of STAT3 in YAMC cells. (Gierut, et al. 2011, Gierut et al, 2012)

1.5 PTK6 and breast cancer

1.5.1 Mammary gland biology and breast cancer

Mammary glands are milk-secreting epidermal appendages that distinguish mammals from other animals. They are composed of multiple cell types, including the epithelial cells that form the ducts and alveolar, the fibroblasts and adipocytes that build up the structure, as well as vascular endothelial cells and immune cells that maintains the physiological functions (Reviewed in Macias et al. 2012). Mammary gland development is regulated by hormones and stimulation such as suckling. Mammary gland epithelium is rudimentary at birth. At the onset of puberty, growth hormone, estrogen and insulin-like growth factor-1 (IGF-1) will promote the growth of ducts. Upon pregnancy, prolactin and progesterone will induce the formation of milk-producing alveoli.

By the time of weaning, the alveolar cells will undergo apoptosis and the mammary gland will remodel back to its adult stage (Keller et al. 2011, Tiede et al. 2011 and reviewed in Macias et al. 2012).

Breast cancer is the most frequently diagnosed cancer in women excluding cancers of the skin, and the second leading cause of cancer-related death in women in US (American Cancer Society 2013). An array of clinical and pathological factors are widely used to categorize the breast cancer patients for prognosis and treatment purpose. Commonly used factors include patient age and family history, tumor size and lymph node status, invasive/non-invasive histological features, and the expression levels and states of molecular markers.

In breast cancer diagnosis, pathologists often analyze breast tumors by histology and determine tumor type and grade according to the morphology after H&E staining. Common types of breast tumors include invasive/infiltrating ductal carcinoma (IDC) and ductal carcinoma in situ (DCIS). IDC refers to the tumor that arose from the milk duct epithelium and has breached the basement membrane and begun to invade the surrounding tissue of the breast. IDC is the most common type of breast cancer and made up about 80% of all the cases, and it has the potential of becoming metastatic breast tumor over time. DCIS refers to breast tumor that was originated from milk duct epithelium and hasn't spread beyond the milk duct into normal surrounding tissue. DCIS is the most common non-invasive type of breast cancer and accounts for about 20% of new breast cancer cases. Although DCIS is non-invasive, it is a sign of elevated risk of developing IDC in the future. Other breast abnormalities such as invasive lobular carcinoma (ILC) and lobular carcinoma in situ (LCIS), are relatively rare comparing to IDC and DCIS (Data compiled from American Cancer Society and www.breastcancer.org).

In molecular marker based breast cancer diagnosis and treatment, expression levels of three proteins are routinely checked with immunohistochemistry assay: estrogen receptor (ER), progesterone receptor (PR) and ERBB2/HER2. Expression status of ER, PR and HER2 determined major intrinsic breast cancer subtypes such as Luminal A (ER/PR positive, HER2 not overexpressed), Luminal B (ER/PR positive, HER2 overexpressed), HER2 (ER/PR negative, HER2 overexpressed) and basal like/triple negative (ER/PR negative, HER2 not overexpressed) (Sorlie et al. 2001, Sorlie et al. 2003, Fan et al. 2006). In these breast cancer subtypes, luminal A tumors have the lowest relapse rate and a higher sensitivity to doxorubicin treatment than luminal B subtype (Gyorffy et al. 2005, Wang et al. 2011), and multiple studies showed that triple negative breast tumors have the worst disease-free and overall survival rate (Diaz et al. 2007, Onitilo et al. 2009). Status of these molecular markers may determine the approach of target therapy: Since the introduction of monoclonal antibody trastuzumab which targets HER2 protein, a better prognosis is expected from HER2 overexpressing breast cancer patients than HER2 negative patients (Brufsky 2010, Dawood et al. 2010, Arteaga et al. 2012). Also, ER positive breast tumors are more likely to response to endocrine therapies while the ER negative patients are unlikely to benefit from the same treatment (Early Breast Cancer Trialists' Collaborative et al. 2011). However, not all breast tumors response to the target therapies even though they express corresponding molecular markers (Morgan et al. 2009, Narayan et al. 2009), and the mechanism for the drug resistance remained to be fully understood. Recent study indicated that tyrosine kinases may have played an important role in the drug resistance (Zhang et al. 2011, Muthuswamy 2011) .

1.5.2 PTK6 and breast cancer

Although expression in normal human breast tissue is low, PTK6 is often overexpressed in breast tumors and breast cancer cell lines (Barker et al. 1997). Overexpression of PTK6 protein

was detected in 86% of invasive ductal breast tumors in a study of human breast tissue microarray (Ostrander et al. 2007), and elevated levels of *PTK6* mRNA was detected in 85% of human breast carcinoma samples (Harvey et al. 2009). Microarray analysis showed *PTK6* mRNA is highly expressed in HER2 and Luminal B (ER/PR+, HER2 overexpressing) breast cancer subtypes and correlated with poor outcome (Irie et al, 2010).

In vitro study showed PTK6 sensitize breast epithelial cells to EGF induced mitogenic effects (Kamalati et al. 1996, Kamalati et al. 2000) and promotes breast tumor cell proliferation (Harvey et al. 2003). The pro-proliferation and pro-migration effect is kinase dependent in MDA-MB-231 cells (Miah et al. 2012). Study in MDA-MB-231 cells showed PTK6 can be regulated by Osteopontin, resulting in regulating of tumor progression and angiogenesis (Chakraborty et al, 2008). PTK6 also promotes IGF-1 induced anchorage independent survival in breast cancer cells (Irie et al, 2010). PTK6 expression is required for heregulin-induced activation of p38 MAPK and ERK5 in T-47D cells (Ostrander et al, 2007). Recent study showed overexpressing human PTK6 in mouse mammary gland with whey acidic protein (WAP) promoter promotes delayed involution and tumor formation associated with activation of p38 MAPK (Lofgren et al. 2011). *In vitro* study showed that PTK6 is a downstream effector of MET, the receptor of hepatocyte growth factor (HGF). Upon HGF stimulation, PTK6 promotes ERK5 dependent cell migration in breast cancer cells (Castro et al. 2010, Locatelli et al. 2011). Further analysis showed that PTK6 can be regulated by HIF-1α/2α (Hypoxia Induced Factors), which are activated by hypoxia often found in solid tumors (Regan Anderson et al. 2013).

The relationship between PTK6 and ERBB family kinases has been heavily investigated shortly after the discovery of PTK6. ERBB family members are receptor tyrosine kinases that bind to many ligands such as EGF, TGF-α, amphiregulin (AR) and neuregulins etc. Four family members

(EGFR, ERBB2/HER2/Neu, ERBB3/HER3 and ERBB4) have been identified. Upon ligand binding, ERBBs form homodimers or heterodimers and phosphorylate tyrosine residues in the tail regions of each receptor. Phosphorylation triggers the downstream signaling events such as pro-proliferation and pro-survival functions (reviewed in Hynes et al. 2009).

PTK6 directly phosphorylates Y845 in the EGFR kinase domain and potentially enhances EGFR kinase activity (Li et al. 2012), and ligand-independent Y845 phosphorylation have been linked to STAT-mediated gene expression (Yang et al. 2008). Knocking down PTK6 in chemo-resistant breast cancer cells sensitizes them to cetuximab, an EGFR blocking antibody (Li et al. 2012). PTK6 expression also enhances ERBB3 mediated PI3K-AKT activation *in vitro* (Kamalati et al. 2000). These data suggest that PTK6 plays a pro-proliferation and pro-survival role in the ERBBs signaling.

ERBB2 doesn't bind to any known ligand, but it is the preferred binding partner of other ERBBs for heterodimerization (reviewed in Hynes et al. 2009). Real-time PCR results suggested PTK6 overexpression is correlated with ERBB2 overexpression in the invasive ductal breast carcinomas (Born et al. 2005). Data obtained from the proximity ligation assay suggested that PTK6 formed protein complexes with ERBB2 in primary breast cancer tissues (Aubele et al. 2010). Expression of PTK6 enhances the ERBB2-induced activation of RAS/MAPK signaling and cyclin E/cdk2 activity and induces cell proliferation of mammary 3-dimensional acini in culture (Xiang et al. 2008), while knockdown of PTK6 in several breast cancer cell lines led to decreased phosphorylation of ERBB2 and PTEN, and inhibited cell migration (Ludyga et al, 2011). Recent studies revealed that PTK6 is more stable in ERBB2-elevated cells because ERBB2 regulates PTK6 stability via up-regulating calpastatin, an inhibitor of calpain, thereby inhibiting calpain-1-mediated proteolysis of PTK6 (Ai et al. 2013), this regulation could contributed to the

simultaneous overexpression of PTK6 and ERBB2 in breast tumors. PTK6 and ERBB2 interaction may interfere with SRC signaling since overexpressing ERBB2 in MCF-7 cells increases PTK6 protein levels and reduces SRC Y416 phosphorylation (Ai et al. 2013). Simultaneously knocking down PTK6 and ERBB2 *in vitro* leads to decreased phosphorylation of pro-survival signaling proteins including MAPK 1/3, ERK 1/2 and p38 MAPK, and reduces the migration, invasion and proliferation of breast cancer cells (Ludyga et al. 2013).

The correlation between PTK6 and ERBB2 overexpression in invasive human ductal breast carcinomas (Born et al. 2005, Aubele et al. 2007, Ostrander et al. 2007, Xiang et al. 2008) and the finding that PTK6 may cooperate with ERBB2 to promote breast tumor cell growth (Xiang et al. 2008) raises the possibility that targeting PTK6 along with ERBB receptors might offer a therapeutic advantage (Harvey et al. 2004, Ludyga et al. 2013). However, although evidence suggested PTK6 promotes proliferation of breast cancer cells, a retrospective study on archival breast cancer samples indicated that high levels of PTK and ERBB2 and ERBB4, but not EGFR and ERBB3 predicted long-term metastasis free survival in breast cancer patients (Aubele et al. 2007, Aubele et al. 2008), suggesting PTK6 de-regulation in breast tumors is more complex than mere overexpression.

II. MATERIAL AND METHODS

2.1 Cell culture

The human embryonic kidney cell line HEK293 and the human breast cancer cell lines MDA-MB-231, SK-BR-3, MCF-7, MDA-MB-453, MDA-MB-468, BT-474, T-47D, human melanoma cell line MDA-MB-435S and the mouse mammary epithelial cell line NMuMG were all obtained from ATCC and cultured according to their guidelines. Immortalized human breast epithelial cell line MCF-10A were obtained from ATCC and cultured in DMEM/F12 with 5% horse serum, 20 ng/ml EGF, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin and 1x Pen/Strep. Detailed characteristics of the breast cancer cell lines used in this thesis are shown in the Table 1. Characteristics of the cell lines used in the experiments are summarized in the table. Data are compiled from Neve et al. 2006, Subik et al. 2010 and www. atcc.org

2.2 Plasmid construction and transfection

MMTV-PTK6 construct was generated by cloning a 2.2 kb PTK6 cDNA fragment into the EcoRI site of rabbit β-globin exon 3 of pKCR-MMTV LTR vector (a gift from Dr. Robert J. Coffey, Matsui, et al. 1990). Transfection of NMuMG cells with MMTV-PTK6 plasmid was performed using Lipofectamine (Invitrogen Corp, Carlsbad, CA). To induce MMTV-PTK6 expression, 0.1 μM of dexamethasone (D8893, Sigma-Aldrich, St. Louis, MO) was added to the media 24 hours prior to harvesting.

2.3 siRNA and shRNA

Pre-designed siRNA targeting PTK6 and negative control Scrambled were purchased from Integrated DNA Technologies. The sequence for PTK6 siRNA is 5'-AGG TTC ACA AAT GTG GAG TGT CTG C-3'. Cells were treated with siRNA in antibiotic-free media and cultured for 48 hours before any following treatment. Lipofectamine 2000 was used for the siRNA transfection.

TABLE I

CHARACTERISTICS OF BREAST CANCER CELL LINES

Cell line	ER	PR	HER2	Breast Cancer Subtype	Cell Type and Tissue
BT-474	**+**	**+**	**+**	**Luminal B**	**Human breast cancer cell**
MCF-7	**+**	**+**	**-**	**Luminal A**	**Human breast cancer cell**
MCF-10A	**-**	**-**	**-**	**N/A**	**Immortalized, non-transformed human mammary gland epithelial cell**
MDA-MB-231	**-**	**-**	**-**	**Basal like**	**Human breast cancer cell**
MDA-MB-453	**-**	**-**	**N/A**	**Unclassified**	**Human breast cancer cell**
MDA-MB-468	**-**	**-**	**-**	**Basal like**	**Human breast cancer cell**
SK-BR-3	**-**	**-**	**+**	**HER2**	**Human breast cancer cell**
T-47D	**+**	**+**	**-**	**Luminal A**	**Human breast cancer cell**

Pre-designed Mission$_{TM}$ TCR shRNA in the pLKO.1 lentirival expression vectors were purchased from Sigma-Aldrich. Lentiviruses expressing TCRN 0000021549 (PTK6 shRNA 49), TCRN 0000021552 (PTK6 shRNA 52) and non-targeting control Scramble were produced in the HEK293FT cells with packaging system HIVtrans and VSVG as previously described (Palka-Hamblin et al. 2009). Cells for PTK6 knockdown were infected with virus in the presence of 5 μg/ml polybrene and cultured for 7-10 days in the selection medium containing puromycin. The minimal dosage of puromycin for selecting stable cell lines was determined case by case prior to the infection. When selection was completed, puromycin concentration was halved for the maintaining medium.

2.4 Real-Time PCR

Total RNA was isolated with TRIzol Reagent (Life Technologies). After DNase (Promega) digestion, cDNA was generated by reverse-transcription with iScript cDNA Synthesis kit (Bio-Rad). Real-time PCR was performed with primers that target PTK6 exon 2 which is specific for full length PTK6. Human cyclophilin was used as internal control. PCR reaction was set up in triplicate with iQ SYBR Green Supermix (Bio-Rad) and ran in MyiQ single-color real-time PCR detection system (Bio-Rad). Primer sequence: PTK6 X2-F: 5' – CGG AAC CGT GGT TCT TTG-3'; PTK6 X2-R: 5' – ACT CGG CTT CTC CGC TGA C-3'; Cyclophilin-F: 5'-GCA GAC AAG GTC CCA AAG ACA G-3'; Cyclophilin-R: 5'-CAC CCT GAC ACA TAA ACC CTG G-3'. Data were collected with Bio-Rad iQ5 software. Averages and standard deviations were calculated with Microsoft Excel 2013.

2.5 Protein lysates, cell fractionation and immunoblotting

For cells: Unless specifically noted, all cells were harvested at 80-85% confluence to minimize the impact of confluency on PTK6 induction. Fresh medium with 10% FBS was added to cells 24

hours prior of harvesting. Cells were lysed with Triton X-100 buffer (20 mM Hepes, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, pH 8.0, 1 mM EGTA, pH 8.0, 10 mM Na-pyrophosphate, 100 mM NaF, 5 mM iodoacetic acid, 1 mM sodium vanadate, 0.2 mM PMSF and proteinase inhibitor cocktail tablet (Roche Diagnostic, Indianapolis, IN). For tissues: Fresh tissues were rinsed in phosphate buffered saline (PBS) and homogenized by tissue homogenizer (Polytron, PT-10, Kinematica) in Triton X-100 buffer.

Cell fractionations were performed using the ProteoExtract Subcellular Proteome Extraction Kit from EMD Millipore according to manufacturer's instructions. 1/10 volume of the total lysates of each fractionation was loaded on SDS-PAGE gel.

Immunoblotting was performed as previously described. PVDF membranes were blocked in Tris-Buffered Saline Tween-20 solution (150 mM NaCl, 20 mM pH 7.5 Tris and 1% Tween 20) with 5% non-fat milk or bovine serum albumin (BSA) (Sigma-Aldrich) for 1 hour at room temperature, then incubated in primary antibody for 1 hour at room temperature or overnight at 4℃ according to antibody manufacturers' recommendations.

2.6 Antibodies

Anti-human PTK6 (C-18) and its blocking peptide; anti-human PTK6 G6; anti-mouse PTK6 C-17; anti-ERBB2/Neu; anti-ERBB3; anti-SP1, anti-FAK, and anti-ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Y342 PTK6 antibody was purchased from Millipore (Billerica, MA). Anti-phospho-Y576/577 FAK, anti-phospho-Y165 p130Cas, anti-total and phospho-Y705 STAT3, anti-total and phospho-T308 and phospho-S473 AKT; anti-total and phospho-Y845, phospho-Y1045, phospho-Y1068 EGFR; Anti-phospho-T202/Y204 ERK1/2, Anti-phospho-T218/Y220 ERK5; Anti-cleaved Caspase 3; anti-cleaved Caspase 9 and anti-

cleaved PARP antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-p130Cas antibody was purchased from BD Biosciences (San Jose, CA); Anti-β-actin (AC-15) and anti-α-tubulin were purchased from Sigma-Aldrich (St. Louis, MO). Sheep anti-mouse and donkey anti-rabbit antibodies were purchased from GE Healthcare Biosciences (Piscataway, NJ). Rabbit IgG was purchased from Vector Laboratories (Burlingame, CA).

2.7 RNA extraction and ribonuclease protection assay

Total RNA was isolated from animal tissues using TRIZOL reagent (GIBCO Invitrogen, CA). Ribonuclease protection assays were performed as described previously (Haegebarth et al. 2004) using [^{32}P] α-CTP-labeled antisense RNA probes. Mouse cyclophilin mRNA was used as loading control and RNA integrity indicator, and the mouse cyclophilin anti-sense probe was synthesized from pTRI-cyclophilin-mouse antisense control template (Ambion, Grand Island, NY).

2.8 Mammosphere culture

Mammospheres were cultured in Corning Costar Ultra-Low attachment multi-well plates (Sigma-Aldrich). Culturing media was 1:1 DMEM/F12 with supplements including 15 ng/ml EGF, 15 ng/ml FGF, 1x B27, 4 μg/ml heparin, and 1x Pen/Strep. Cells were tripsinzed, spinned down and washed twice with sterile PBS, then suspended in the mammosphere media and counted by Countess automated cell counter (Invitrogen) before seeding. Cells were plated in triplicate. An aliquot of cells were subjected to immunoblotting to verify successful knockdown of endogenous PTK6.

2.9 Immunohistochemistry and immunofluorescence

Vectastain ABC kit (Vector Laboratories) and 3, 3'-Diaminobenzidine tetrahydrochloride tablets (DAB) (Sigma-Aldrich) were used for immunohistochemistry. Antigen retrieval was done in sub-

boiling 0.01 M sodium citrate for 20 minutes. Samples were incubated in 3% H_2O_2 prepared in methanol for 10 minutes to quench endogenous peroxidase, and then washed with PBS and blocked with serum at room temp for 1 hour or 4℃ overnight, followed by primary antibody incubation.

For immunofluorescence, slides were blocked with 3% BSA in TNT buffer (0.1 M Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 hour and incubated with primary antibodies at 4℃ overnight. After washing in TNT buffer, slides were incubated with biotinylated anti-rabbit secondary antibodies and then fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Laboratories). FITC was diluted 1:400 in TNB (Tris-NaCl-blocking buffer). Slides were mounted with mounting medium with DAPI from Vector Laboratories. Slides were read on a Zeiss LSM 700 confocal microscope and pictures were taken with manufacture's imaging software ZEN (Zeiss Efficient Navigation). Exposure times for FITC were fixed across the samples for each antibody, while times for DAPI may vary depending on the staining strength.

2.10 Animals

MMTV-H-RAS [FVB.Cg-Tg(MMTV-vHaras)SH1Led/J, stock number 004363] and MMTV-ERBB2 mice [FVB-(MMTV-ERBB2)NK1Mul/J, stock number 005038] were purchased from Jackson Laboratories (Bar Harbor, ME). MMTV-PyMT induced mouse mammary gland tumors were provided by Dr. Pradip Raychaudhuri (University of Illinois at Chicago).

MMTV-PTK6 transgenic mice were generated in the FVB/NJ inbred strain (Jackson Laboratories) and non-transgenic FVB/NJ mice used as controls were purchased from the same facility. Transgenic animals were generated by microinjection of MMTV-PTK6 construct into fertilized FVB/NJ eggs, performed by Dr. Roberta Franks from UIC Research Resource Center. Tail DNA

was subjected to PCR analysis with primers that were specific for the transgene (BRK-Forward: 5'- GCT ATG TGC CCC ACA ACT ACC -3', BRK-Reverse: 5'- CCT GCA GAG CGT GAA CTC -3'). Nulliparous females were never housed with males after weaning. Multiparous females were kept in breeding and underwent at least 2, but generally 3-4 pregnancies. Mice showed signs of discomfort, lose more than 15% of body weight or developed tumors larger than 2 cm in diameter were sacrificed as they met endpoint criteria of the protocol approved by the UIC Institutional Animal Care and Use Committee.

C57BL/6J *Ptk6* null mice were described previously (Haegebarth et al. 2006). To generate *Ptk6* null mice in FVB/NJ background for mating with FVB/NJ MMTV-ERBB2 mice, wild type FVB/NJ mice were crossed with C57BL/6J *Ptk6* null mice and then backcrossed with FVB/NJ mice for 7 generations. *Ptk6* $^{-/-}$ mice were genotyped with two sets of primers. Sik-New-Forward (5' -ATG GTG TCT TGG GAC AAG GCT CAC CTG-3') and Sik-NEW-Reverse (5'-CAT CCA GCA GGG TGG CCC ACC ACC ACA-3') will produce a ~150 base pair band in *Ptk6* $^{+/+}$ or *Ptk6*$^{+/-}$ mouse, while Sik-OLD-Forward (5'-CAT ACA CTT CAT TCT CAG TAT-3') and Sik-OLD-Reverse (5'-ACG TGG CTG TCC AGA CAT AG-3') will produce a ~1500 base pair band in *Ptk6*$^{+/-}$ or *Ptk6*$^{-/-}$ mouse. ERBB2 transgene were genotyped by primer pair Neu-Forward (5'-TTT CCT GCA GCA GCC TAC GC-3') and Neu-Reverse (5'-CGG AAC CCA CAT CAG GCC-3'). PCR program was set as following: 94 ℃ 100 seconds, (94 ℃ 45 seconds-63 ℃ 45 seconds-72 ℃ 60 seconds) X 30 cycles, 72 ℃ 10 minutes, and then hold at 10 ℃.

2. 11 <u>Tissue preparation and microarray</u>

For whole mounts: mouse mammary glands were harvested, spread on glass slides, air dried for 5 minutes and then fixed in Pen-fix solution (Richard-Allan Scientific, Kalamazoo, MI) for 24 hours. Samples were then passed through graded ethanol, acetone, and rehydrated. Tissues were stained

in carmine alum for 3 days and then dehydrated in ethanol followed by clearing in xylene (Fisher Scientific, Fair Lawn, NJ). Stained whole mounts were stored in xylene during examination and photographing using a dissection microscope.

For microscopic sections: tissues were fixed in 10% buffered formalin (Fisher Scientific) for 24 hours and then transferred to 70% ethanol prior to routine processing. Paraffin embedded tissues were sectioned at 5 microns and stained with hematoxylin and eosin. Whole mount analysis, postmortem examination and histopathologic analysis of tissue sections were performed by a veterinary pathologist (Susan Ball-Kell, DVM, PhD).

For normal human breast tissue samples: the normal human mammary gland tissue arrays have been described (Asztalos et al. 2010). Samples on the TMAs were obtained from women between 18 and 45 years of age who underwent a reduction mammoplasty or breast biopsy with benign findings at the University of Illinois at Chicago Hospital. 67 cores from 27 patients with benign findings were analyzed. For human breast cancer samples: paraffin-embedded tissues were obtained from patients diagnosed with breast cancer between 18 to 45 years of age from the University of Illinois at Chicago Hospital, Northwestern Memorial Hospital (Chicago) and Allina Hospitals and Clinics (Minneapolis, MN) and processed as previously described (Asztalos et al. 2010). The breast cancer TMA was constructed with triplicate 2.0 mm diameter cores placed adjacent to each other resulting in 131 cores from 45 patients on the TMA. These studies were approved by the Institutional Review Boards of each institution. The tumors were first analyzed using routine H&E morphology of the tissue microarrays to determine in-situ and invasive cancers. In-situ tumors were separated into low and high grades 1-3 using the Van Nuys system (Silverstein et al. 1995), while invasive mammary carcinomas were stratified into grades 1-3 using the Nottingham combined histologic grading system (Bloom et al. 1957, Elston et al. 1991).

2.12 Proliferation assay

For cells: cells were cultured in triplicate and lysed with trypsin. When counted with Countess automated cell counter, cells were stained with 0.1% concentration of Trypan Blue for 30 seconds and read by the machine. Only live cells were documented. The CellTiter-Glo luminescent cell viability assay is used to determine the number of viable cells based on quantitation of the ATP present, an indicator of metabolically active cells. When measured with CellTiter-Glo luminescent cell viability assay, cells were mixed with same volume of CellTiter-Glo reagent and agitated for 2 minutes, then incubated at room temperature for 10 minutes. The mixture was then subjected to analysis by the Promega GloMax luminometer.

For animals: Animals were injected intraperitoneally with 5-bromo-2'-deoxyuridine (BrdU) (Sigma) in PBS at 50 μg/g of body weight two hours prior to sacrificing. BrdU incorporation was detected using anti-BrdU (BD Bioscience) antibody and the Mouse-on-Mouse (M.O.M) immunodetection Kit (Vector Laboratories). For each sample, cell counting was performed on 5 pictures taken randomly from different areas to represent the average distribution of BrdU positive cells.

2.13 Apoptosis assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed with ApopTag Fluorescein *in Situ* Apoptosis Detection Kit (Millipore), all procedures were performed according to the manufacturer's protocol.

2.14 Statistics

Statistical analysis was performed in consultation with Dr. Hui Xie, a statistician in the UIC Design and Analysis Core. Univariate analysis was initially conducted to summarize the tumor data.

Categorical data are presented as percentages and Pearson Chi-square tests are used to compare frequency distributions among different experimental groups. When appropriate, the more powerful Cochran-Armitage Trend tests are applied. Continuous data are presented as means and standard deviations. Two-sample t-tests are used to compare the mean values between two groups. Linear regression models are fitted to the data to describe the relationship between normalized tumor size and age, and Wald tests are used to compare regression lines from different experimental groups. All analyses were performed using SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC). Densitometry analysis of immunoblotting results was performed with ImageJ 1.45s (NIH) (Rasband 1997-2011). Quantitative data are shown as the mean ± SD. P-values were determined using the 2-tailed Student's T test (Microsoft Excel, 2010). A difference was considered statistically significant if the p-value was equal to or less than 0.05.

2.15 Survival analysis

Original data were collected by TCGA group from The Cancer Genome Atlas, Office of Cancer Genomics, National Cancer Institute, National Institutes of Health. 532 invasive breast carcinoma, 61 paired normal breast tissue and 3 paired metastatic samples were analyzed. Sample data includes age, histology, TNM stage, ER/PR/ERBB2 status, sex, stage, and others. Array measured 20,423 genes, 111,123 reporters with mRNA. Data Link: http://tcga-data.nci.nih.gov/tcga/ No associated paper was published by the time we analyzed the data (2011/09/02).

For our study, datasets containing 447 breast cancer samples with patient survival information was extracted from Oncomine database (The Cancer Genome Atlas-Invasive Breast Carcinoma Gene Expression Data). Patients were arbitrarily categorized into "high", "medium" and "low" groups according to their PTK6 mRNA level. The survival curve was estimated using the Kaplan-Meier

method and the differences among three groups was tested using the log-rank test. The analysis was performed using SAS 9.2.2010.

III. RESULTS

3.1. Re-examining PTK6 expression in normal breast tissue and breast tumors

3.1.1 Background

PTK6 cDNA was originally cloned from a metastatic human breast tumor and breast cancer cell lines T-47D and MCF-7 (Mitchell et al. 1994). Since then, PTK6 expression has been studied extensively in breast tumors and cancer cell lines. Immunoblots showed that high levels of PTK6 protein were detected in the breast cancer cell lines ZR75-30, BT-474, T-47D, MDA-MB-453 and MDA-MB-361, and moderate levels of PTK6 were found in SK-BR-3 and MDA-MB-231. Low levels of PTK6 were observed in PMC 42, MDA-MB-157 and MDA-MB-468, and Cal51 cells did not have detectable level of PTK6 (Mitchell et al. 1994, Barker et al. 1997, Harvey et al. 2004). The expression level of PTK6 does not relate to the phase of cell cycle or proliferation rate (Mitchell et al. 1994), but it may be affected by the confluency and/or differentiation as shown in Caco-2 cells (Llor et al. 1999). Although human PTK6 is localized on a chromosomal region that is often found to be amplified in breast carcinomas and ovarian carcinomas (Park et al. 1997), the high PTK6 expression in breast cancer cell lines and breast carcinomas was unlikely to be caused by gene amplification, but rather by over-expression (Aubele et al. 2009). The PTK6 cDNA sequence derived from tumor tissue is identical to the sequence derived from normal PTK6 genomic sequence, suggesting the overexpression of PTK6 in breast tumor is not due to gene mutation (Mitchell et al. 1994, Mitchell et al. 1997).

After the discovery of PTK6 in breast tumor tissue, studies were extended to normal breast tissue sample from reduction surgery and cell lines derived from normal human mammary epithelia, as well as MCF-10A, an immortalized but non-transformed breast epithelial cell line. Lysates of reduction mammoplasty tissue were probed with ICR100 antibody developed by Crompton lab

and the PTK6 signals were extremely low or undetectable, comparing to those of breast carcinoma samples (Mitchell et al. 1994). Interestingly, a moderate level of PTK6 was detected in MCF10A cells, higher than some of the breast cancer cell lines such as MDA-MB-468 (Mitchell et al. 1994). However, it was still much lower than many of the breast cancer cell lines such as T-47D and BT-474, and was considered negligible in some experiments.

With all these findings, it has been commonly accepted that PTK6 is not expressed in normal human mammary glands but it is overexpressed in the majority of breast tumors and breast cancer cell lines. However, our findings suggest that PTK6 protein is actually expressed in the normal breast epithelium, while in breast tumors, PTK6 protein exhibits changes in expression levels, subcellular localization and activation. The change of PTK6 expression from normal to tumor tissue is more likely to be of de-regulation of activation and overexpression/loss of expression than merely induction.

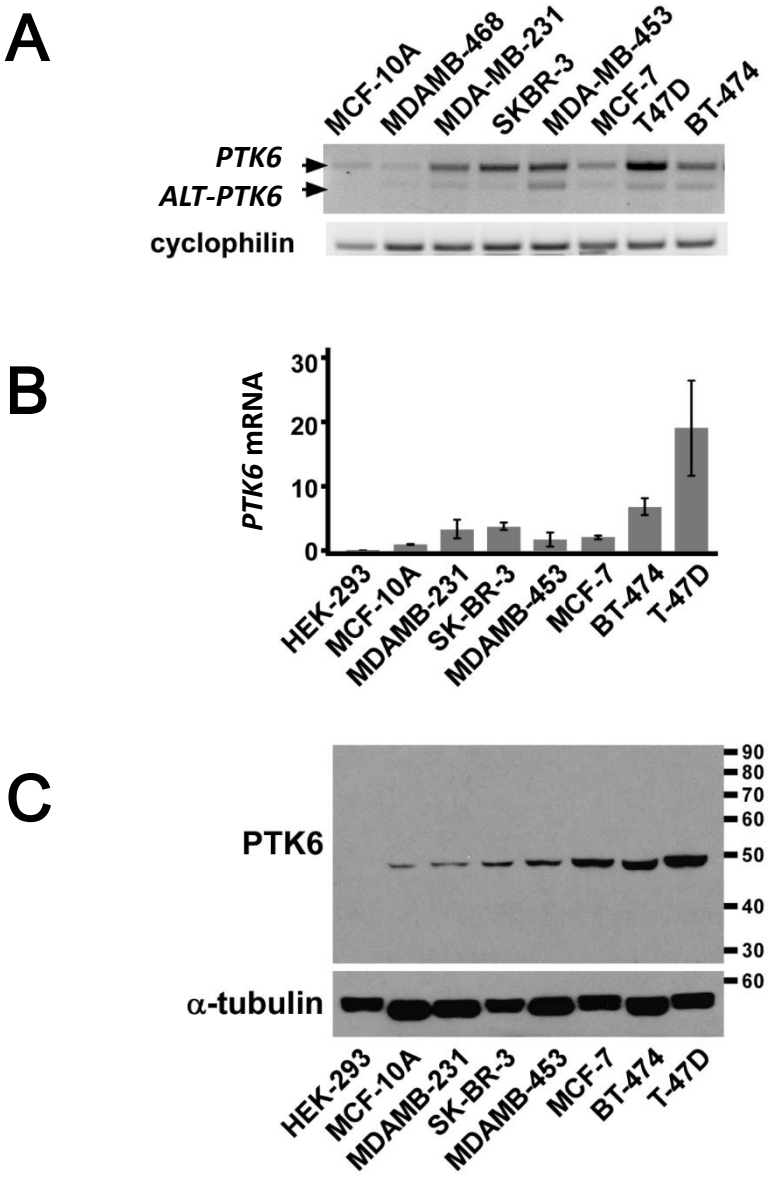
3.1.2 PTK6 is expressed in MCF-10A and overexpressed in multiple breast cancer cell lines.

MCF-10A cells arose from spontaneous immortalization of human fibrocystic mammary tissue after extended cultivation in low calcium concentrations (Soule et al. 1990). They are non-tumorigenic in nude mice, can grow and differentiate in 3D collagen culture, and their growth is hormones and growth factors dependent (Soule et al. 1990, Tait et al. 1990, Debnath et al. 2003, Underwood et al. 2006). Since MCF-10A cells are immortalized but not transformed and still possess the characteristics of normal mammary gland epithelium, they are commonly used as a normal control to compare with tumorigenic breast cancer cell lines.

It is well documented that PTK6 is highly expressed in many breast cancer cell lines. However, different results have been reported in regard of MCF-10A cells: Low to moderate levels of PTK6 have been detected in MCF-10A cells in some experiments (Mitchell et al. 1994, Xiang et al. 2008, Irie et al. 2010), while in other studies MCF-10A cells seemed to be PTK6 negative (Miah et al. 2012, Regan et al. 2013). To examine PTK6 expression in MCF-10A cells, PCR amplification and immunoblotting were performed. Multiple cell lines were maintained according to provider's protocol and harvested at 80-85% confluency to minimize the possible induction of PTK6 by confluence. Total transcripts were transcribed into cDNA with random hexamers. After PCR amplification, *PTK6* transcripts were detected in all the cell lines analyzed, including MCF-10A (Figure 3 A). Primers used in PCR flanked exon 1 & 3 and amplified both full length *PTK6* and *ALT-PTK6*. *ALT-PTK6* is detected along with full length *PTK6* in all the cell lines except MCF-10A which might due to the low level of expression. Real-time PCR was performed with a pair of primers that target full length *PTK6* specific exon 2, and *PTK6* was again detected in MCF-10A cells (Figure 3 B). Immunoblotting also detected endogenous PTK6 expression in MCF-10A cells (Figure 3 C). However, comparing to the breast tumor cell lines, MCF-10A has the lowest levels of both PTK6 mRNA and protein. These findings demonstrate that PTK6 is expressed in non-transformed normal breast epithelial cells and up-regulated in some breast cancer cell lines. The protein levels of endogenous PTK6 are consistent with previous studies (Barker et al. 1997, Irie et al. 2010). Interestingly, mRNA levels of endogenous PTK6 do not always correlate with the protein levels: moderate levels of PTK6 mRNA were detected in MDA-MB-231 and SK-BR-3, higher than those in MCF-7 and MDAMB-453, but PTK6 protein levels of MDA-MB-231 and SK-BR-3 were much lower than MCF-7 and MDAMB-453. This finding agrees with the result that PTK6 protein may be regulated post-transcriptionally (Irie et al. 2010).

Figure 3: PTK6 is expressed in non-transformed breast epithelial cell line and overexpressed in breast cancer cell lines

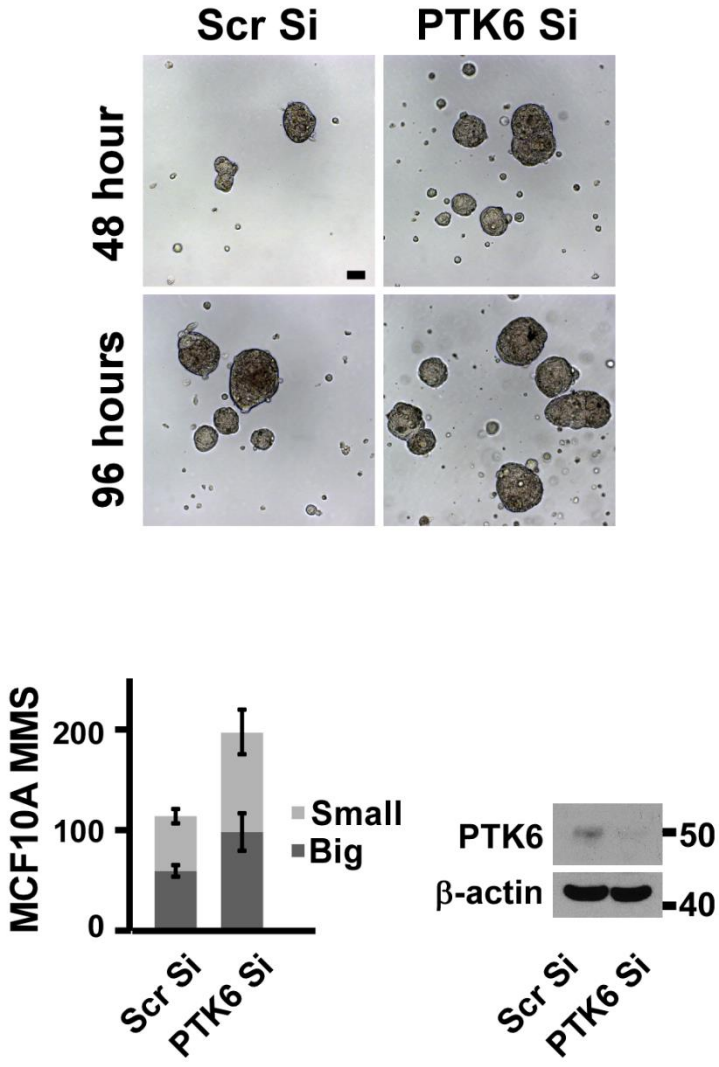
(A). Semi-quantitative reverse-transcription PCR was performed with a set of primers specific to exon 1 and 3 which amplify both full length and *ALT-PTK6*. Full-length *PTK6* and *ALT-PTK6* mRNA co-existed in breast cancer cell lines. Human cyclophilin was used as loading control. **(B).** Full length *PTK6* transcripts in breast cancer cell lines were analyzed using quantitative real-time PCR. *PTK6* mRNA levels were normalized to endogenous cyclophilin mRNA levels for each cell line, and then compared to MCF-10A which was set as the standard. *PTK6* mRNA levels are shown as folds (MCF-10A = 1), *PTK6* null HEK293 cell was used as negative control. **(C).** Immunoblotting was performed with total cell lysates of the breast cancer cell lines. Endogenous PTK6 was detected by PTK6 G6 antibody manufactured by Santa Cruz. α-tubulin was used as loading control.



To explore the functions of PTK6 in normal human mammary gland, mammosphere formation assay was performed on MCF-10A cells. Mammosphere assay is a cell culture method developed by analogy with studies on neuronal stem cells (Dontu et al. 2003). In this culture system, cells are grown under non-adherent conditions and those capable of surviving and proliferating would form clusters of cells termed "mammospheres" (Dontu et al. 2003, Shaw et al. 2012). Mammospheres are enriched in mammary stem/progenitor cells capable of self-renewal and multi-lineage differentiation, they contain cells capable of generating functional ductal-alveolar structures and they are able to reconstitute the mammary tree in the cleared mammary fat pad of NOD/SCID mice when combined with human mammary fibroblasts (Liu et al. 2005). Mammosphere assay is an easy to perform approach to enrich stem-like cells and has gained increasing popularities in breast cancer research (Grimshaw et al. 2008, Cioce et al. 2010, Creighton et al. 2010, Guttilla et al. 2011, Yan et al. 2011, Hinoharaa et al. 2012, Rota et al. 2012, Shaw et al. 2012, Xie et al. 2012). Most of the studies were performed on breast cancer cell lines such as MCF-7, T-47D, MDA-MB-453 and MDA-MB-231, but MCF-10A has also been shown to form mammospheres in culture and the sphere forming capacity can be altered by cytokine stimulation, genotoxic stress and transcriptional factors regulation (Liu, G. et al. 2009, Liu, M. et al. 2009, Botlagunta et al. 2010, Asiedu et al. 2011).

In our study, to determine the contribution of PTK6 to stem cell characteristics, MCF-10A cells were transfected with siRNA that targets *PTK6* mRNA to silence the endogenous PTK6, and then subjected to mammosphere culture (Figure 4). Non-targeting siRNA with a scrambled nucleotide sequence (Scr Si) was used as negative control. Mammospheres were examined under an inverted microscope every other day. By 48 hours post seeding, mammospheres of different sizes were visible in both cell cultures. Increased number of mammospheres formed in PTK6 knock-down

Figure 4: Silencing endogenous *PTK6* promotes mammosphere formation in MCF-10A cells MCF-10A cells were transfected with PTK6 siRNA (PTK6 Si) to silence the endogenous *PTK6* or Scrambled siRNA (Scr Si) as negative control. 48 hours after siRNA transfection, cells were seeded in non-adhesive plates containing mammosphere forming medium. Mammospheres were divided into small (20-50 μm) and big (>50 μm) categories when counted on day 6 (bottom left). Numbers and error bars represent the average value and standard deviation of triplicate cultures. Size bar = 50 μm. Immunoblotting of the lysates prepared from the remainder of the siRNA transfected cells showed the successful PTK6 silencing (bottom right).



cells and these spheres were larger in size. This pattern persisted throughout the whole culturing period. Mammospheres were counted 6 days post seeding because after day 6 spheres will continue to grow and aggregate into huge clusters if in close proximity and making the count inaccurate. Loss of endogenous PTK6 resulted in an increased number of mammosphere formation which applied to both small (20-50 μm) and large (>50 μm) spheres, suggesting PTK6 may play a role in maintaining the differentiating characteristics in normal breast epithelium, and knocking down PTK6 could cause the normal cells to become more "stem like".

3.1.3 PTK6 is expressed in normal human breast tissue

Comparing to the abundant research in breast tumors and breast cancer cell lines, the role of PTK6 in normal breast is poorly investigated, possibly due to the common notion that PTK6 is not expressed in normal breast tissue. However, knockdown of PTK6 in MCF-10A cells promoted mammosphere formation, leading us to think PTK6 may have physiological function in normal human mammary gland. A recent study also showed that *PTK6* transcripts are detectable in non-neoplastic human mammary glands (Harvey et al. 2009). To examine PTK6 expression in normal mammary glands, we obtained breast tissue microarray slides from Dr. Debra A. Tonetti and performed immunohistochemistry on 76 cores from 27 patients. The TMA cores contained normal mammary glands obtained from breast reduction or benign biopsies. Antibodies against total human PTK6 and phospho-tyrosine-342 PTK6 (PTK6 P-Y342) were used to detect the endogenous PTK6. Slides were read and scored by Dr. Rajyasree Emmadi and Dr. Elizabeth L. Wiley (Department of Pathology, UIC), two board certificated pathologists specialized in human mammary gland pathology.

The human mammary gland epithelia are composed of a layer of luminal cells surrounded by a layer of myoepithelial cells. The ductal epithelial network is embedded in an extracellular matrix

of stromal cells. Milk-secreting alveolar luminal cells are formed upon pregnancy (Visvader et al. 2009, Lim et al. 2011). PTK6 was detected in luminal cells of almost all the cores that contained mammary gland tissue (66 out of 67) (Figure 5 & Table 2). Signal strength ranged from weak (1+) to medium (2+), and localization was cytoplasmic, nuclear or both. Weak PTK6 was also detected in the myoepithelial cells, predominately in the cytoplasm and in a few cases in the nucleus, as shown in the cytoplasm of prominent myoepithelial cells (Figure 5 upper right corner, outlined by dashed lines), and in the nucleus of the vacuolated myoepithelial cells (Figure 5 lower right corner, outlined by dashed lines). PTK6 expression levels may vary even in similar gland structures from different core suggesting PTK6 may be tightly regulated by signals from the environment. We were not able to determine if menstrual cycle or pre/post menopause states played a role in PTK6 expression/localization as these states could not be determined from the morphology alone.

Active PTK6 P-Y342 was not detected in the normal mammary glands. Some weak staining was observed in some samples, mostly accumulated in the lumen and at one side of the cells, and it was later proved to be non-specific staining. The exception was found in apocrine metaplasia (Figure. 9), in which both total and active PTK6 were detected in the nucleus of the luminal cells. Apocrine metaplasia is commonly detected in the female breast after the age of 25. It was suggested to be a manifestation of epithelial unrest, and a reflection of an underlying stimulus that renders the breast more susceptible to neoplasia (Reviewed in Wells et al. 2007).

Results were consistent and repeatable across TMA slides (each solid-lined box represents a different patient). Detecting PTK6 in normal human mammary gland led us to re-think if PTK6 is expressed in normal mouse mammary gland. Previous study showed that PTK6 was not expressed in mouse mammary gland including virgin, pregnant, lactating and involuting breast tissue by

Figure 5. PTK6 is expressed but not activated in normal human mammary gland
Immunohistochemistry and immunofluorescence assays were performed on serial sections of human breast TMAs. Antibodies against total human PTK6 and PTK6 P-Y342 were used to detect endogenous PTK6. Corresponding areas from the same patient were shown in the same solid-lined boxes. PTK6 was detected in the luminal cells and can be cytoplasmic, nuclear or a both (brown DAB staining). PTK6 was also detected in the myoepithelial cells (outlined by dashed lines), predominately in the cytoplasm and in a few cases in the nucleus, as shown in the cytoplasm of prominent myoepithelial cells (upper right corner) and in the nucleus of the vacuolated myoepithelial cells (lower right corner). However, active PTK6 P-Y342 (green FITC staining) was not detected in the normal mammary glands. Size bar = 50 μm

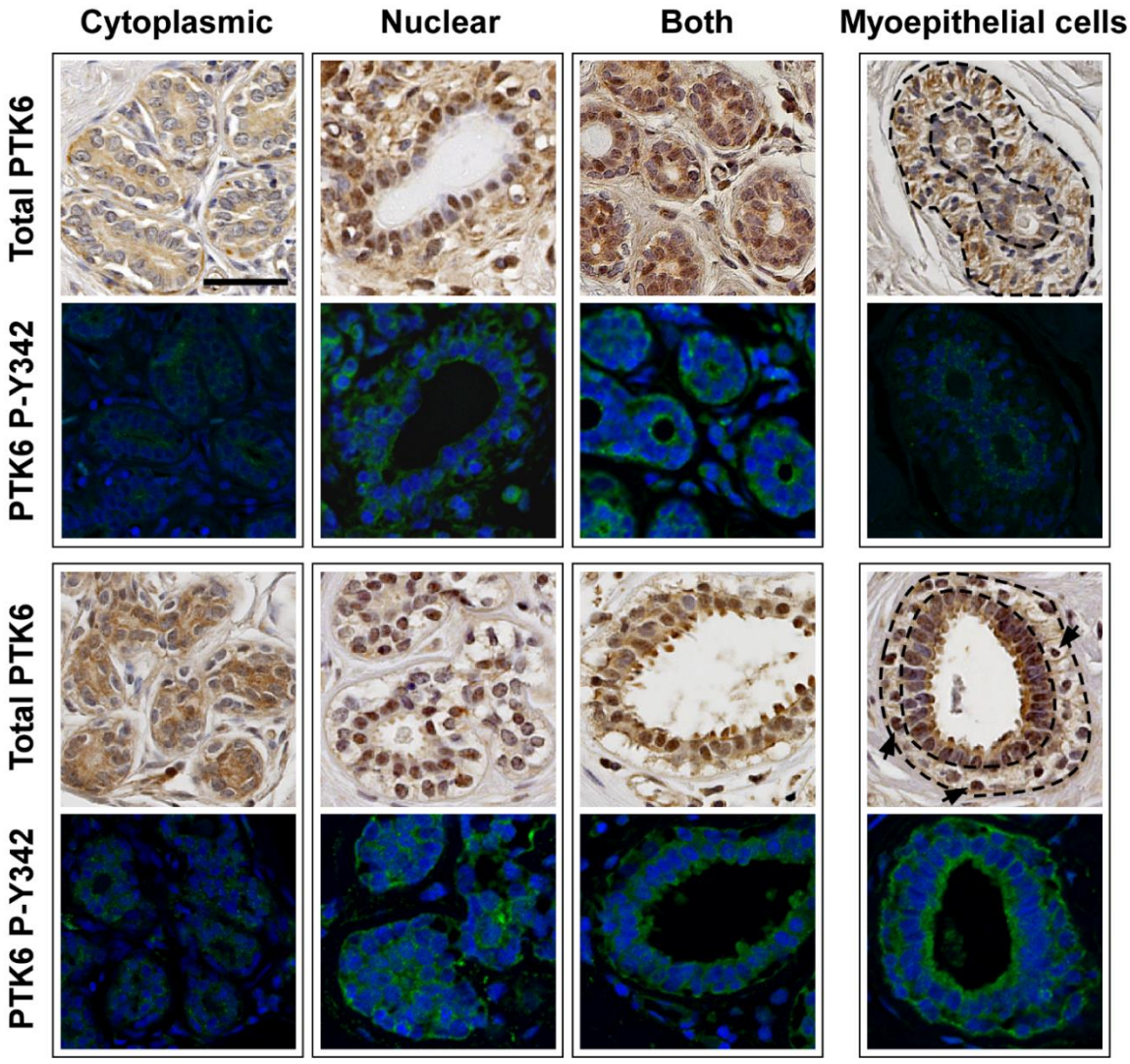


TABLE II

PTK6 EXPRESSION AND DISTRIBUTION IN NORMAL HUMAN MAMMARY GLAND

Total PTK6 expression in normal human mammary glands						
Cell type and subcellular localization		PTK6 positive patients	Signal strength			
			0	1+	2+	3+
Luminal cells	Cytoplasm	27 (100%)	0 (0%)	13 (48.1%)	13 (48.1%)	1 (3.7%)
	Nucleus	18 (66.7%)	10 (37.0%)	5 (18.5%)	12 (44.4%)	0 (0%)
Myoepithelial cells	Cytoplasm	25 (92.6%)	2 (7.4%)	22 (81.5%)	3 (11.1%)	0 (0%)
	Nucleus	5 (18.5%)	22 (81.5%)	3 (11.1%)	2 (7.4%)	0 (0%)

[a] Patient number =27, core number =76. Percentages were calculated on patient number rather than core number.

[b] If a patient had different PTK6 staining level among the cores, the highest number was used (e.g. if three cores from the same patient were scored as 1+, 2+, 2+, then the patient would be counted as a 2+).

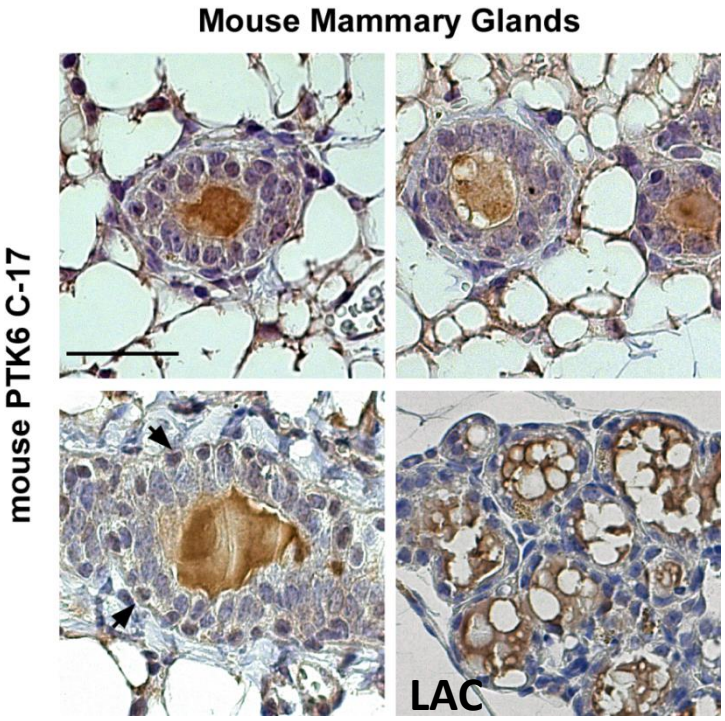
[c] Cytoplasmic and nuclear signals were not mutually exclusive and some samples were counted in both categories if they have mixed pattern.

RNase protection assays (Llor et al. 1999). To validate this result, we performed immunohistochemistry on mouse mammary glands with an antibody against mouse PTK6 (PTK6 C-17, Santa Cruz Biotechnology). Endogenous PTK6 was not detected in any cells, regardless of the mammary gland to be normal or lactating (Figure 6), confirming the fact that unlike in human, PTK6 is not expressed in normal mouse mammary glands.

In addition, disrupting *Ptk6* gene expression in mouse does not seem to affect the biological function of mammary gland. In our study we generated *Ptk6* null mouse in multiple strains such as C57BL/6J and FVB/NJ, and *Ptk6* $^{-/-}$ mice did not exhibit any defects in lactation or involution throughout several pregnancies. No significant morphology difference between the mammary glands of *Ptk6* $^{+/+}$ and *Ptk6* $^{-/-}$ mice was detected by whole mount staining either.

The difference in PTK6 expression between human and mouse may be caused by the species difference. Although human and mouse mammary gland share certain similarities, they each have their unique structures and developmental stages. For example, the human mammary gland is characterized by a branching network of ducts that terminate in clusters of small ductile that constitute the terminal ductal lobular units (TDLUs). TDLUs develop early and persist into the post-menopause days, while the mouse counterpart lobule-alveolar units only develop during pregnancy with hormonal stimulation and disappear after weaning (Cardiff 1998). Moreover, the human breast parenchyma is more fibrous than the mouse stroma, which contains predominantly adipocytes (Reviewed in Visvader 2009). PTK6 could be regulated by human specific signals correlated with these structural difference, or by other mechanisms yet to be discovered. In addition, PTK6 can be induced by stress like irradiation (Haegebarth et al. 2009) and hypoxia (Anderson et al. 2013). Difference in lifespan between human and mouse may also cause different exposure levels to stress and stimulation, which may contribute to the different PTK6 expression patterns.

Figure 6: Endogenous PTK6 is not detected in normal mouse mammary glands
Mouse PTK6 (C-17, Santa Cruz) antibody was used to detect endogenous mouse PTK6, and while this antibody produced background staining by reacting to the milk proteins inside the lumen, no epithelial cell stained positive for PTK6, no matter luminal or myoepithelial (arrow) cells, normal ductal cells or lactating alveolar cells (lower right, LAC). Scale bar = 50 μm.



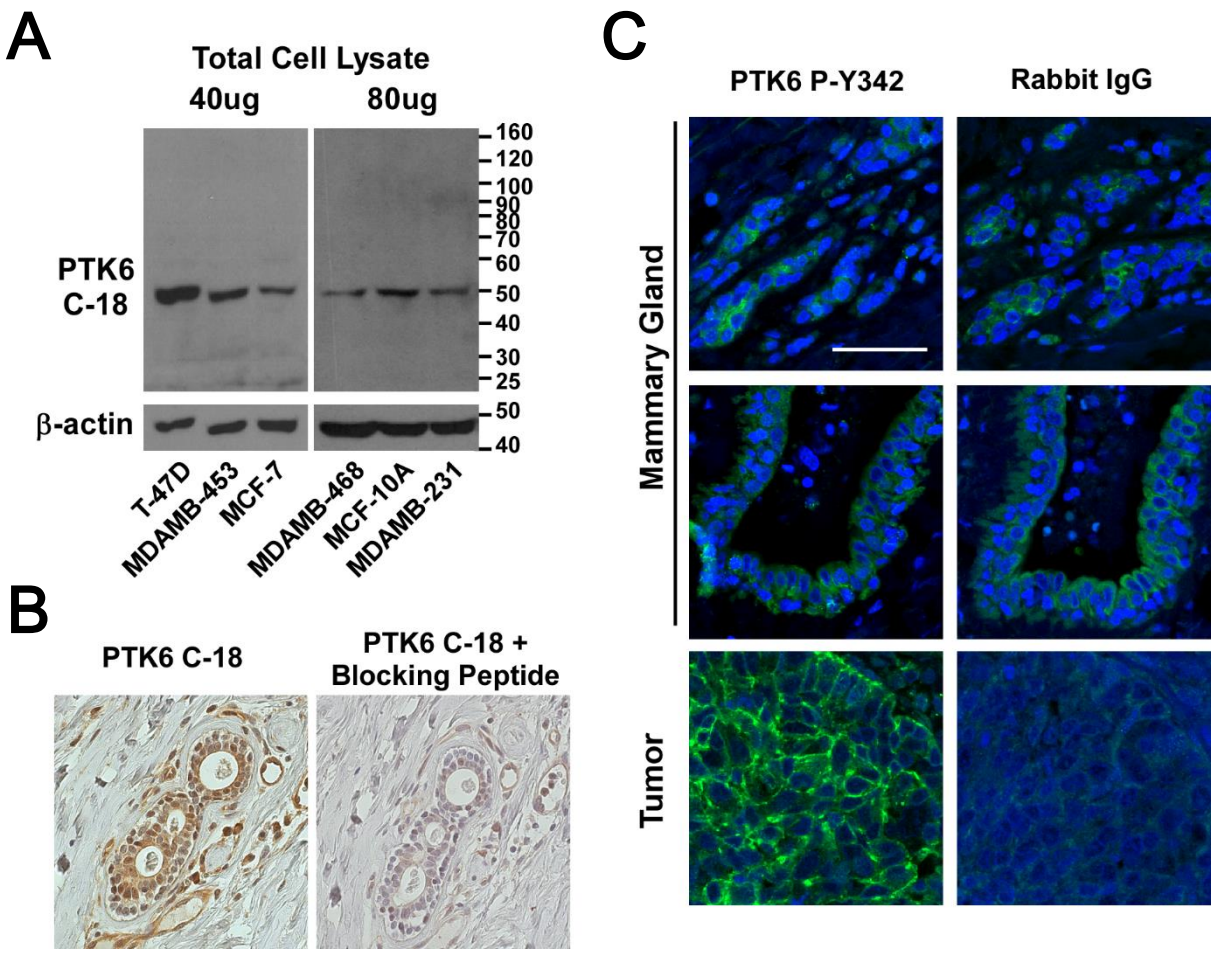
3.1.4 Validating the specificity of human PTK6 antibodies

To determine the signals detected in the normal human mammary glands are real PTK6 signals, human PTK6 C-18 antibody was tested with immunoblotting and blocking peptide competition assay. Total cell lysates of human breast cell lines were probed with 1:2000 dilution of PTK6 C-18 antibody to assert antibody specificity. 40 μg of the lysates were loaded for PTK6 medium/high-expressing cells MCF-7, T-47D and MDA-MB-453, and 80 μg were loaded for low-expressing cells MCF-10A, MDA-MB-468 and MDA-MB-231.The whole membrane was shown in (Figure 7 A). Only one ~50 kDa band specific for PTK6 showed up in each lane, suggesting the unspecific binding was minimal to none.

Peptide competition assay was performed to examine the specificity of PTK6 antibody. PTK6 C-18 was pre-incubated with blocking peptide (SC-1188p, Santa Cruz Biotechnologies) at 1:5 ratio of weight for 2 hours at room temperature, then applied to the human mammary gland sections to incubate at room temperature for 1 hour as primary antibody. The control C-18 antibody without blocking peptide was also sit at room temperature for 2 hours before used in the immunohistochemistry experiment to minimize the difference caused by antibody deterioration if any. Without peptide competition, both cytoplasmic and nuclear PTK6 can be observed in the luminal cells and some nuclear staining was observed in the myoepithelial cells too, with peptide competition, PTK6 signal was greatly reduced in all the epithelial cells, decreased from medium strength to barely detectable, proving that the signals detected in normal human mammary glands represented real PTK6 protein expression (Figure 7 B).

Our lab showed that P-Y342 antibody is capable of specifically detecting active PTK6 in immunoblotting and immunohistochemistry experiments previously (Zheng et al. 2013). To assert

Figure 7. Validating the specificity of PTK6 antibodies
(A). Total cell lysate of breast cancer cells and MCF-10A cells were probed with human PTK6 C-18 antibody, the same antibody used in the immunohistochemistry on normal breast TMAs. Whole membrane was shown. β-Actin was used as loading control. **(B).** Peptide competition assay was performed on serial section of human breast tissue fixed in paraffin block. Immunohistochemistry staining was performed with normal antibody (PTK6 C-18, left) or pre-incubated antibody (PTK6 C-18 + blocking peptide). **(C).** Serial sections of human breast tumor containing some mammary gland structure were stained with PTK6 P-Y342 antibody and rabbit IgG to assert the background noise. Pictures were taken from different areas of the same sample, and the gain time of FITC was kept consistent. Size bar =50 μm



the level of background of mammary gland and breast tumor tissue with this P-Y342 rabbit polyclonal antibody, a staining with a rabbit IgG as primary antibody was performed on human breast tissue that contained both tumor and normal glands. P-Y342 antibody and rabbit IgG were both diluted to 0.5 μg/100 μl and incubated overnight at 4 ℃. Same gain time of FITC was used when taking pictures. Both IgG and P-Y342 produced some weak signals lining the mammary gland cells (Figure 7C, mammary gland), and was regarded as non-specific background noise. However, no signal was detected in the tumor areas by IgG staining, while the signal strength was striking with P-Y342 antibody (Figure 7C, tumor).

3.1.5 PTK6 is overexpressed and activated in breast tumors

Although it is well known that PTK6 is overexpressed in human breast tumors, the activation status of PTK6 in breast tumors has not been reported previously. It is unknown if the kinase activity of PTK6 is playing a role in breast tumorigenesis in a physiologically relevant context. To determine the activation status of PTK6 in breast tumors, immunohistochemistry and immunofluorescence were performed on breast tumor TMA slides containing 131 cores from 45 patients, using the same condition as normal breast TMA staining. Each patient has 3 different cores (although some cores were missing or contained no useful tissue). PTK6 signal strength was scored from 0 to 3 by Dr. Emmadi (Figure 8). Total PTK6 was detected in 95.6% of the patients (87.5% if calculated by the number of cores) (Table 3), most of which exhibited medium (2+) to strong (3+) levels of signals (Figure 9 & 10). Tumor subcategories were determined by analyzing corresponding H&E staining. The majority of the samples were invasive ductal carcinomas, both high and low grade, with a few ductal carcinomas in situ, lobular carcinomas in situ and mammary glands with micro invasions. Highest cytoplasmic PTK6 staining was detected in invasive ductal carcinomas, and highest nuclear signals were detected in lobular carcinomas in situ. Although some nuclear staining of

Figure 8. PTK6 signal strength scoring system

Each core of the TMAs was given a score from 0 to 3 based on the signal strength of PTK6. 0: no signal, 1: weak, 2: medium, 3: strong. Size bar =50 μm

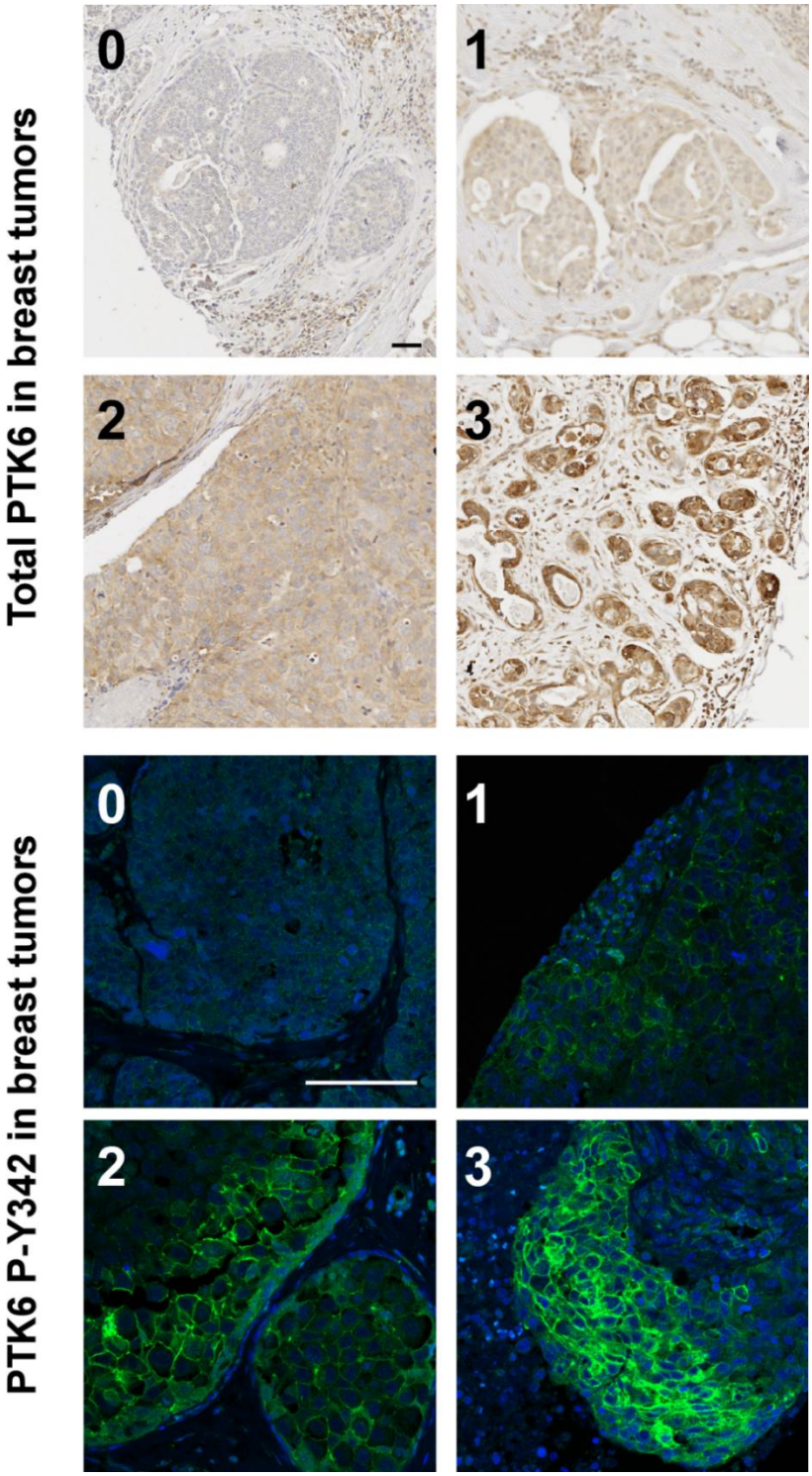


TABLE III

PTK6 EXPRESSION AND DISTRIBUTION IN HUMAN BREAST TUMOR

PTK6 expression and activation in human breast tumors						
Antibodies and subcellular localization		PTK6 positive patients	Signal strength			
			0	1+	2+	3+
Total PTK6	Cytoplasm	43 (95.6%)	2 (4.4%)	13 (28.9%)	28 (62.2%)	2 (4.4%)
	Nucleus	20 (44.4%)	25 (55.6%)	7 (15.6%)	12 (26.7%)	1 (2.2%)
PTK6 P-Y342	Membrane	18 (40%)	25 (55.6%)	12 (26.7%)	4 (8.9%)	2 (4.4%)
	Cytoplasm	14 (31.1%)	29 (64.4%)	11 (24.4%)	3 (6.7%)	0 (0%)
	Nucleus	5 (11.1%)	38 (84.4%)	4 (8.9%)	1 (2.2%)	0 (0%)

[a] Patient number =45, core number =131. Percentages were calculated on patient number rather than core number.

[b] If a patient had different PTK6 staining level among the cores, the highest number was used.

total PTK6 can be found in the low grade carcinomas (Figure 9, arrows), it was absent from most of the high grade carcinomas and PTK6 appears to be cytoplasmic and membranous. Similar expression patterns can be observed in the ductal carcinoma in situ: PTK6 is nuclear/cytoplasmic in low grade tumors and became more cytoplasmic/membranous in higher grade tumors (Figure 9).

Interestingly, overexpression of PTK6 did not necessarily mean that it was activated: although more than 90% of patients overexpressed PTK6, only half of the patients were positive for active PTK6. Membrane localized active PTK6 was detected in about 40% of the patients, and the signals ranged from weak (26.7%) to strong (4.4%). Cytoplasmic active PTK6 was also found in 31.1% of the patients, but the signal strength was not as strong as the membrane staining. Nuclear active PTK6 was only detected in the lobular ductal carcinoma patient, and the signal was moderate to strong in some individual cells. Active PTK6 appeared to correlate with the grades of tumors: In the low grade ductal carcinomas, P-Y342 signal was low to non-detectable, but in the high grade invasive ductal carcinomas, strong PY-342 PTK6 signal was detected at the membrane of tumors cells that have breached basement membrane. In the DCIS, active PTK6 is localized at the membrane of those cells close to the basement membrane (Figure 9, FITC).

PTK6 expression was detected in lobular carcinoma in situ (LCIS). Unlike ductal carcinomas, PTK6 was mostly nuclear localized in the lobular carcinomas. As shown in Figure 10A, medium to strong levels of total PTK6 were found in the nucleus of lobular carcinomas in situ of both low and high grade, while the membrane and cytoplasmic staining is very low or undetectable. Just like total PTK6, active PTK6 accumulated in the nucleus, ranging from weak (white arrow) in low grade LCIS to strong in high grade LCIS. Most of the high grade LCIS cells were positive for nuclear PTK6 but some PTK6 negative cells were also observed. The difference between LCIS

Figure 9: Active PTK6 is localized at membrane in high grade ductal carcinomas
Immunohistochemistry and immunofluorescence were performed on breast tumor TMAs using the same condition as normal breast TMAs. Total PTK6 (DAB brown) and P-Y342 PTK6 (FITC green) were shown in pairs circled by solid-lined box, each box represent a different patient and two patients were shown for each tumor subtype. The only exception is the DCIS-HG, pictures were taken from two different cores of the same patient due to the lack of DCIS-HG samples. Nuclear staining of PTK6 are indicated by arrows. Size bar = 50 μm. IDC: invasive ductal carcinoma. DCIS: ductal carcinoma in situ. LG: low grade. HG: High grade.

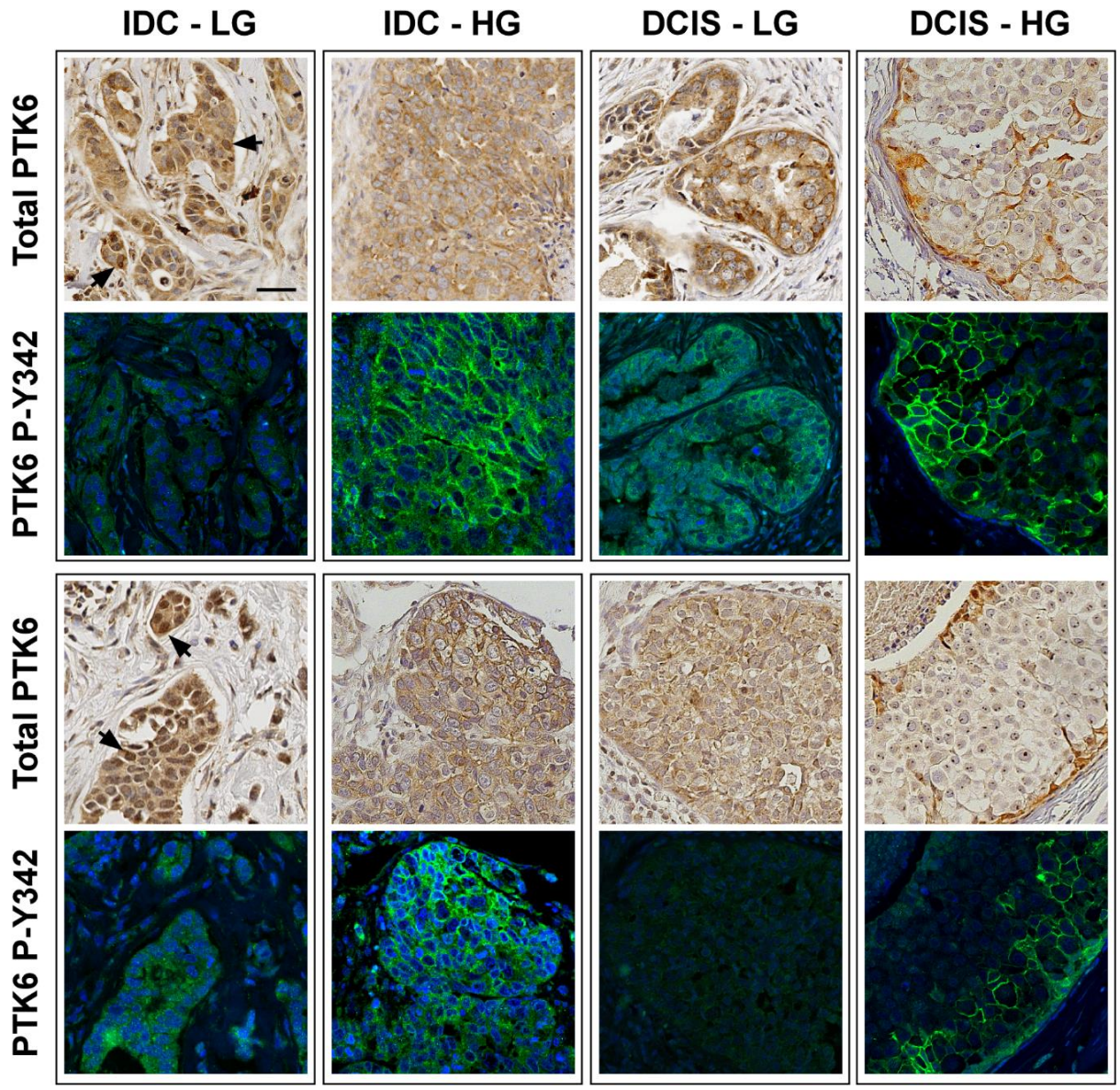
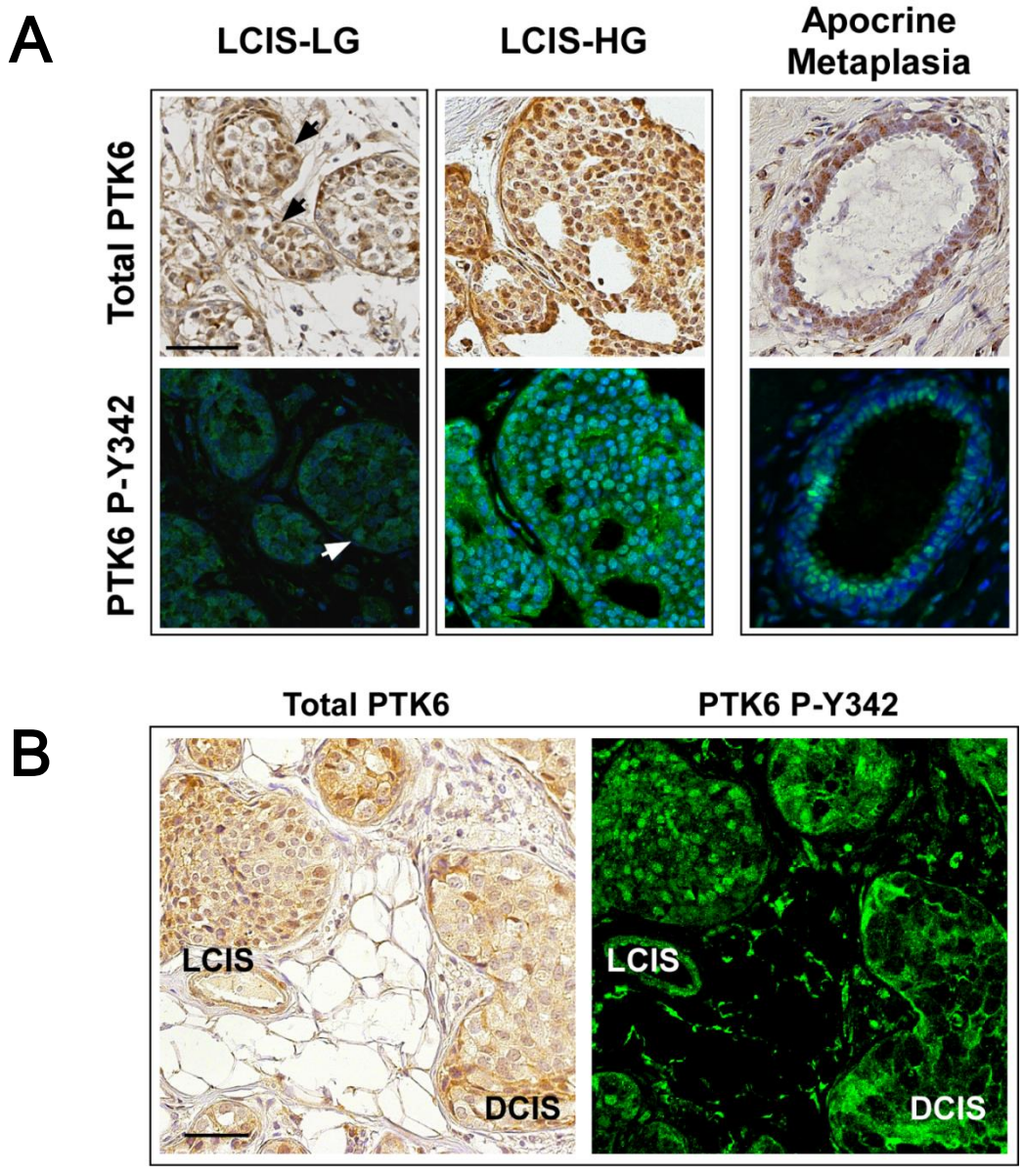


Figure 10. Active PTK6 is localized in nucleus in lobular carcinomas and apocrine metaplasia PTK6 expression was detected in lobular carcinoma in situ. **(A). Left:** Nuclear PTK6 was found in the lobular carcinoma in situ of both low and high grade. Weak nuclear signals were pointed out with arrows. **Right:** Picture shows a mammary gland with apocrine metaplasia with typical "snout" structure in the lumen. Both total and active PTK6 localized in the nucleus of the luminal cells. **(B).** A tumor sample containing both LCIS and DCIS is stained by total PTK6 (Left) and PTK6 PY-342 (Right, FITC only) and exhibited the different PTK6 subcellular localization: nuclear in LCIS and membranous in DCIS. (Size bar = 50 μm)



and DCIS was shown by a representative core containing both LCIS and DCIS (Figure 10B): active PTK6 is nuclear in LCIS and at the membrane in DCIS.

Previous studies have shown that targeting PTK6 to the membrane activates PTK6 and enhances its oncogenic functions in vitro (Ie Kim et al. 2009, Zheng et al. 2013). Results from our TMA study supported these findings in a physiologically relevant way: PTK6 is more active in high grade tumors, and often localized at membrane. In addition, results obtained from tumors are mirrored by results from a breast cancer cell line study (Figure 11): Breast cancer cell lines MDAMB-453 and T-47D, as well as breast epithelial cell line MCF-10A were fractionated into cytoplasmic (C), membrane/organelle (M) and nuclear (N) compartments. Immunoblotting was performed with PTK6 C-18 antibody to study the subcellular localization of the endogenous PTK6. In the highly tumorigenic MDA-MB-453 and T-47D cells, total PTK6 was mostly localized in the cytoplasm but a decent amount of PTK6 was also detected in the membrane/organelle fraction, while in the non-tumorigenic MCF-10A cells, PTK6 was only detected in the cytoplasm, supporting the hypothesis that membrane (active) PTK6 may be involved in breast tumorigenesis.

3.1.6 PTK6 is only activated in the malignant tissue but not in the normal mammary glands from the same patient

A group of three cores was harvested from each patient, and some groups contain both malignant and benign tissues. When analyzing PTK6 expression from the same patient, we found that the active PTK6 is only detectable in the tumor tissue but not in the normal adjacent gland, although the total PTK6 level may not change much from normal to tumor tissue (Figure 12). This finding suggests that the activation of PTK6 might be more indicative than total PTK6 expression in breast tumor prognosis, and worth more exploration to evaluate it role as a diagnostic marker.

Figure 11. Increased membrane localization of PTK6 in breast cancer cell lines than in non-transformed cell line

(A). Breast cancer cell lines MDA-MB-453, T-47D and breast epithelial cell line MCF-10A were fractionated into cytoplasmic (C), membrane/organelle (M) and nuclear (N) compartments. PTK6 C-18 antibody was used to detect endogenous PTK6. Receptor kinase EGFR and ERBB2 were used as membrane markers, AKT as cytoplasmic marker, and SP1 as nuclear marker. In breast tumor cell lines MDA-MB-453 and T-47D, total PTK6 is mostly localized in the cytoplasm but a decent amount of PTK6 is also detected in the membrane/organelle fraction, while in the non-transformed MCF-10A cells PTK6 was only detected in the cytoplasm. **(B).** Immunofluorescence assay showed endogenous PTK6 (FITC green) is cytoplasmic in MCF-10A cells.

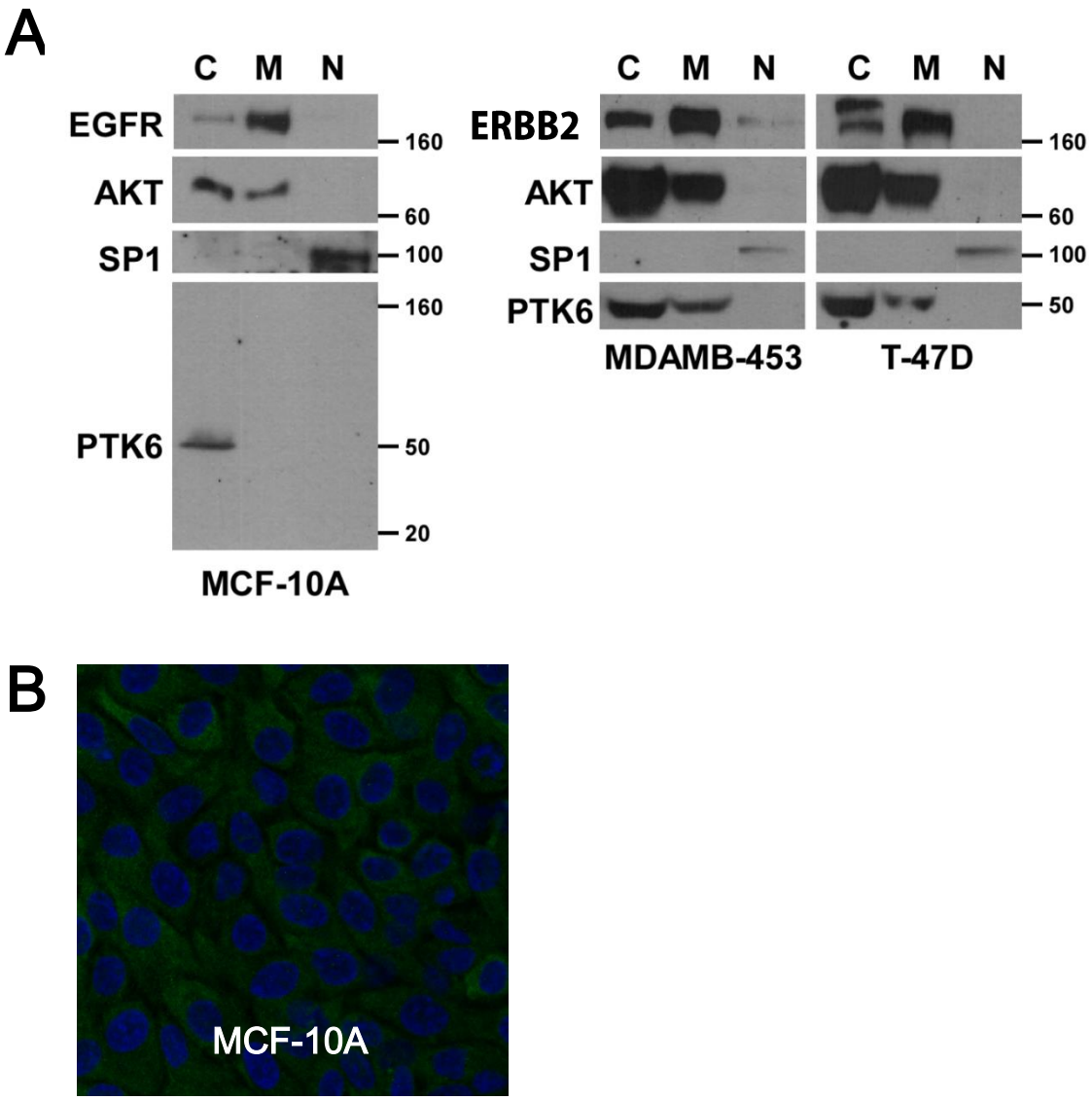
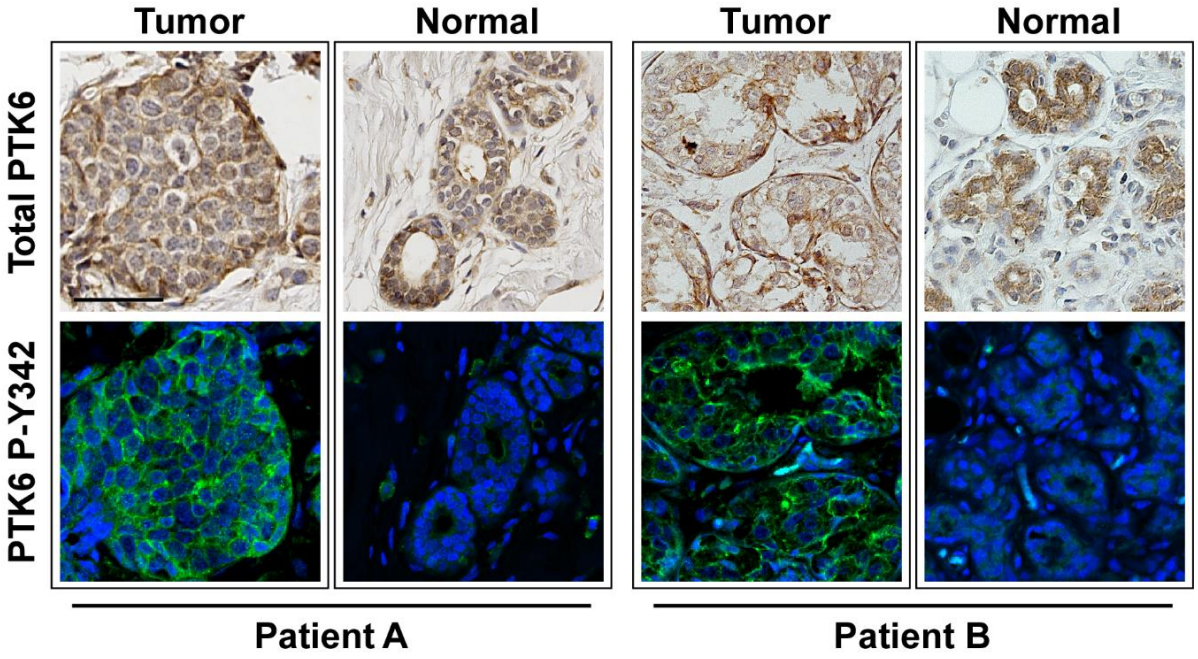


Figure 12. PTK6 is activated in the malignant tissue but not in the normal mammary glands from the same patient

A group of three cores were isolated from each patient, and some groups contain both malignant and benign tissues. Total and active PTK6 of corresponding areas are shown in pairs circled by solid-lined boxes. Active PTK6 is detected in the tumor areas but not in the normal mammary gland areas from the same patient. Two different patients (A & B) were shown here. Size bar =50 μm.



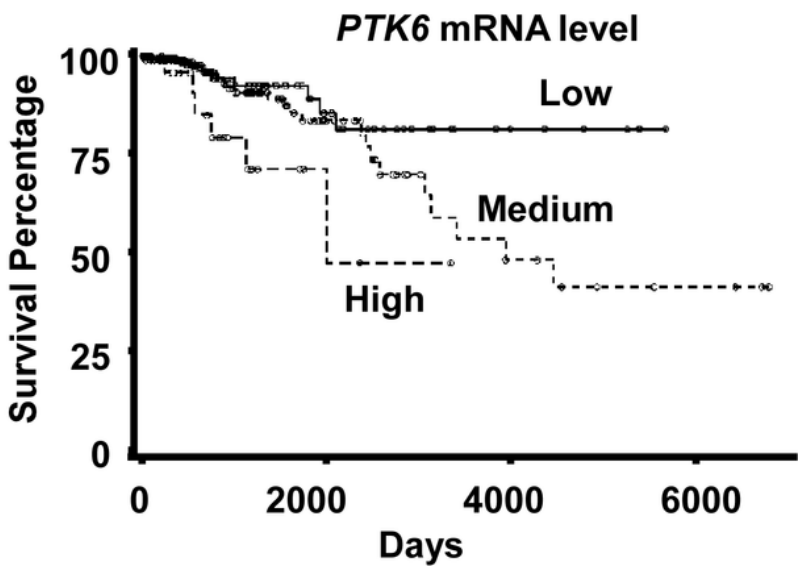
3.1.7 PTK6 mRNA levels correlate with breast cancer survival rate

The relationship of breast tumor survival and PTK6 expression was studied with data obtained from the Oncomine database (Figure 13). Original experiments were performed by TCGA group (Cancer Genome Atlas, 2012). *PTK6* mRNA levels were divided into the following categories: "high" >8.5, "medium" 2.3-8.5, "low"<2.3. The survival curve was estimated using the Kaplan-Meier method plotting survival probability (%) against time (days), and it showed that high *PTK6* mRNA correlates with poor prognosis, and low *PTK6* mRNA group has best survival rates. The differences among three groups were tested using the log-rank test and the p-value (0.0107 by Log-Rank, 0.0241 by Wilcoxon) showed the correlation of *PTK6* mRNA level and survival rate was statistically significant. Similar result was reported very recently (Anderson et al. 2013). Compared to Anderson's analysis, we analyzed a larger sample size (447 vs 117) and had a longer tumor-free follow-up period (20 years vs 12 years). We also divided PTK6 into 3 categories (high, medium, low) instead of two (high and low). However, we do not have the metastasis information. Levels of *PTK6* transcripts may serve as a prognostic marker for breast cancer when the status of PTK6 protein activation is unavailable.

Figure 13. High *PTK6* mRNA level is correlated with poor survival rate

(A). The relationship of breast tumor survival rate and *PTK6* transcripts was studied with data obtained from Oncomine database (The Cancer Genome Atlas-Invasive Breast Carcinoma Gene Expression Data). *PTK6* mRNA levels were arbitrarily categorized into following categories: "high" >8.5, "medium" 2.3-8.5, "low"<2.3. The survival curve was estimated using the Kaplan-Meier method plotting survival probability (%) against time (days) and it showed that high *PTK6* mRNA correlates with poor prognosis, and low *PTK6* group has best survival rates. The analysis was performed using SAS 9.2.2010. The differences among three groups were tested using the log-rank test and the p-value showed the correlation of PTK6 mRNA level and survival rate was statistically significant. Sample size =447. The detailed numbers of each group are shown in **(B)**

A



B

Category	PTK6 Value	Total Number	Deceased	Alive
Low	< 2.3	140	8	132
Medium	2.3 - 8.5	274	24	250
High	> 8.5	33	6	27
Total		447	38	409

3.2 PTK6 regulates breast tumorigenesis in mouse models

3.2.1 Background

PTK6 is expressed throughout the alimentary canal and in skin in differentiated epithelial cells (Vasioukhin et al. 1995), and has been shown to promote differentiation of small intestinal enterocytes (Haegebarth et al. 2006) and keratinocytes (Vasioukhin et al. 1997, Wang et al. 2005). Interestingly, while PTK6 expression and functions in normal epithelia suggested it might have tumor suppressor roles, disruption of the mouse *Ptk6* gene conferred resistance to carcinogens and impaired activation of the STAT3 transcription factor in the mouse colon, suggesting PTK6 may play oncogenic roles in certain context. STAT3, a transcription factor that plays essential roles in the development of a variety of tumor types, is a substrate of PTK6 and its activation is promoted by tyrosine phosphorylation (Liu et al. 2006, Gao et al. 2012).

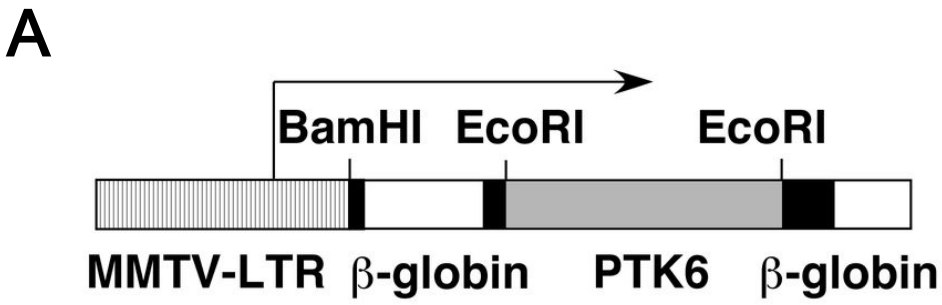
PTK6 is not expressed in mouse mammary gland at any developmental stage (Llor et al. 1999), and disruption of *Ptk6* gene does not affect the functions of mammary gland, as the *Ptk6* $^{-/-}$ mice lactate and undergo involution normally. However, PTK6 is overexpressed in the majority of breast tumors and regulates pro-survival and pro-proliferation signaling pathways. The mechanism of this up-regulation of PTK6 expression is not fully understood. Recent study showed that in some breast tumors PTK6 expression is induced by hypoxia (Anderson et al. 2013), suggesting that PTK6 may be a mediator of hypoxia-induced breast cancer progression. In our study, overexpression of PTK6 in human breast tumors led us to hypothesize that ectopic expression of human PTK6 might promote mammary gland tumorigenesis in mice. To explore contributions of PTK6 to development of breast cancer in vivo, we generated multiple lines of transgenic mice containing the human *PTK6* gene expressed under control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR).

3.2.2 Production and characterization of MMTV-PTK6 transgenic mice

To generate MMTV-PTK6 transgenic animals, PTK6 coding sequences were cloned into an expression vector containing the MMTV-LTR promoter (Matsui et al. 1990) (Figure 14 A). The MMTV promoter has been extensively used to target transgene expression to the mammary gland *in vivo* (Hennighausen 2000). We determined that the MMTV-PTK6 construct, which is inducible by dexamethasone in tissue culture cell lines, could be expressed in mouse normal murine mammary gland (NMuMG) cells at levels comparable to that observed for PTK6 in human breast tumor cell lines (Figure 14 B, NMuMG + Tg).

Vector sequences were removed from the MMTV-PTK6 expression cassette prior to its microinjection into fertilized FVB/NJ eggs (by Dr. Robert Franks from Research Resource Center, UIC). Several transgenic mice were born and human *PTK6* integration was identified by southern blot (Figure 15 A). Those mice tested positive for human *PTK6* were selected as founders and set up for breeding. Offspring of these founders were sacrificed and lysates from mature female mammary glands were subject to immunoblotting probed by human PTK6 antibody (Figure 15 B). Lysates of salivary glands and male testes were also examined because MMTV-LTR is known to promoter expression in salivary gland and seminal vesicle, skin, and various cells of the immune system (Sinn et al. 1987). Three lines (B28, B33 and B35) with mammary gland MMTV activation were chosen for the study. The B28, B33, and B35 lines express low, medium and high levels of human PTK6 mRNA (Figure 16 A) and protein (Figure 16 B), respectively. The MMTV LTR drives transgene expression in the mammary gland of virgin adult, pregnant, and postpartum mice (Bouchard et al. 1989, Sinn et al. 1987, Pierce et al. 1993), and expression occurs in ductal and alveolar cells of the mammary gland (Hennighausen 2000). Using immunohistochemistry (Figure 16 C), we detected transgene expression in mammary glands from both virgin and multiparous

Figure 14. Generating PTK6 construct with mammary gland specific expression
(A). A schematic diagram of the MMTV-PTK6 construct is shown. A 2.2 kb human *PTK6* cDNA (grey region) was inserted into the third exon of the rabbit β-globin gene under the control of the MMTV LTR (striped region). **(B).** Expression of the MMTV-PTK6 construct was transfected into NMuMG cells stimulated with dexamethasone. Transgenic PTK6 protein levels (NMuMG +Tg) are comparable to that produced in human breast cancer cell lines MCF7, MDA-MB-231 and MDA-MB 453. PTK6 was not detected in the MDA-MB-435 cell line. Expression of β-actin was examined as a loading control.



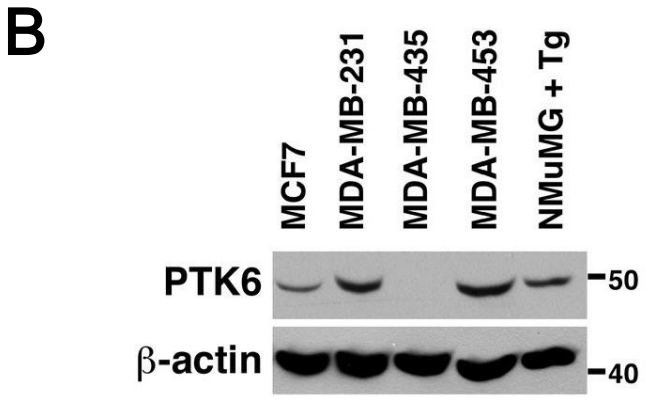


Figure 15. Selecting transgenic founders

(A). *PTK6* integration was detected by southern blot with a probe targeting human specific *PTK6* sequence. 8 out of 35 founders tested positive for *PTK6* integration and were put into breeding. **(B).** Offspring of these founders was analyzed for PTK6 protein expression. Lysates were prepared from mammary gland and salivary gland of female pups, and testes and salivary gland of male pups. Three lines with mammary gland MMTV activation were chosen for further study. (B33, B35, and B28, which is not shown in this figure)

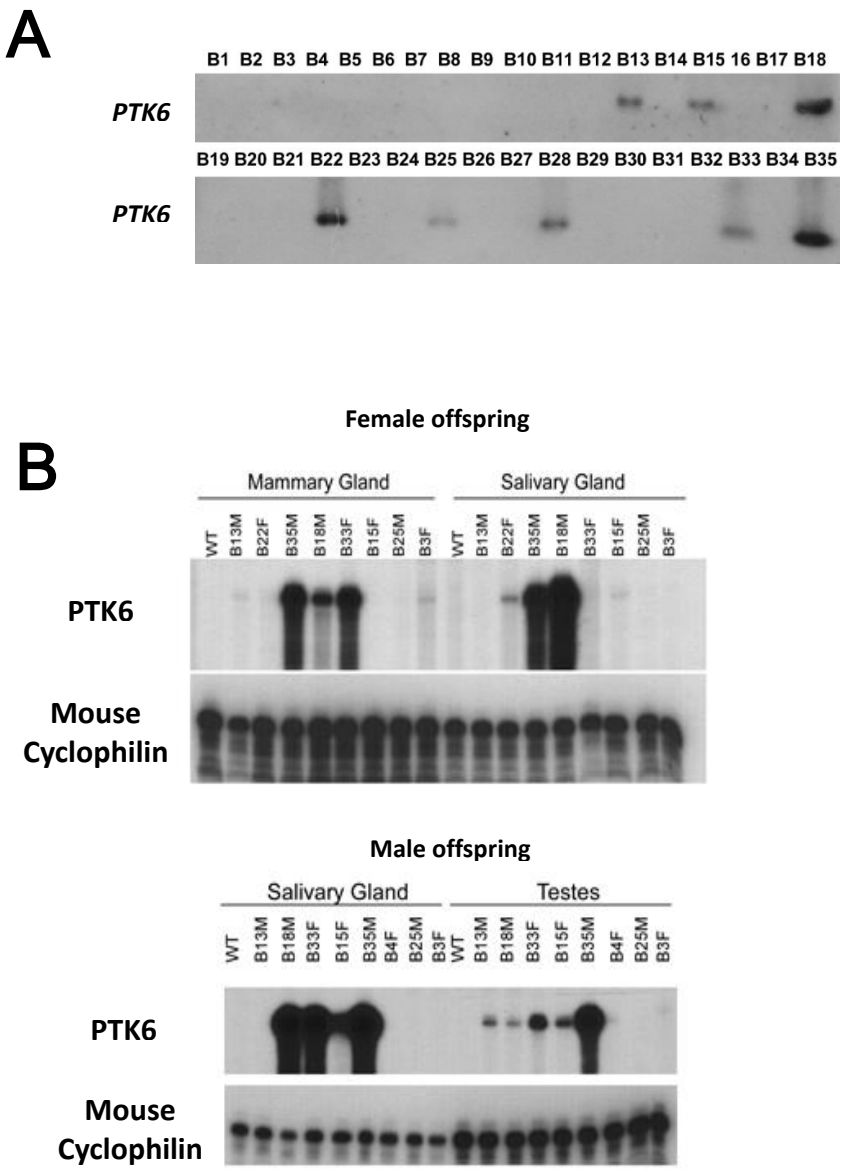
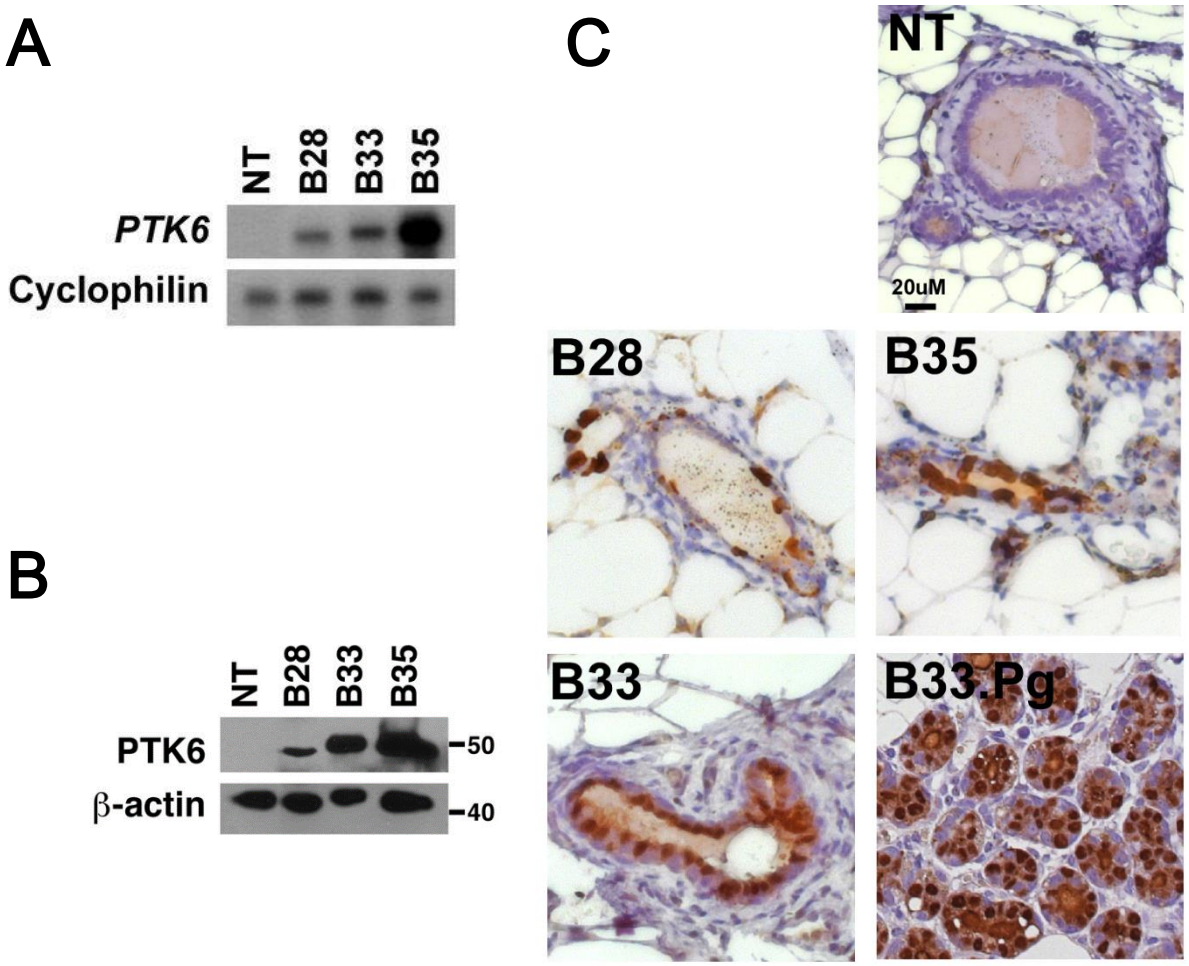


Figure 16. Ectopic PTK6 expression is detected in mammary glands

(A). Ribonuclease protection assays were performed with RNAs prepared from mammary glands of three transgenic lines (B28, B33 and B35) and nontransgenic control mice (NT). *PTK6* mRNA was detectable in the three transgenic lines but not in NT animals. Mouse cyclophilin was used as loading control. **(B).** Ectopic PTK6 expression was detected in transgenic mammary glands by immunoblot. Levels of ectopic PTK6 protein expression correlated with the levels of *PTK6* mRNA shown in A. **(C).** Immunohistochemistry demonstrates expression of ectopic human PTK6 in the transgenic mammary gland epithelial cells, as shown in the virgin animals (B28, B33, B35) and pregnant animals (B33.Pg). The nontransgenic mammary gland stained negative for PTK6. Size bar = 20 μm.



female mice. Ectopic human PTK6 was detected in nuclei and cytoplasm of mammary gland epithelial cells in all three established transgenic lines, resembling the expression pattern of PTK6 in normal human mammary glands.

3.2.3 PTK6 promotes tumorigenesis in the mouse mammary gland

Three independent mouse PTK6 transgenic lines were maintained and monitored for spontaneous tumorigenesis over a 2.5 year period. MMTV-PTK6 transgenic mice developed more than twice as many tumors as nontransgenic littermate controls, with an average latency of 21 months. However, tumors that formed in the PTK6 transgenic and nontransgenic control mice were similar in size and histology. Data are summarized in Table 4 and Figure 17. Hyperplastic alveolar nodules (HAN) were observed in PTK6 transgenic animals as early as 70 weeks of age, and were frequently detected in aging animals (Figure 18, top panels). Transgenic mice developed multiple mammary gland tumors. An example of a mouse from the B33 line with tumors in its right inguinal and left thoracic mammary is shown at 105 weeks of age (Figure 18, upper right corner). Ectopic PTK6 expression was detected in nulliparous and multiparous mammary glands and tumors using a human PTK6 specific antibody (Figure 18, lower panels)

3.2.4 Active ectopic PTK6 promotes STAT3 activation in normal mammary gland and mammary gland tumors.

Activation of PTK6 can be monitored using an antibody specific for phosphorylation of tyrosine residue 342 located in its catalytic domain. We expressed wild type human PTK6, which may or may not be active, and has both kinase-dependent and -independent functions. Using immunofluorescence, we examined PTK6 activation in mammary glands of transgenic mice and nontransgenic controls. Active PTK6 (P-Y342) can be detected by twelve weeks of age, with levels increasing and becoming more membrane associated at 40 weeks of age (Figure. 19, top panels).

Figure 17. PTK6 transgenic animals display increased susceptibility to mammary gland tumor development.

A non-transgenic FVB/NJ control (NT) and three independently developed MMTV-PTK6 transgenic mouse lines (B28, B33, B35) were maintained for 2.5 years and mammary gland tumorigenesis was monitored. Transgenic mice developed multiple breast tumors during this period, and non-transgenic animals also developed spontaneous breast tumors. The breast tumor occurrence was shown as the percentage of animals that developed breast tumors relative to the total animal number. The Cochran-Armitage Trend test validated the hypothesis that increased numbers of MMTV-PTK6 transgenic animals develop breast tumors than non-transgenic mice (p=0.042).

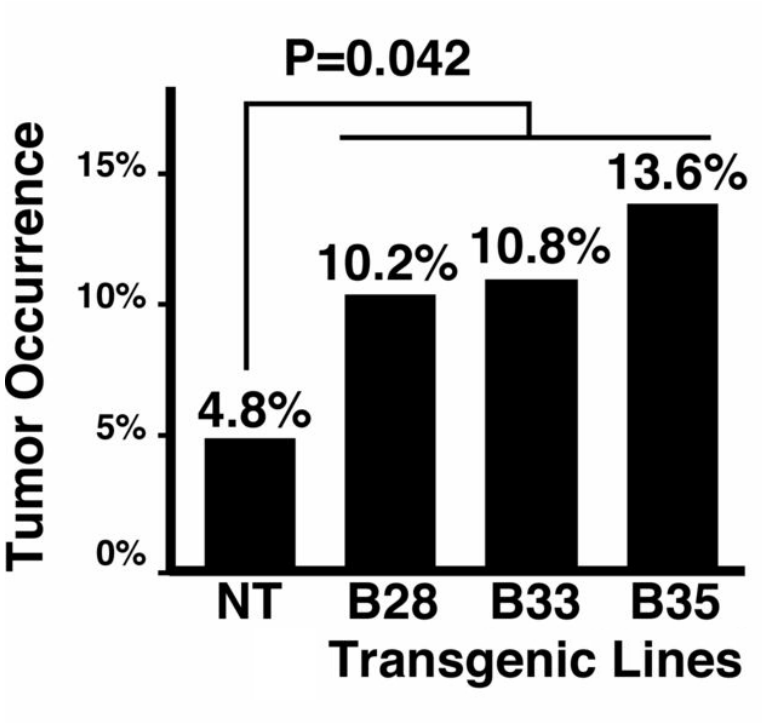


Figure 18. MMTV-PTK6 transgenic animals develop neoplastic hyperplasia and breast tumors.

Transgenic animals developed mammary gland lesions and breast tumors at older age. **Top panels:** Whole mount staining was performed on the thoracic mammary glands of transgenic animals. Age and parous status were noted in the figure. Hyperplastic alveolar nodules (HAN) (black arrow) were observed in animals as early as 70 weeks of age, and were frequently detected in the aging animals. Multiple mammary gland tumors (red arrow) were found in the animal of 105 weeks. **Bottom panels:** Immunohistochemistry assay performed on the corresponding area confirmed PTK6 expression in the hyperplastic epithelial cells.

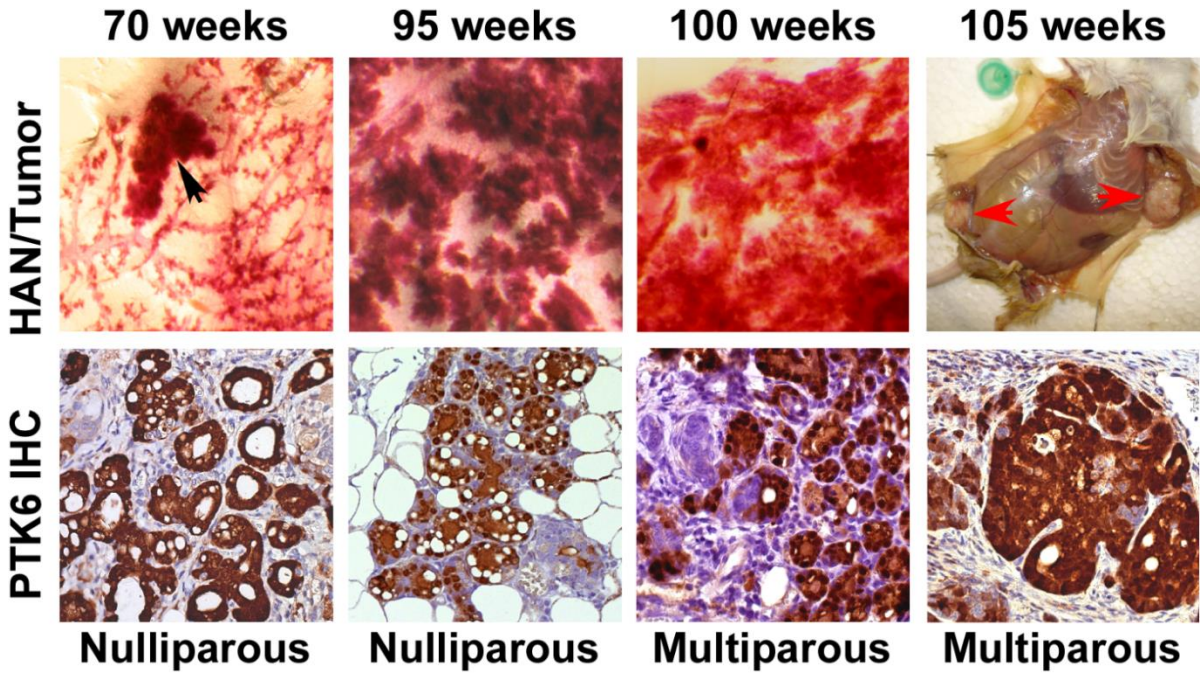


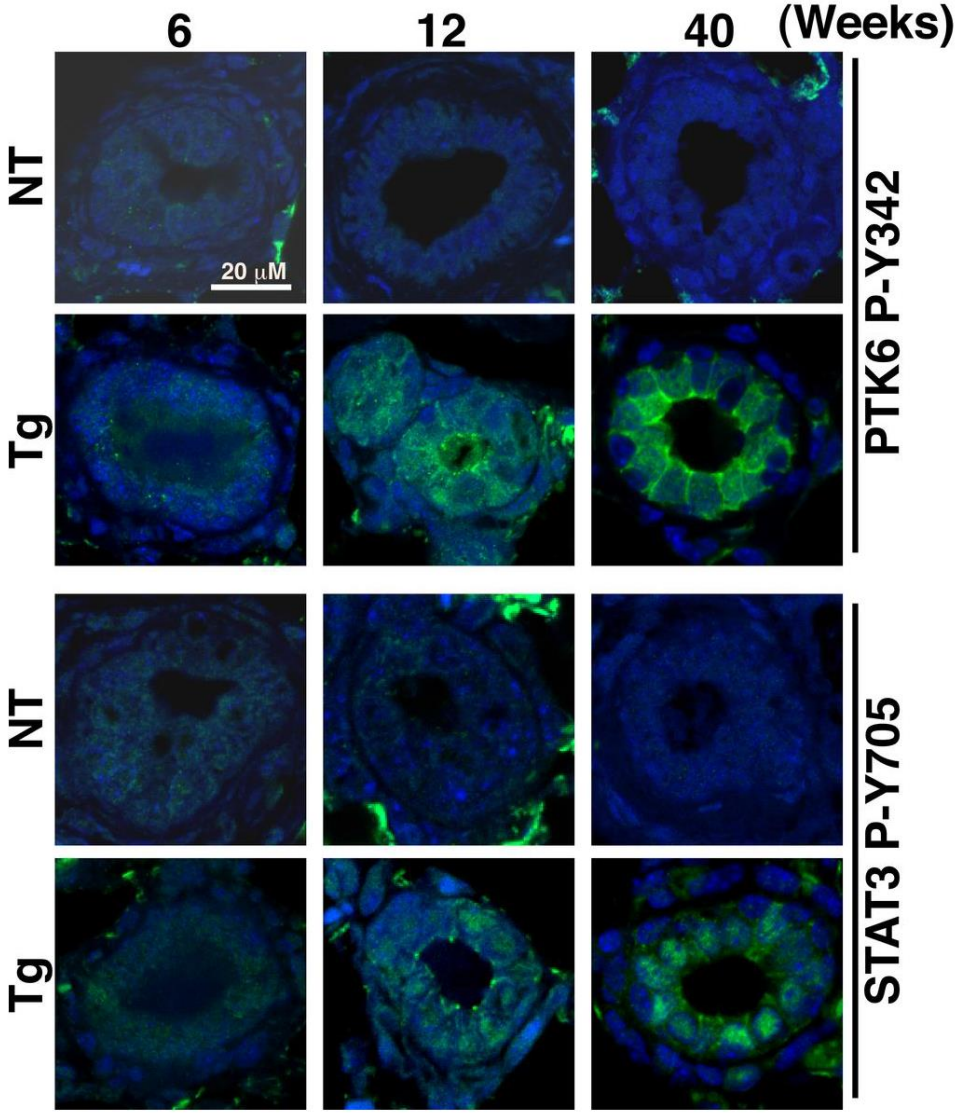
TABLE IV

TUMOR OCCURRENC7E AND LATENCY IN PTK6 TRANSGENIC AND NON-TRANSGENIC (NT) ANIMALS

Transgenic Line	Number of Tumors	Number of Animals	Tumor Occurrence (%)	Avg. Tumor Weight (g)	Avg. Latency (weeks)
NT Controls	5	104	4.81	3.7 ± 1.1	93
PTK6 B28	5	49	10.2	4.9 ± 2.6	88
PTK6 B33	7	65	10.77	2.6 ± 2.0	92
PTK6 B35	3	22	13.64	3.6 ± 1.2	88

Figure 19. Ectopic PTK6 is active and promotes STAT3 phosphorylation at tyrosine residue 705 in the mouse mammary gland.

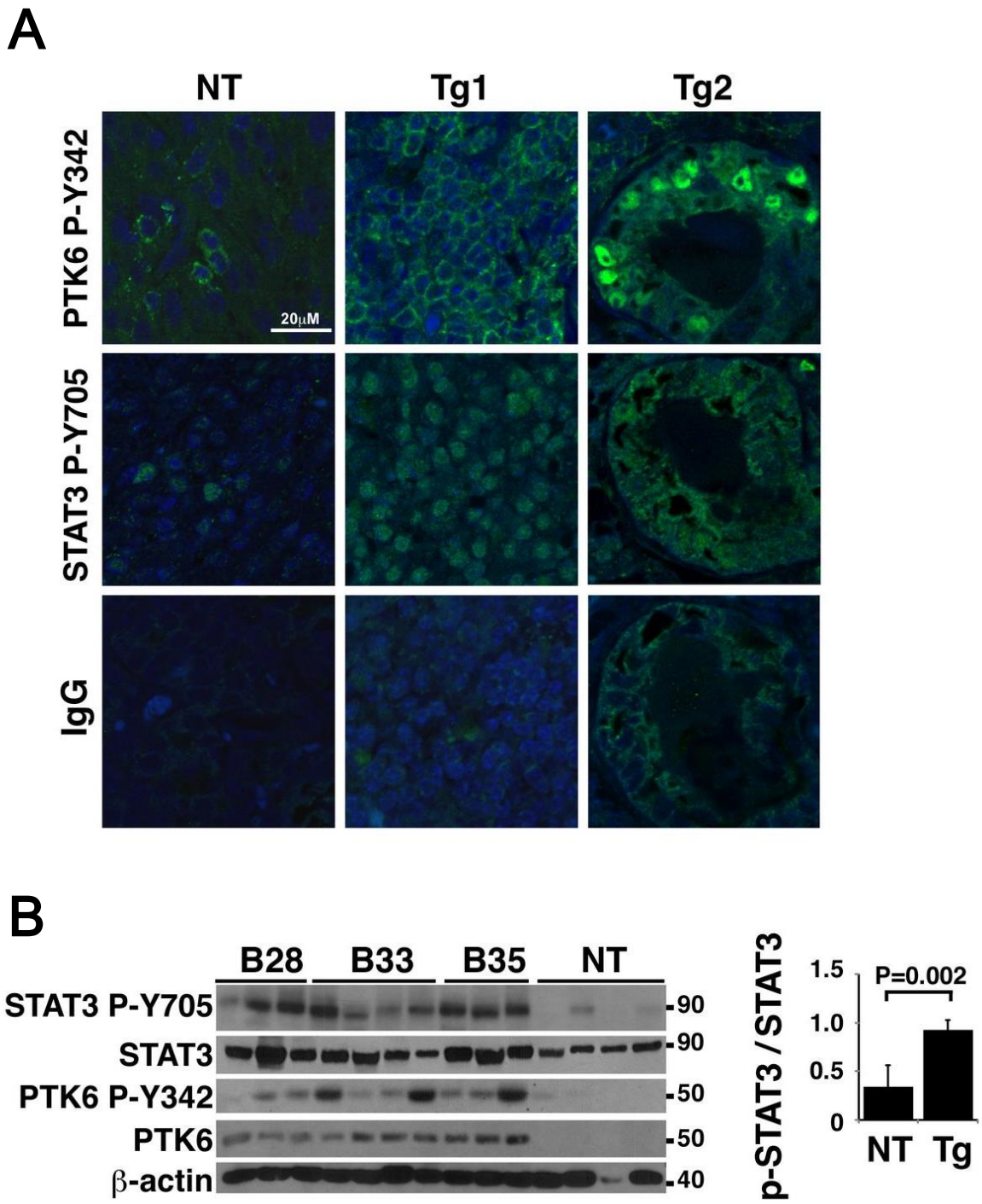
Immunofluorescence assays were performed on mammary gland serial sections from age-matched nontransgenic (NT) or transgenic (Tg) animals using antibodies specific for active PTK6 (P-Y342) and active STAT3 (P-Y705). PTK6 was not expressed in prepubescent transgenic animals up to 6 weeks of age, and STAT3 phosphorylation was minimal. Upon maturity, PTK6 was expressed and activated in Tg mammary glands, and STAT3 phosphorylation and translocation to the nucleus was observed (12 weeks of age). At 40 weeks of age, ectopic PTK6 remained active and STAT3 displayed activating phosphorylation and nuclear localization in Tg animals, but this phosphorylation was not detected in NT mammary glands. Primary antibody binding was detected with FITC (green) and sections were counterstained with DAPI (blue). The size bar represents 20 μm.



The STAT3 transcription factor is a substrate of PTK6 (Liu, L. et al. 2006). In addition to playing distinct roles in mammary gland development and involution, STAT3 promotes expression of genes that regulate cell proliferation, survival, and tumor metastasis in the mammary gland (reviewed in Haricharan et al. 2013). Phosphorylation of STAT3 on tyrosine residue 705 (P-Y705) promotes its dimerization and activation, and was impaired in the normal mouse colon and human colon cancer cells following PTK6 knockout and knockdown, respectively (Gierut, J. J. et al. 2012). We found that levels of active P-Y705 STAT3 correlated with expression of the PTK6 transgene (Figure 19, bottom panels). Nuclear localization of active STAT3 was prominent in non-involuting transgenic mammary glands at 40 weeks of age, but was not detected in nontransgenic controls.

Prominent activation of STAT3 was detected in tumors from PTK6 transgenic mice using both immunohistochemistry and immunoblotting (Figure 20). Localization of active PTK6 (P-Y342) at the plasma membrane correlated with increased activation and nuclear localization of STAT3 (P-Y705) (Figure 20, Tg1), while activation of PTK6 in the nucleus did not lead to significant activation and nuclear localization of STAT3 (Figure 20, Tg2). Total cell lysates were prepared from tumors that formed in the three independently derived transgenic strains B28, B33, B35, and nontransgenic (NT) mice. Immunoblotting was performed with antibodies specific for active STAT3 (P-Y705), total STAT3, active PTK6 (P-Y342), total human PTK6, and β-actin as a control. The antibody used to detect PTK6 expressed from the transgene is specific for the human protein and does not recognize mouse PTK6. Each lane represents a tumor that formed in an individual mouse of the indicated strain. A significant increase in STAT3 activation (P-Y705) was detected in individual tumors from PTK6 transgenic mice compared with tumors that developed in nontransgenic controls (Figure 20 B). The possible mechanism of PTK6 promoting breast tumor

Figure 20. Active PTK6 and STAT3 are expressed in mouse mammary gland tumors.
(A). Breast tumors that developed in nontransgenic (NT) and PTK6 transgenic lines (Tg) were analyzed for expression of active PTK6 and active STAT3 using immunofluorescence. Morphologically similar areas of NT and Tg tumors are shown. P-PTK6 and P-STAT3 signals were low and sporadic in NT tumors. Membrane associated active PTK6 (P-Y342) correlated with active nuclear STAT3 (P-Y705) in tumors from PTK6 transgenic mice (Tg1). Active PTK6 could be found at the membrane (Tg1) or sometimes within the nucleus (Tg2). The tumor in Tg1 was an adenocarcinoma composed of small glandular structures with small lumens consistent with an acinar pattern, and the nuclear activated PTK6 appeared in the acinar cells. Background staining was monitored using IgG as a control. The size bar represents 20μm. **(B).** Immunoblotting was performed with total cell lysates prepared from tumors isolated from multiple B28, B33, and B35 animals, as well as tumors that developed in nontransgenic control mice. Each lane represents a unique tumor sample from an individual mouse. STAT3 activation was consistently observed in tumor samples from PTK6 transgenic mice. For quantitation, P-STAT3 levels were normalized to total STAT3 levels in nontransgenic and transgenic mice (right panel). Immunoblotting for PTK6 was performed using an antibody specific for the human protein expressed by the transgene.


A
NT
Tg1
Tg2
PTK6 P-Y342
20μM
STAT3 P-Y705
IgG
B
B28
B33
B35
NT
STAT3 P-Y705
STAT3
PTK6 P-Y342
PTK6
β-actin
90
90
50
50
40
p-STAT3 /STAT3
1.5
1.0
0.5
0
P=0.002
NT
Tg

formation is summarized in Figure 21: PTK6 can be activated by stimulation of extracellular signals like EGF and HGF. Once activated (Y342 phosphorylation), PTK6 can phosphorylate its substrates, most likely at the membrane. STAT3, being a substrate of PTK6, is constitutively phosphorylated and activated in PTK6 overexpressing mammary glands. Phosphorylation of STAT3 results in its dimerization and translocation to nucleus, in which STAT3 can perform its function as a transcriptional regulator. Activated STAT3 promotes pro-survival and pro-proliferation signals, and eventually leads to breast tumor formation in mammary glands.

3.2.5 Induction of PTK6 in mouse tumors of different origins

Immunoblotting and immunocytochemistry using anti-mouse PTK6 antibodies demonstrate PTK6 expression in tumors. We did not detect expression of endogenous PTK6 in the normal nontransgenic mouse mammary gland (Figure 22 A-D; NT MG). However, endogenous mouse PTK6 expression is induced in a variety of mouse mammary gland tumors, including spontaneous tumors that form in nontransgenic mice (Figure. 22 A, NT TU), and tumors from transgenic mice that express human PTK6 (Figure. 22 A, E), ERBB2 (Figure. 22 B, E), activated H-RAS (Figure 22 C, E) or polyoma Middle T (Figure 22 D, E) in the mammary gland. The antibody used to detect mouse PTK6 (PTK6 C-17, Santa Cruz Biotechnology) was generated from a carboxy-terminal peptide that is not conserved between human and mouse, and the anti-human and anti-mouse PTK6 antibodies used are species specific. Interestingly, diverse patterns of PTK6 intracellular localization were observed, although many tumors displayed nuclear endogenous PTK6 localization. These data indicate that induction of PTK6 in breast cancer is conserved between humans and mice.

Figure 21. Model of PTK6 regulating mammary gland tumorigenesis

PTK6 can be activated by stimulation of extracellular signals like EGF and HGF. Once activated (Y342 phosphorylation), PTK6 can phosphorylate its substrates, most likely at the membrane. STAT3, being a substrate of PTK6, is constitutively phosphorylated and activated in PTK6 overexpressing mammary glands. Phosphorylation of STAT3 results in its dimerization and translocation to nucleus, in which STAT3 can perform its function as a transcriptional regulator. Activated STAT3 promotes pro-survival and pro-proliferation signals, and eventually leads to breast tumor formation in mammary glands.

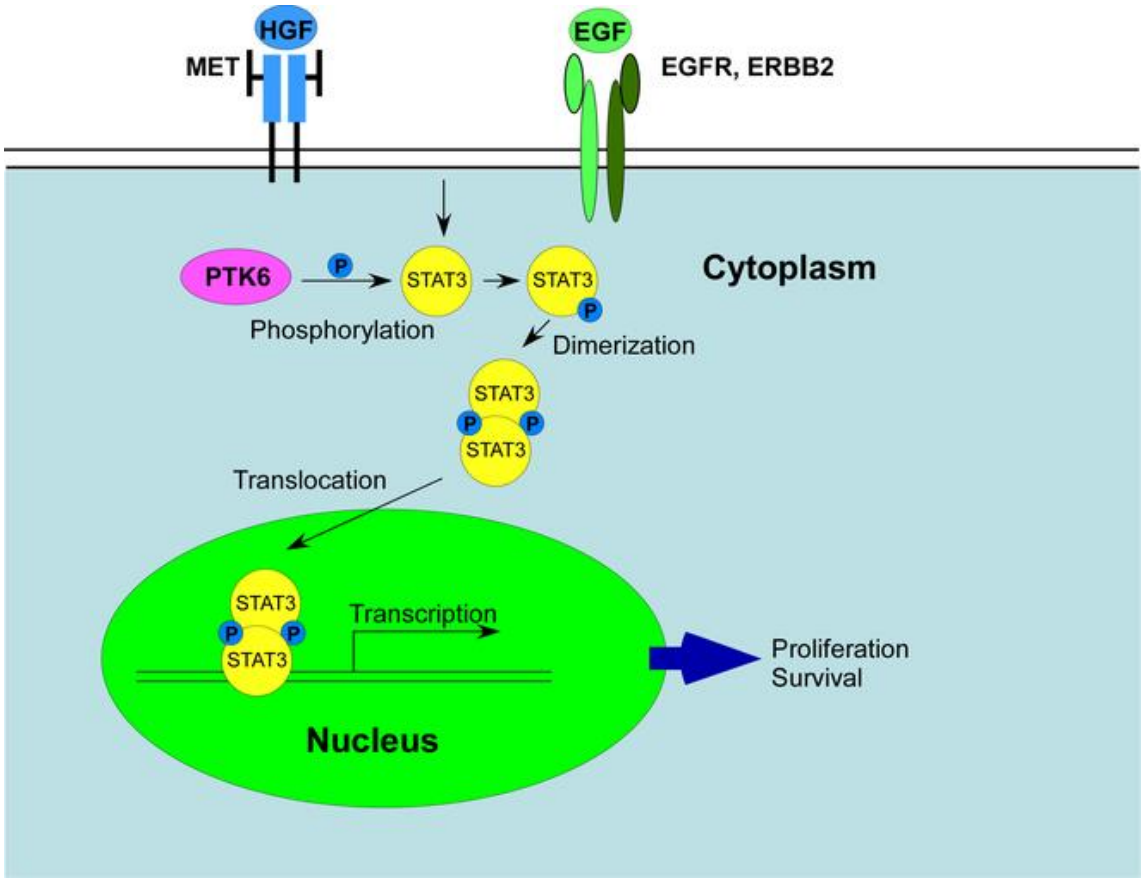
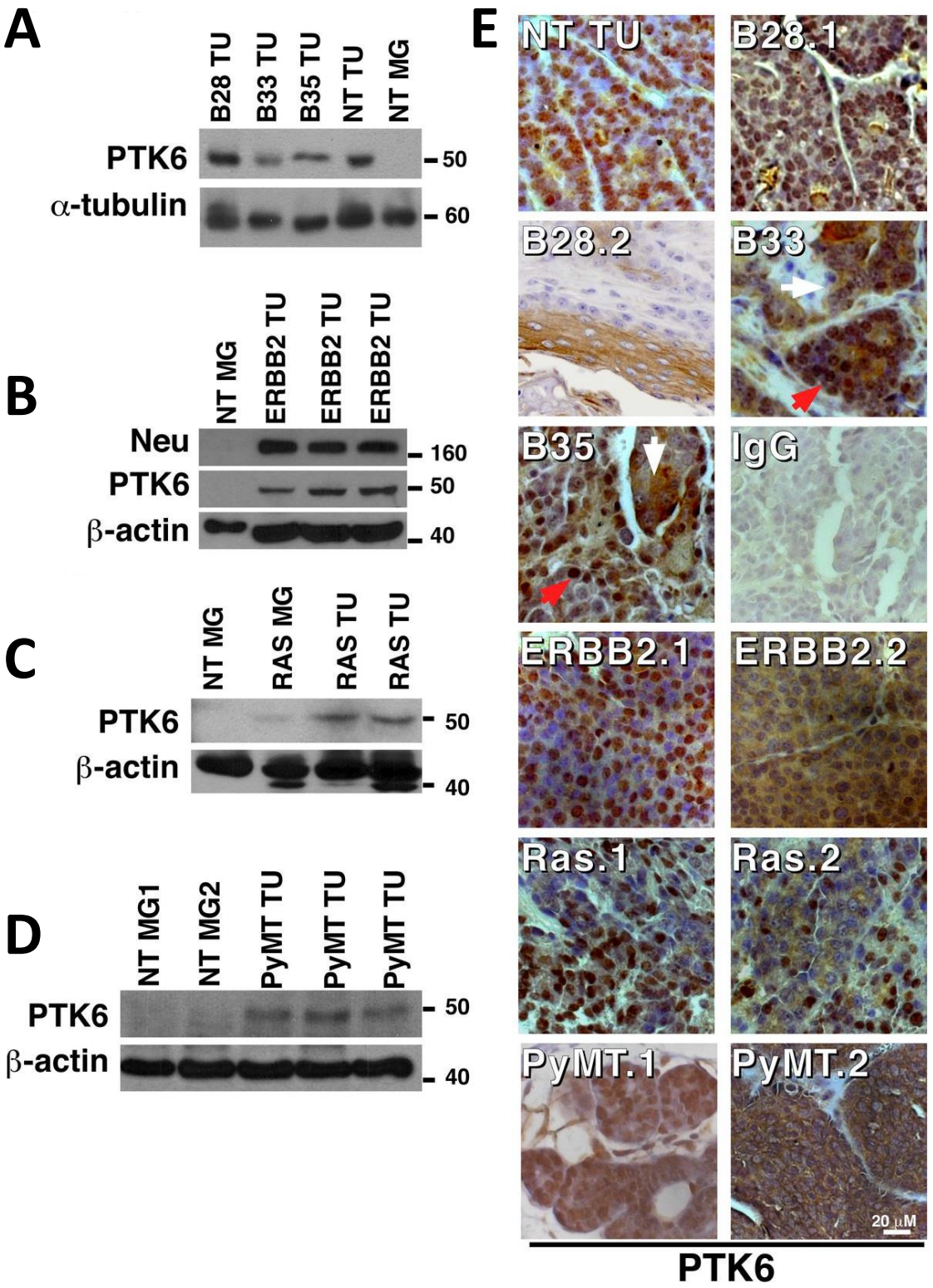


Figure 22. Endogenous mouse PTK6 is induced in mammary gland tumors of different origins.

(A-D). Mouse PTK6 protein expression was detected using immunoblotting and an antibody specific for mouse PTK6 that does not cross-react with the human PTK6 encoded by the transgene. Non-transgenic mammary gland was used as negative control. Expression of α-tubulin or β-actin was examined as loading controls. Induction of the endogenous PTK6 protein was detected in all mouse mammary gland tumors examined, including tumors that formed in nontransgenic control mice (NT TU), MMTV-PTK6 **(A)** MMTV-ERBB2 **(B)** MMTV-H-Ras **(C)** MMTV-PyMT **(D)** transgenic animals. **(E).** Immunohistochemistry was used to examine endogenous PTK6 expression in mouse tumors of different origins. PTK6 is predominately nuclear in more differentiated acinar cells (NT, PyMT1) and in areas of tumors from different transgenic strains (B28.1, ERBB2.1, Ras.1), but it can also be cytoplasmic as in ERBB2.2, Ras.2 and PyMT.2. Variation in PTK6 intracellular localization is sometimes observed in adjacent regions of the same tumors, as indicated by red (nuclear) and white (cytoplasmic/membrane) arrows in B33 and B35. Besides the neoplastic epithelial cells, endogenous PTK6 was also detected in the cytoplasm of the cuboidal shaped epithelial cells lining the ductules and keratin producing stratified squamous epithelial cells (B28.2). Sections were stained with normal rabbit IgG as a negative control (IgG).


A
B28 TU
B33 TU
B35 TU
NT TU
NT MG
PTK6
α-tubulin
50
60
B
NT MG
ERBB2 TU
ERBB2 TU
ERBB2 TU
Neu
PTK6
β-actin
160
50
40
C
NT MG
RAS MG
RAS TU
RAS TU
PTK6
β-actin
50
40
D
NT MG1
NT MG2
PyMT TU
PyMT TU
PyMT TU
PTK6
β-actin
50
40
E
NT TU
B28.1
B28.2
B33
B35
IgG
ERBB2.1
ERBB2.2
Ras.1
Ras.2
PyMT.1
PyMT.2
20 μM
PTK6

3.2.6 Enhanced proliferation is counteracted by increased apoptosis in ERBB2/PTK6 double transgenic mice

Several studies have indicated that PTK6 and ERBB2 are co-expressed in human breast tumors and PTK6 promotes ERBB2 oncogenic signaling in human breast tumor cell lines (Xiang et al. 2008, Ludyga et al. 2011, Ludyga et al. 2013, Ai et al. 2013,). Figure 23 shows that several breast cancer cell lines express PTK6 and ERBB2 simultaneously.

We hypothesize that introduction of MMTV-PTK6 would accelerate and/or augment ERBB2 induced mammary gland tumorigenesis in the mouse. We crossed the MMTV-PTK6 transgenic strains with the MMTV-ERBB2 line, which expresses the activated rat *ERBB2* (*c-neu*) gene and is prone to developing mammary gland tumors. In our colony, approximately 80% of MMTV-ERBB2 transgenic mice develop mammary gland tumors within 8 months just as reported (Muller et al.1988, Guy et al. 1996). Co-expression of activated ERBB2 and PTK6 did not significantly influence occurrence or size of tumors that developed. Unexpectedly, regression analysis suggested that PTK6 expression may delay tumor initiation and increase latency (Figure 24).

To examine the impact that co-expressing PTK6 with ERBB2 has on proliferation, we examined BrdU incorporation in tumors. Although tumor size was not increased (Figure 25 A), a significant increase in cell proliferation was detected in tumors that formed in the double ERBB2/PTK6 transgenic mice, compared with single ERBB2 transgenic animals (Figure 25 B). To determine if an increase in programmed cell death might offset the increase in cell proliferation observed, TUNEL assays were performed to detect apoptotic cells. Increased levels of TUNEL positive apoptotic cells were detected in ERBB2/PTK6 double transgenic mice (Figure 25 B). Increased apoptosis could counteract the observed increase in cell proliferation and explain the lack of increased tumor size in vivo.

Figure 23: PTK6 is often co-overexpressed with ERBB2 in breast tumor
Endogenous PTK6 and ERBB2 protein levels of breast cancer cells were examined, and several cell lines overexpress PTK6 and ERBB2 simultaneously (underlined): PTK6 is overexpressed in BT-474, SK-BR-3, T-47D and MDA-MB-453, while ERBB2 is overexpressed in BT-474, SK-BR-3 and MDA-MB-453.

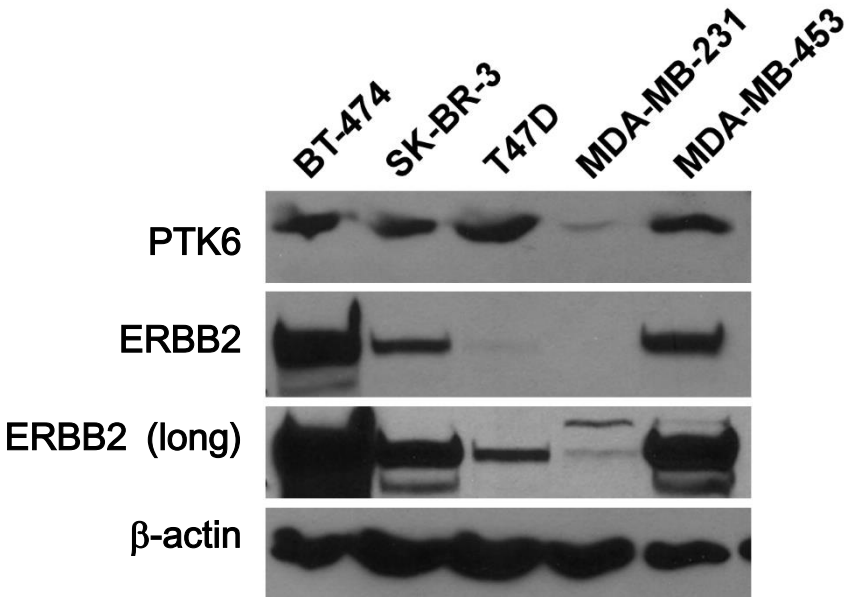


Figure 24. Transgenic expression of PTK6 in the MMTV-ERBB2 mouse model does not enhance ERBB2 driven tumorigenesis

Breast tumors from ERBB2 (n=28) and ERBB2/PTK6 mice (n=18) were harvested and analyzed at various time points; total tumor weight is plotted against age (days). Tumor occurrence between ERBB2 (B2) and ERBB2/PTK6 (B2/PTK6) animals did not show a statistically significant difference (p=0.70). However, regression analysis suggested a delay in tumor initiation in ERBB2/PTK6 animals (p<0.001).

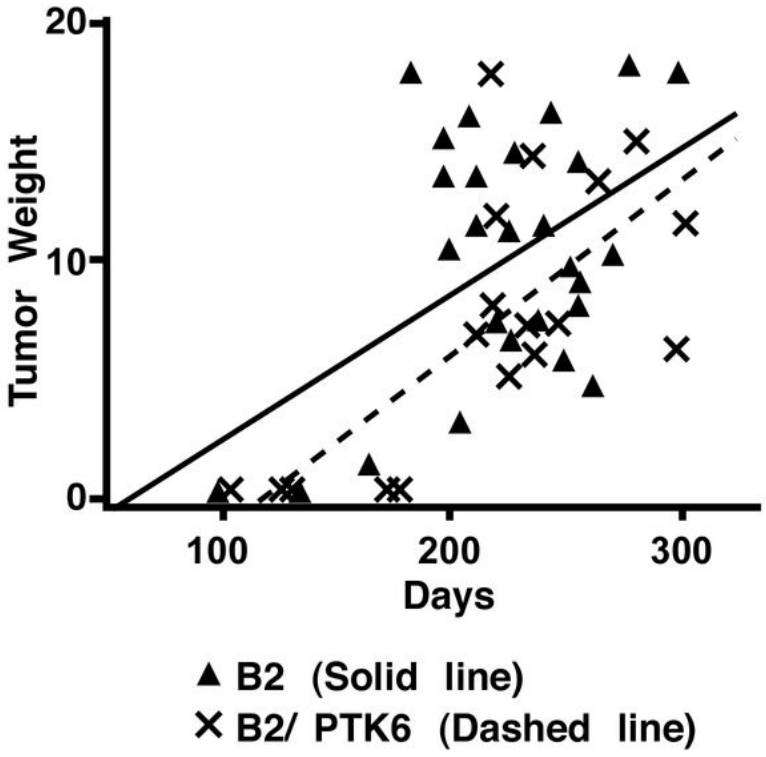
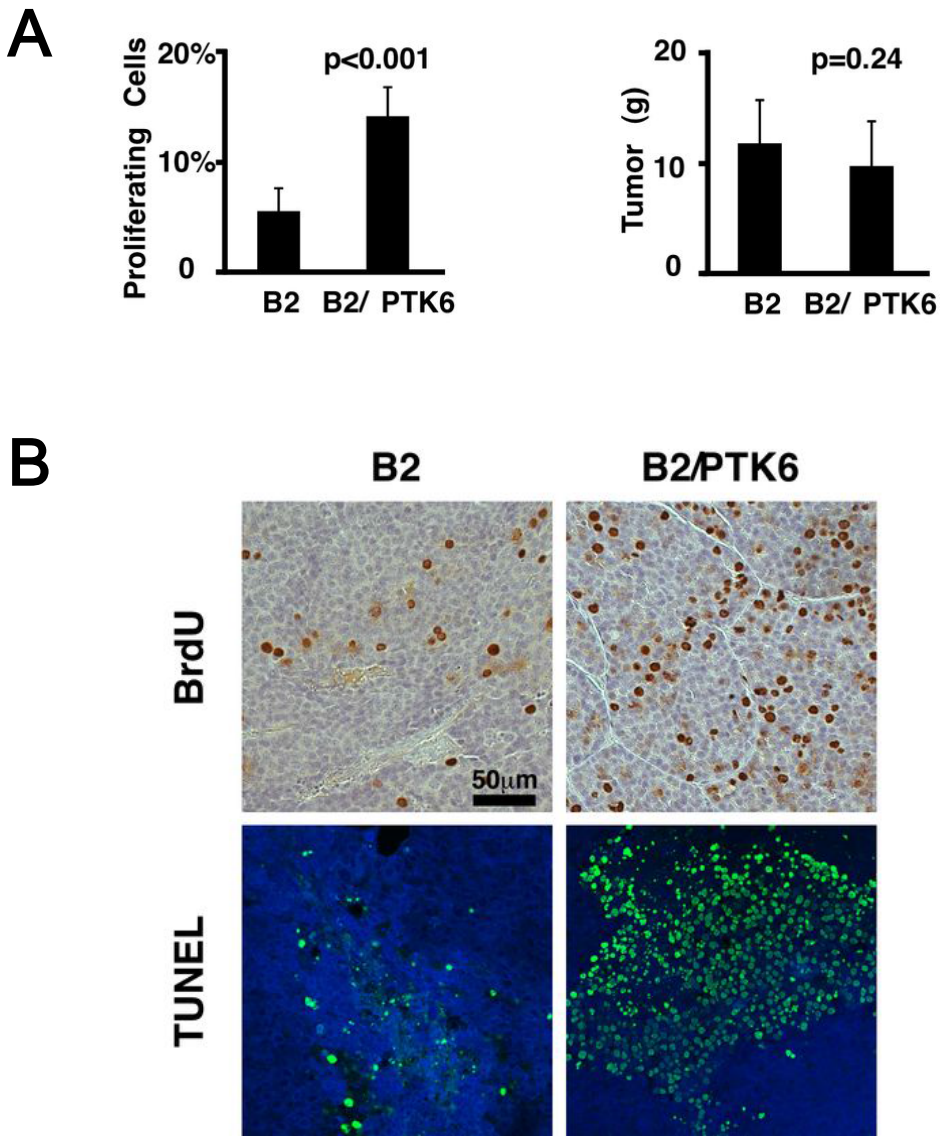


Figure 25. ERBB2/PTK6 transgenic animals exhibited higher levels of proliferation and increased apoptosis

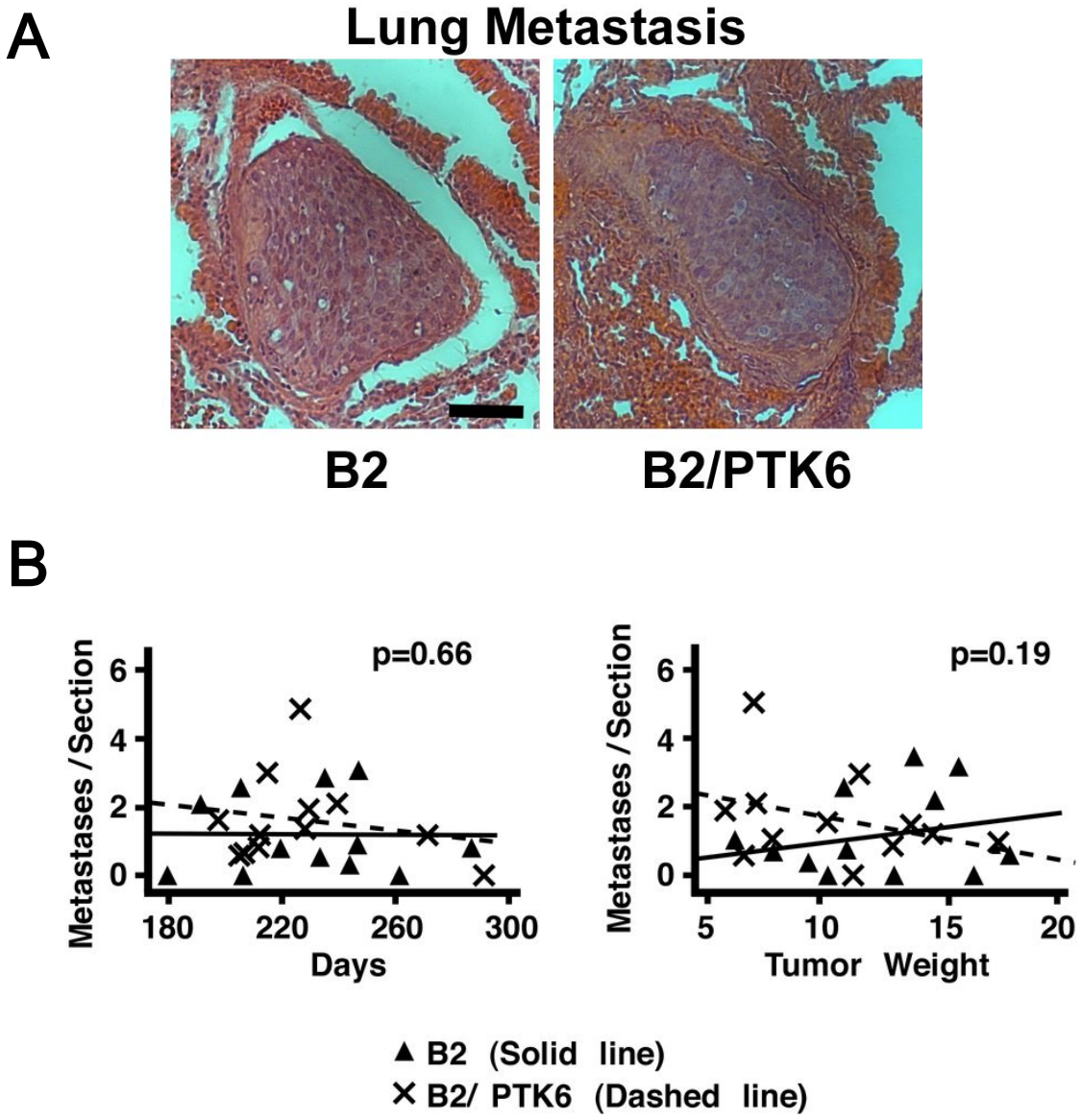
(A). Proliferation in tumors that developed in ERBB2 (n=10) and ERBB2/PTK6 (n=10) transgenic mice was examined using BrdU labeling. ERBB2/PTK6 tumors exhibited higher levels of proliferation than ERBB2 tumors, even in tumors of the same size. The number of BrdU labeled epithelial cells in ERBB2/PTK6 tumors is three-fold higher than in ERBB2 tumors ($p<0.001$), but the tumor size between these two groups is not significantly different ($p=0.24$) **(B).** Although ERBB2/PTK6 tumors display higher levels of proliferation (BrdU incorporation), they also exhibit increased apoptosis. Increased apoptosis is detected in tumors that formed in ERBB2/PTK6 double transgenic animals compared with tumors that formed in ERBB2 transgenic mice. Apoptosis was analyzed using the TUNEL assay in several pairs of equivalently-sized ERBB2 and ERBB2/PTK6 tumors.



ERBB2 induced tumors metastasize to the lung in transgenic mice (reviewed in Ursini-Siegel et al. 2007). In our study lung metastasis was observed in both ERBB2 and ERBB2/PTK6 mice (Figure 26 A). Lungs of ERBB2 and ERBB2/PTK6 animals were harvested from animals with tumors that had reached humane endpoint size, cut into 0.5 x 0.5 cm^2 pieces, fixed, embedded in paraffin and sectioned. Sections were stained with hematoxylin and eosin, and tumor emboli were counted and normalized with the area of the lung (represented by the number of 0.5 x 0.5 cm^2 lung pieces). Metastases were quantitated in twelve mice of each genotype (ERBB2 versus ERBB2/PTK6 transgenics), and correlations of lung metastasis with animal age and primary breast tumor weight were analyzed (Figure. 26 B). We did not detect a significant difference in the timing or size of metastases between the two groups.

Figure 26. Transgenic expression of PTK6 in the MMTV-ERBB2 mouse model does not enhance lung metastasis

(A). Lung metastasis was detected in both ERBB2 and ERBB2/PTK6 transgenic animals. **(B).** we examine lung metastases in single ERBB2 and double ERBB2/PTK6 transgenic mice and correlated development of metastases with age and primary breast tumor weight. Linear regression models were fitted to the data and Wald tests were conducted to compare regression slopes between the ERBB2 and ERBB2/PTK6 transgenic groups, resulting in p-values of 0.66 and 0.19, respectively, suggesting that the slopes from two experimental groups are not statistically different from each other.



3.3 PTK6 ablation impairs ERBB2 induced breast tumorigenesis in mice

3.3.1 Background

Approximately 25% of human breast tumors overexpress ERBB2. PTK6 has been indicated to co-overexpress with ERBB2 in the invasive ductal carcinomas (Born et al. 2005). Recent studies showed that ERBB2 regulates PTK6 protein stability(Ai et al. 2013), and simultaneously knocking down PTK6 and ERBB2 *in vitro* leads to significant decreased phosphorylation of pro-survival signaling proteins like ERK1/2 and p38 comparing to single knockdown of PTK6 or ERBB2 (Ludyga et al. 2013).

MMTV-ERBB2 mouse model was developed by Dr. Leder's lab from Harvard Medical School (Guy et al. 1996, Muller et al. 1988) in FVB/NJ strain, and it has been widely used in breast cancer research (reviewed in Andrechek et al. 2000, Hutchinson et al. 2000). MMTV-ERBB2 transgene contains the MMTV-promoter, a SV40 polyadenylation site sequence, and a 4.6kb cDNA from the rat ERBB2 with a point mutation Val664 Glu which renders the gene active. In these animals (Jackson Laboratory Catalog# 005038), female mice exhibited diffuse, polyclonal tumor masses and the tumor morphogenesis corresponds to transgene expression (Muller et al. 1988). About 20% of MMTV-ERBB2 mice develop mammary tumors by the 175 days of age, and the tumor incidence increases to 83% by the 210 days of age (Rao et al. 2000). In our study, the timeline of MMTV-ERBB2 induced mammary gland tumorigenesis is consistent with previous reports. We also detected the induction of endogenous PTK6 in all the MMTV-ERBB2-induced breast tumors (Figure 22). This finding raised the questions such as: Is PTK6 expression required for ERBB2 induced tumorigenesis? Will disrupting *Ptk6* gene expression inhibit breast tumor formation in ERBB2 transgenic mouse? To answer these questions, we crossed MMTV-ERBB2 mice with *Ptk6*

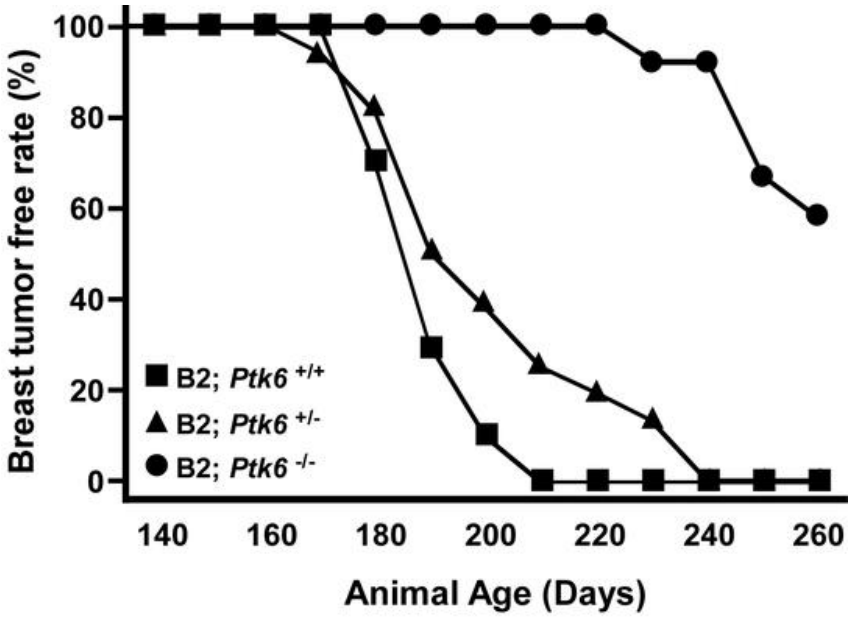
null mice and monitored breast tumor development. We hypothesized that PTK6 ablation will delay ERBB2-induced breast tumor formation, or decrease breast tumor proliferation rate.

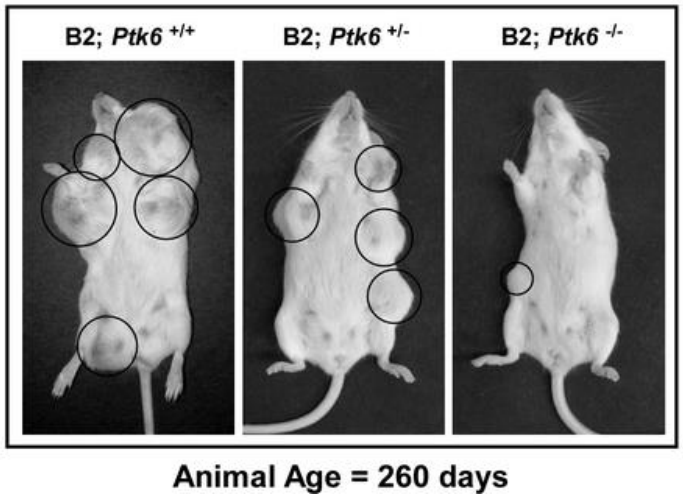
3.3.2 ERBB2-induced breast tumor formation is delayed in *Ptk6* null mice

MMTV-ERBB2 is a potent breast tumor oncogene. Mammary gland hyperplasia can be observed in 4 month old female animals. ERBB2 induced breast tumors usually become palpable around 6 months. Once tumors are palpable, the tumor-bearing animals would reach their endpoints within 2-3 months.

We bred MMTV-ERBB2 animals (B2) with *Ptk6* null (*Ptk6* $^{-/-}$) mice and genotyped the offspring by PCR. Both ERBB2 and *Ptk6* null breeders are from FVB/NJ strain. Three lines of animals (B2; *Ptk6* $^{+/+}$, B2; *Ptk6* $^{+/-}$ and B2; *Ptk6* $^{-/-}$) were maintained and examined at least once a week (Figure 27). We began to detect palpable tumors in B2; *Ptk6* $^{+/+}$ animals around 170 days, and by day 220 all of the B2; *Ptk6* +/+ animals developed one or multiple breast tumors (n=10). B2; *Ptk6* $^{+/-}$ started to develop breast tumor almost at the same time as B2; *Ptk6* $^{+/+}$ animals, but by day 210 there were 25% of the B2; *Ptk6* $^{+/-}$ animals remained tumor free, and not until day 240 did B2; *Ptk6* +/- animals become 0% tumor free (n=16). The most interesting result was observed in B2; *Ptk6* $^{-/-}$ (n=12): No tumors were detected in any of the animals by day 210 when all the B2; *Ptk6* $^{+/+}$ mice carried breast tumors. 92% percent of B2; *Ptk6* $^{-/-}$ animals remained tumor free up to 240 days when all the B2; *Ptk6* $^{+/-}$ mice developed breast tumors. By the end of cutoff date of 260 days, 58% of the B2; *Ptk6* $^{-/-}$ animals remained tumor free, and a few of them remained tumor free over 360 days. However, *Ptk6* ablation alone is not sufficient to abolish ERBB2 induced breast tumors as many of the B2; *Ptk6* $^{-/-}$ animals developed breast tumors after 12 months, but in *Ptk6* knockout mice the ERBB2 induced tumor initiation time was greatly delayed (170 days in B2; *Ptk6* $^{+/+}$ versus 220 days in B2; *Ptk6*$^{-/-}$) and the survival time was greatly increased (8 - 9 month in B2; *Ptk6*$^{+/+}$

Figure 27: MMTV-ERBB2 induced breast tumor formation is delayed in *Ptk6* null mice
Top: MMTV-ERBB2 transgene was expressed in *Ptk6* wild type (B2; *Ptk6*$^{+/+}$), heterozygous (B2; *Ptk6*$^{+/-}$) and null (B2; *Ptk6*$^{-/-}$) mice. Breast tumor formation was monitored by palpating mammary glands at least once a week, and the tumor free time in the figure indicates the time before tumor become palpable. Tumorigenesis in these three lines of animals were compared in top panel. Numbers of animals analyzed: B2; *Ptk6*$^{+/+}$, n=10; B2; *Ptk6*$^{+/-}$, n=16; B2; *Ptk6*$^{-/-}$, n=12. **Below:** Comparison of animals of same age but with different genotypes. Tumors were circles. Animal age: 260 days.





versus 10 - 12+ months in B2; *Ptk6*$^{-/-}$). All the animals used in the study were virgins to eliminate the effect of pregnancy on mammary glands, nevertheless, the B2; *Ptk6*$^{-/-}$ mice that underwent multiple pregnancies still exhibited significant delay in breast tumor formation comparing to age-matched multiparous B2; *Ptk6*$^{+/+}$ mice (data not shown).

3.3.3 Disrupting *Ptk6* gene affects ERBB2 induced proliferation in breast epithelium

We noticed that although the initiation time of ERBB2 induced breast tumor was greatly delayed in B2; *Ptk6*$^{-/-}$ animals, once the palpable tumors were formed, animals would reach their endpoint with 2-3 months, which is about the same progression time as B2; *Ptk6*$^{+/+}$ and B2, *Ptk6*$^{+/-}$ animals. To examine the effect of PTK6 ablation on breast tumor proliferation, immunofluorescence and BrdU assay were performed on mammary gland and breast tumor sections. We did not detect a significant difference in ERBB2 expression in the tumor (Figure 28, top panels TU), however, we noticed a decrease of ERBB2 expression in the mammary glands of B2; *Ptk6*$^{-/-}$ animals (Figure 28, top panels MG) comparing to B2; *Ptk6* $^{+/+}$ mammary glands. This possible finding needs to be confirmed with more samples and be supported by immunoblotting of mammary gland lysates. BrdU staining showed that B2; *Ptk6*$^{-/-}$ mice have much less proliferating cells (Figure 28, bottom panel MG) than in the B2; *Ptk6*$^{+/+}$ mammary glands. This finding explained the delayed initiation time because ERBB2 induced proliferation was impaired in B2; *Ptk6*$^{-/-}$ mammary glands. Interestingly, in weight-matched breast tumors, the number of proliferating cells was comparable in B2; *Ptk6*$^{+/+}$ and B2; *Ptk6*$^{-/-}$ tumors (Figure 28, bottom panel TU), this result explained why the tumor progression time is similar between B2; *Ptk6*$^{-/-}$ and B2; *Ptk6*$^{+/+}$ because the proliferation rate is similar in tumors. We further examined protein expression in breast tumor using immunoblotting. Lysates of two B2; *Ptk6*$^{+/+}$ and two B2; *Ptk6*$^{-/-}$ breast tumors were probed for mouse PTK6, ERBB2, ERBB3, phospho and total STAT3, and loading control β-actin (Figure 29). PTK6 induction was

Figure 28: MMTV-ERBB2 induced proliferation is inhibited in mammary glands in *Ptk6* null mice

Mammary glands and breast tumors were harvested from age matched mice of B2; *Ptk6* $^{+/+}$ and B2; *Ptk6* $^{-/-}$ genotype. Immunofluorescence assay was performed with ERBB2 antibody to examine transgenic ERBB2 expression, and the proliferation cells in these tissues were detected by staining with BrdU antibody. MG: Mammary gland. TU: Breast tumor. Size bar =50 μm

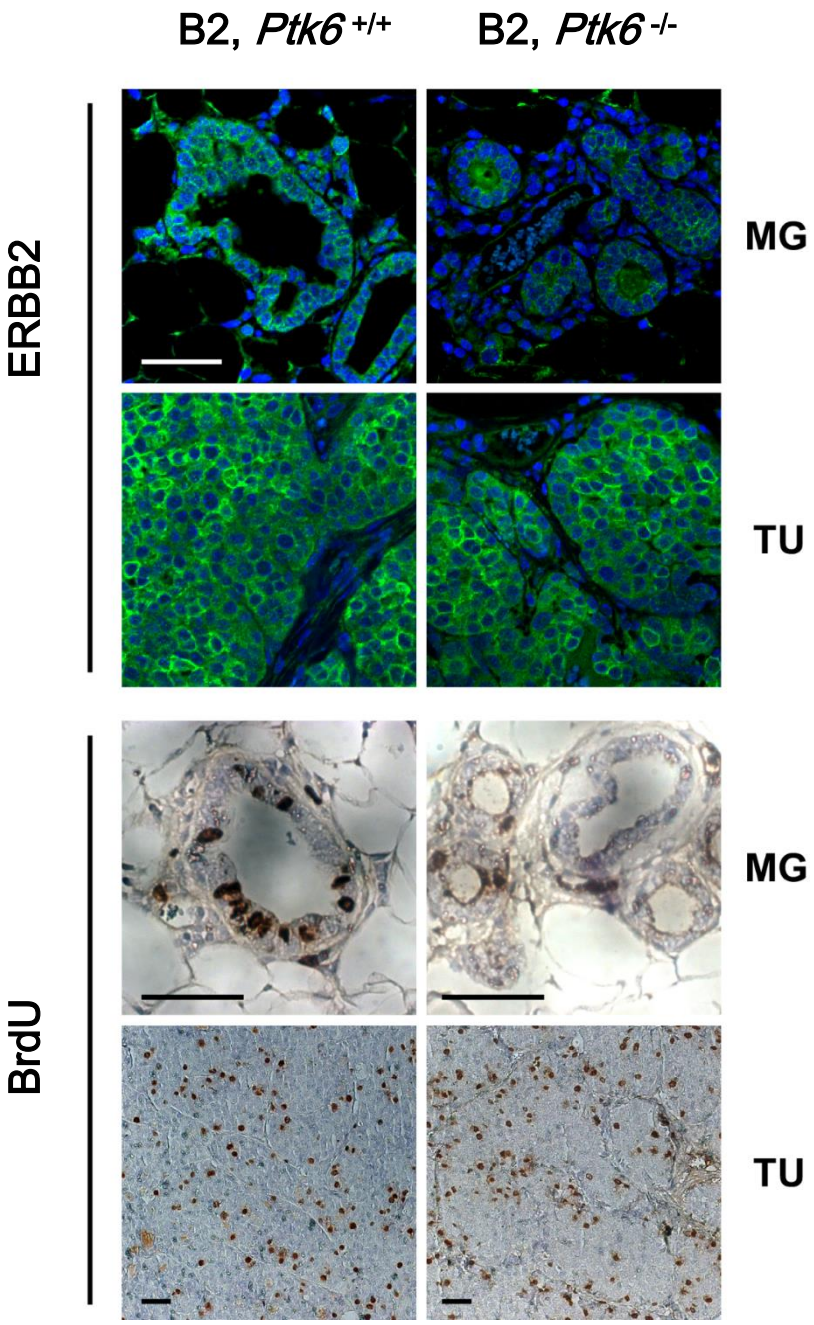
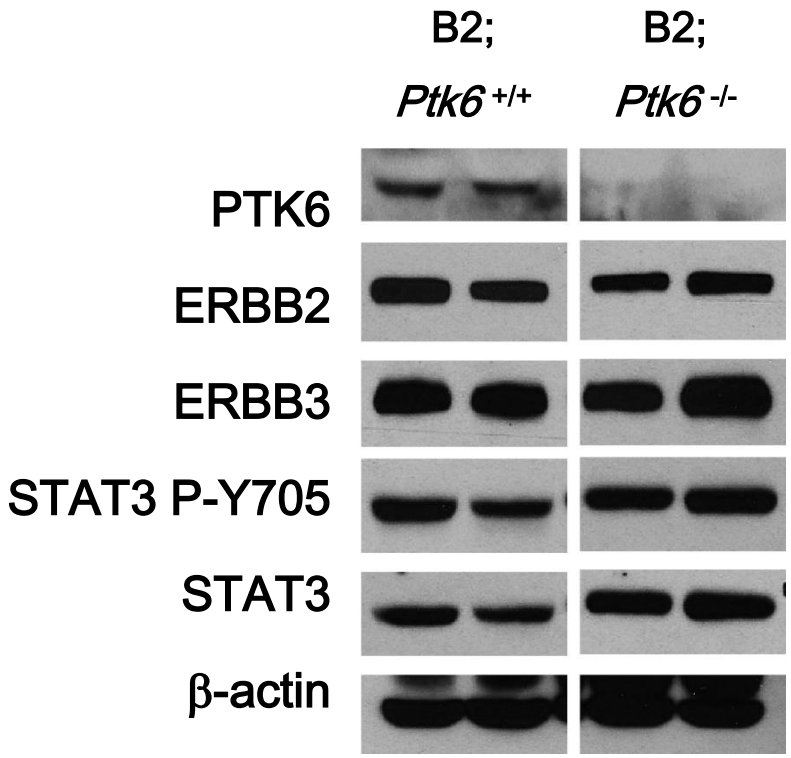


Figure 29: Disrupting endogenous PTK6 expression does not alter the total ERBB2 expression level in breast tumors

Total cell lysates of breast tumors harvested from B2; *Ptk6*$^{+/+}$ and B2; *Ptk6*$^{-/-}$ mice were probed with ERBB2, ERBB3, mouse PTK6, phospho and total STAT3, and β-actin. No significant changes were observed between ERBB2 induced *Ptk6* $^{+/+}$ and *Ptk6*$^{-/-}$ tumors.

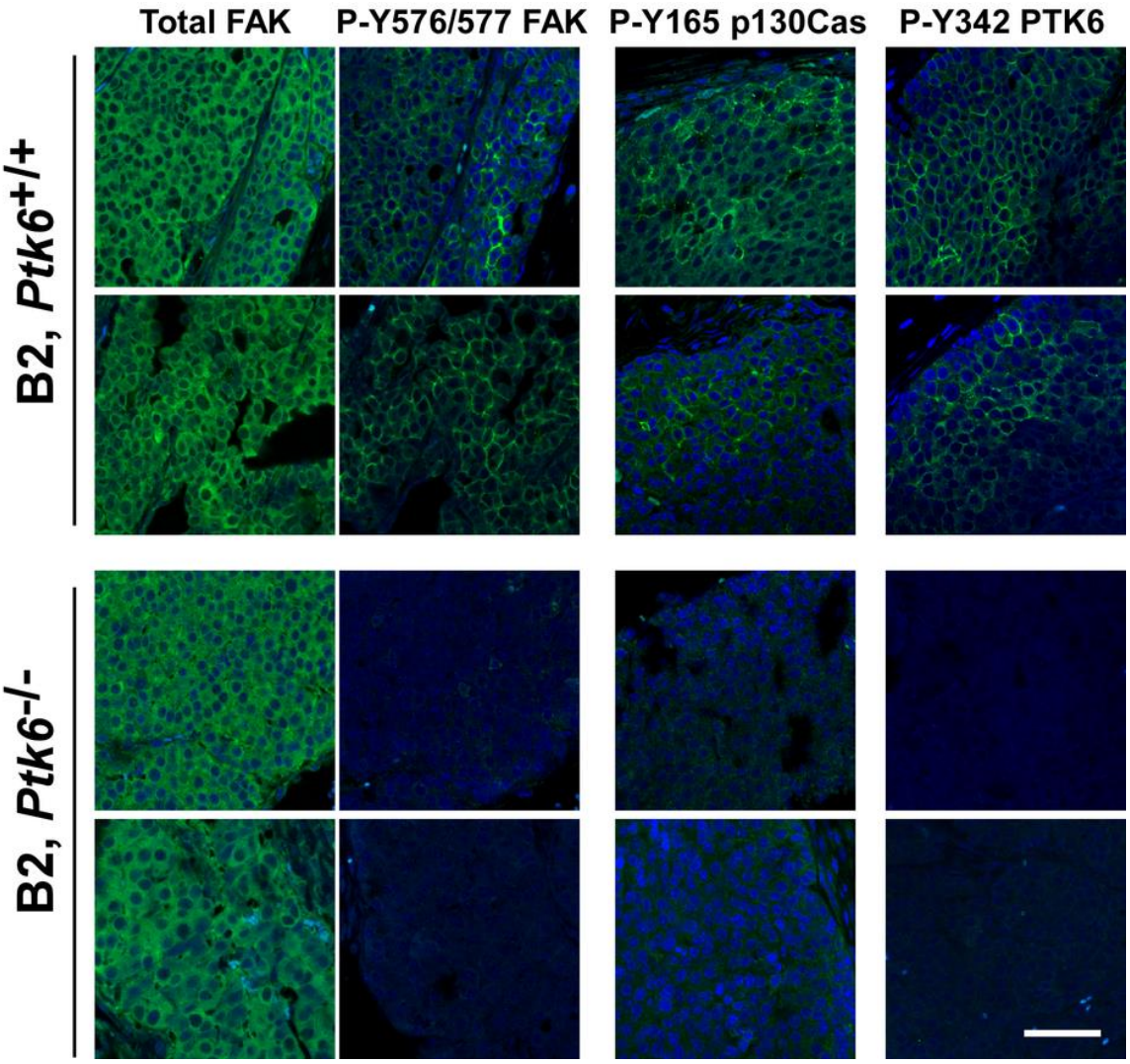


detected in B2; *Ptk6*$^{+/+}$ but not in B2; *Ptk6*$^{-/-}$ animals. ERBB2 and ERBB3 expression in these tumors was not affected by the absence of endogenous *Ptk6*, suggesting PTK6 induction may be important during tumor initiation stage but may play different role than promoting proliferation in formed breast tumors.

3.3.4 PTK6 ablation in ERBB2 induced breast tumors decreased activation of FAK and p130Cas

Both FAK and p130Cas have been suggested to play roles in ERBB2-mediated breast tumor formation. A correlation between elevated expression of FAK and ERBB2 overexpression has been identified in human breast tumors (Xu et al. 2000, Lark et al. 2005), and FAK activation has been observed in ERBB2 overexpressing human breast cancer cell lines (Ignatoski et al. 1999, Schmitz et al. 2005). Studies showed FAK is expressed in ERBB2-positive breast cancer and contributes to the proliferative and invasive potentials (Lahlou et al. 2012). p130Cas is required for ERBB2 transformation and promotes invasiveness of ERBB2 transformed mammary acini (Cabodi et al. 2010, Tornillo et al. 2011). Our lab found that PTK6 directly phosphorylates FAK and p130Cas in prostate cancer cells and promotes cell migration (Zheng et al. 2011, Zheng et al. 2012). We hypothesized that *Ptk6* disruption will decrease ERBB2 induced FAK and p130Cas activation. Two B2; *Ptk6*$^{+/+}$ and two B2; *Ptk6*$^{-/-}$ tumors of similar size and weight were compared (Figure 30). In B2; *Ptk6*$^{+/+}$ tumors, P-FAK and P-p130Cas signals were detected near the border of tumors, supposedly in the proliferating/invading zone. Active PTK6 was also detected in the same areas, correlated with the activation of FAK and p130Cas. In B2; *Ptk6*$^{-/-}$ tumors, active PTK6 signal was non-detectable, and P-FAK and P-p130Cas signals were minimal to none, suggesting the absence of PTK6 may affect the migration potential of ERBB2 induced tumors. Levels of total FAK (Figure 30) and total p130Cas (Data not shown) were comparable.

Figure 30: Activation of FAK and p130Cas is decreased in *Ptk6* null, ERBB2 induced tumors
Signaling molecules involved in cell migration and tumor metastasis were examined in ERBB2 induced breast tumors of similar size and weight. Genotypes of the animals are labeled in the figure. Gain time for FITC green is constant across all pictures. Size bar =50 μm



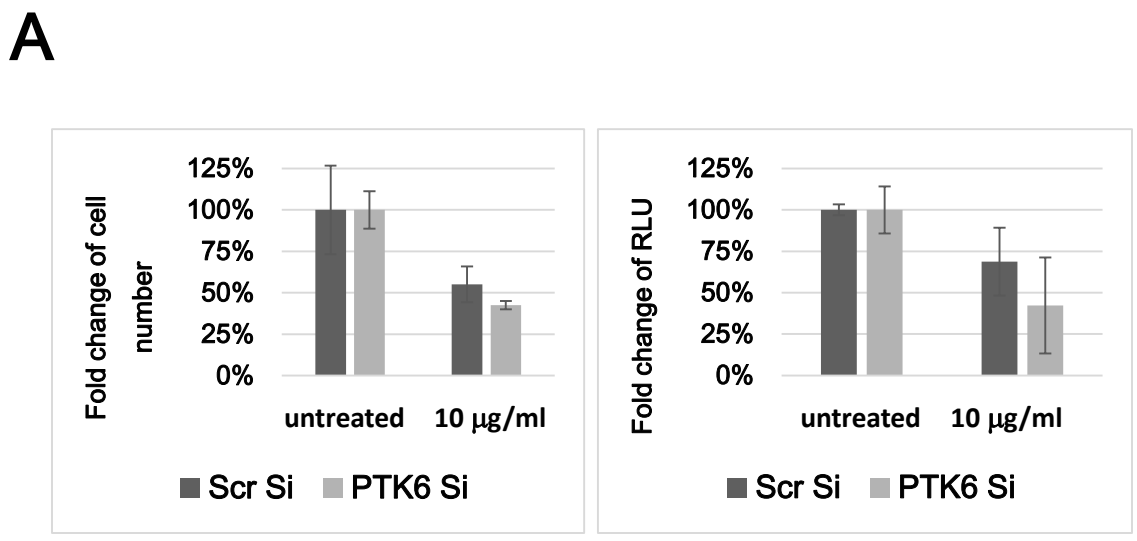
3.3.5 Silencing PTK6 in ERBB2 overexpressing breast cancer cell lines sensitizes them to trastuzumab treatment and other chemo-therapy

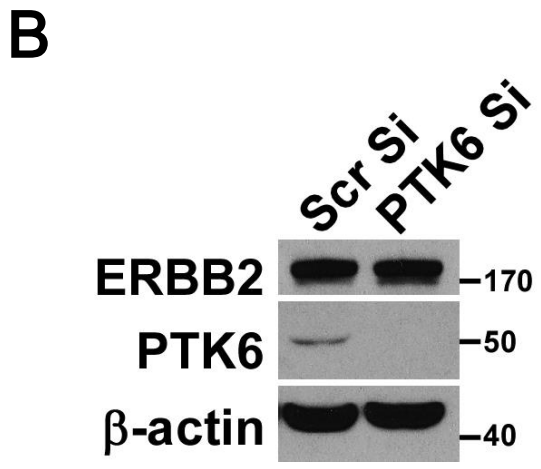
ERBB2 overexpressing tumors used to have poor prognosis before the introduction of the monoclonal antibody trastuzumab (also known as Herceptin). Trastuzumab binds to the extracellular segment of the ERBB2 receptor and cause cells to undergo cell cycle arrest (Cho et al. 2003). However, not all breast tumors response to trastuzumab treatment, and active SRC has been identified in acquired and *de novo* trastuzumab-resistant cells. Down-regulating SRC in these cells sensitizes them to trastuzumab induced growth inhibition (Muthuswamy 2011, Zhang et al. 2011). Since PTK6 is often overexpressed in ERBB2 overexpressing cell lines and involved in ERBB2 mediated signaling pathways(Born et al. 2005, Aubele et al. 2007, Xiang et al. 2008, Aubele et al. 2010, Ai et al. 2013), we hypothesized that knockdown of PTK6 will sensitize cells to trastuzumab treatment.

ERBB2 overexpressing SK-BR-3 cells were transfected with PTK6 siRNA, and serum starved for 24 hours prior to trastuzumab treatment. 10 μg/ml of trastuzumab was added to the medium containing 10% FBS and incubated for 4 days. It was known that knocking down PTK6 in breast cancer cells may inhibit cell proliferation, to take the impact of growth inhibition by PTK6 down-regulation into consideration, SK-BR-3 cells that have been transfected with siRNA but not treated with trastuzumab were cultured as standards and measured by the end of day 4 too. Numbers of viable cells were measured by automated cell counter (measures cell number) and luminometer (measures the luminescent signal proportional to the amount of ATP present) then normalized to standards. An increased trastuzumab induced growth inhibition effect was detected in PTK6 knockdown SK-BR-3 cells (Figure 31), suggesting down-regulating PTK6 could potentially be beneficial when treating ERBB2 positive breast tumors with trastuzumab.

Figure 31: Knocking down PTK6 sensitizes SK-BR-3 cells to trastuzumab induced proliferation inhibition

(A). SK-BR-3 cells were transfected with PTK6 SiRNA to knock down endogenous PTK6 (Scrambled SiRNA as negative control), then treated with 10 μg/ml trastuzumab for 4 days. Cells that have been transfected with siRNA but not treated with trastuzumab were used as standards (untreated) to normalize any proliferation inhibition effect conferred by knocking down PTK6 alone. Numbers of viable trastuzumab treated cells were measured by automated cell counter and luminometer then normalized to standards. An increased trastuzumab induced growth inhibition effect was detected in PTK6 knockdown SK-BR-3 cells. **(B).** PTK6 knockdown was verified by immunoblotting.





3.4 Evaluating PTK6 as a therapeutic target in breast cancer treatment

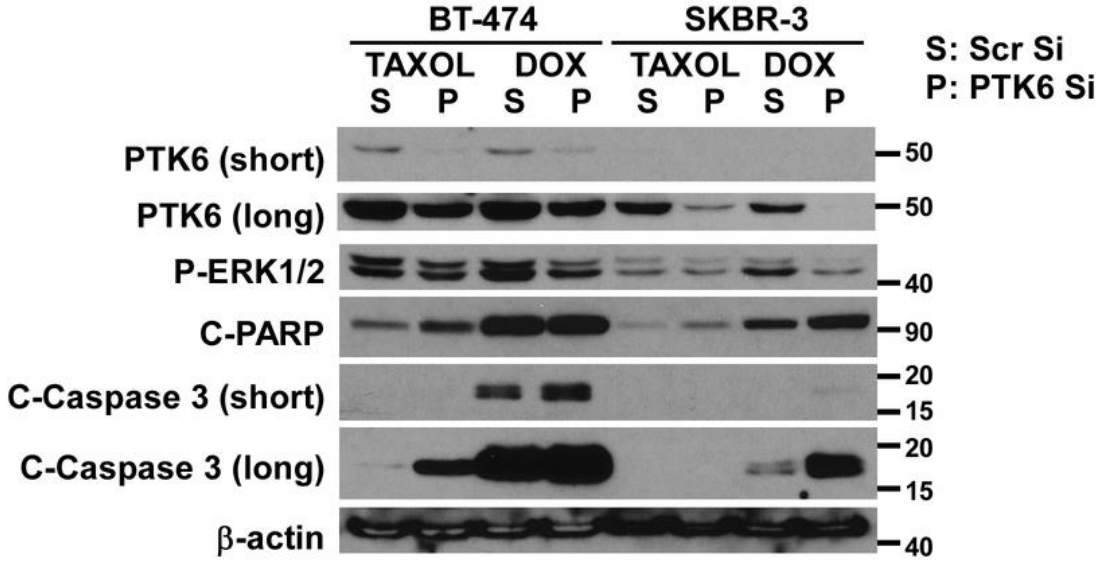
3.4.1 Background

Since PTK6 is overexpressed in the majority of human breast cancers, it has been considered as a potential therapeutic target for a long time (Harvey et al. 2003). Supporting data has accumulated over the years: Silencing endogenous PTK6 with siRNA decreases breast cancer cell proliferation (Harvey et al. 2003). Down-regulating PTK6 in colon cancer cells enhances apoptosis following DNA damage induced by γ-irradiation, doxorubicin and 5-fluorouracil (Gierut et al. 2012). In ERBB2 induced tumors, overexpressing PTK6 confers resistance to the ERBB2 kinase inhibitor Lapatinib, suggesting targeting PTK6 may sensitize breast tumor to ERBB2 inhibitors (Xiang et al. 2008). Knocking down PTK6 in EMT-1, a cancer cell line derived from metastatic breast tumor and expresses high level of activated ERBB2, would induce apoptosis and partially reverse the epithelial-to-mesenchymal phenotype (Ai et al. 2013). Recent study explored the effect of simultaneously knocking-down PTK6 and ERBB2 in breast cancer cells and observed greater decreased of pro-survival signaling than knocking-down PTK6 or ERBB2 alone (Ludyga et al. 2013). It has not been well studies if PTK6 knockdown has similar effects for both ERBB2 overexpressing and non-overexpressing cells, and we studied multiple breast cancer cells including BT-474, SK-BR-3 and MCF-7 to evaluate if knockdown PTK6 is beneficial for cells with different ERBB2 expression profiles.

3.4.2 Knockdown of PTK6 sensitizes breast cancer cells to chemo and irradiation therapy

Paclitaxel (Taxol) is a mitotic inhibitor and doxorubicin (Dox) is a DNA-damaging agents. Both Taxol and Dox are FDA-approved breast cancer drugs. Two cell lines that represent HER2 overexpressing breast cancer subtypes, Luminal B (BT-474) and HER2 (SK-BR-3), were treated with Taxol and Dox (Figure 32). Taxol and Dox was dissolved in DMSO. When treating cells with

Figure 32: Knocking down PTK6 sensitizes ERBB2 overexpressing cells to chemotherapy
ERBB2 overexpression cells BT-474 and SK-BR-3 were transfected with siRNA to knock down PTK6, then subjected to Taxol and Dox treatment for 24 hours. Final concentrations were: Taxol 15 μM in both BT-474 and SK-BR-3, Dox: 15 μM in BT-474 and 2 μM in SK-BR-3. Pro-survival signaling molecules and apoptosis markers were examined. P: PTK6 siRNA. S: Scramble siRNA, negative control.



Taxol or Dox solution, same amount of DMSO was added to the control cells to provide the background toxicity of DMSO. No significant cell death was detected in DMSO treated cells. Cells were transfect with PTK6 or Scramble siRNA prior to the chemo-treatment. Comparing to DMSO treatment, PTK6 is induced after exposed to DNA damaging agent like Taxol and Dox (Data not shown). Both BT-474 and SK-BR-3 are not sensitive to Taxol treatment, when PTK6 is down-regulated by siRNA, BT-474 became more sensitive to Taxol treatment. But SK-BR-3 is still resistant to Taxol treatment. However, SK-BR-3 is more sensitive to Dox as 2 μM of Dox treatment induced PARP and Caspase 3 cleavage, which were augmented by PTK6 knockdown. Similar result was observed in BT-474 cells with 15 μM of Dox treatment. In addition, phosphorylation of pro-survival protein ERK 1/2 decreased in PTK6 knocked down cells. These results suggested that targeting PTK6 will sensitize ERBB2-overexpressing breast cancer cells to chemotherapy.

A similar effect was observed in Luminal A type of breast cancer cells, MCF-7. Taxol treatment activated pro-survival signaling proteins shown by the phosphorylation of certain proteins (AKT S473, AKT T308, ERK1/2 T202/Y204), and PTK6 knockdown decreased the levels of AKT and ERK1/2 phosphorylation while the total AKT and ERK1/2 were not altered (Figure 33 A, left). Taxol treatment also caused cell death in MCF-7 cells, and increased levels of apoptosis markers (cleaved Caspase 9, cleaved PARP) were detected in PTK6 knockdown cells.

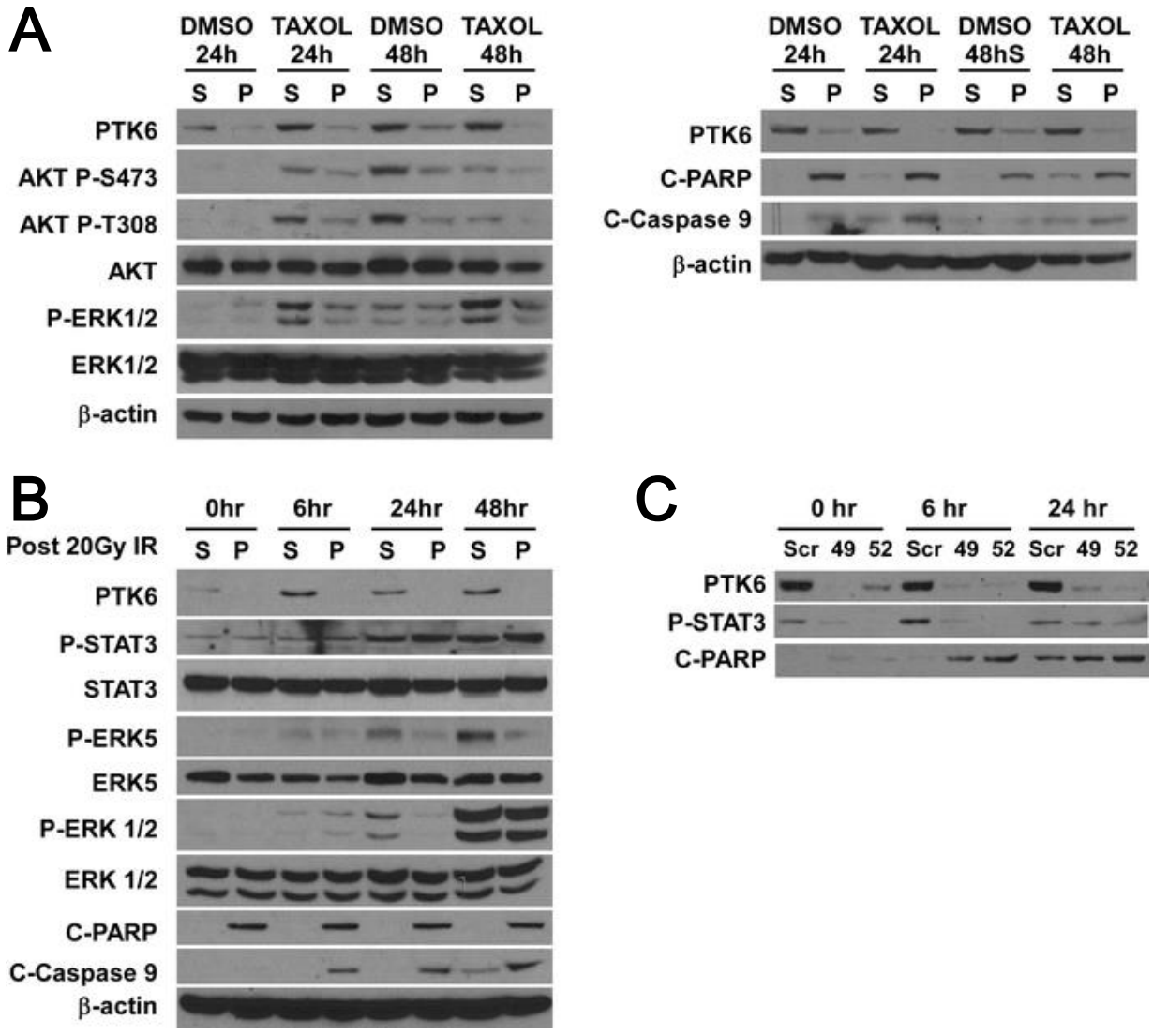
Another common DNA-damaging agent for breast cancer treatment is γ-irradiation. We studied the effect of knockdown PTK6 in combination with γ-irradiation treatment in MCF-7 cells (Figure 33 B). Endogenous PTK6 was transiently knocked down with siRNA, cells were then irradiated with 20Gy of γ–irradiation. PTK6 was induced by irradiation and promoted phosphorylation on pro-survival signaling proteins like ERK5 and ERK1/2. Phosphorylation of these proteins were

impaired when PTK6 was knocked down. Knocking down PTK6 also sensitize MCF-7 cells from irradiation induced apoptosis, as shown by increased levels of cleaved PARP and cleaved Caspase 9. Interestingly, STAT3 phosphorylation did not seem to be inhibited when PTK6 was transiently knocked down by siRNA, but when PTK6 is stably knocked down by two short hairpin RNA constructs (49 and 52) with lentivirus, irradiation induced STAT3 activation was greatly diminished, suggesting short term and long term knockdown of PTK6 may have different effects .

These results suggested that down-regulating PTK6 in breast cancer cells sensitizes them to anti-cancer therapies. Knockdown of PTK6 impairs the activation of pro-survival signaling and increases apoptosis regardless of ERBB2 status. PTK6 could be a valuable therapeutic target in breast cancer treatment in combination with other DNA-damaging agents.

Figure 33: Down-regulating PTK6 increases chemo and irradiation sensitivity in MCF-7 cells

(A). MCF-7 cells were transfected with PTK6 siRNA and then treated with 10 μM paclitaxel. Paclitaxel was dissolved in DMSO. Molecules involved in pro-survival pathways were shown in the left and apoptosis markers were shown in the right. **(B).** Endogenous PTK6 in MCF-7 was knocked down with siRNA, cells were then irradiated with 20Gy of γ–irradiation. P: PTK6 siRNA. S: Scramble siRNA, negative control. **(C).** Endogenous PTK6 was stably knockdown by shRNA. 49 &52: two shRNA constructs targeting PTK6. Scr: Scramble shRNA, negative control**.**



IV. DISCUSSION

4.1 PTK6 is expressed in normal human mammary gland and activated in breast tumors

We discovered that PTK6 is expressed but not activated in normal human mammary gland, and in breast tumors PTK6 is often overexpressed, activated and translocated from nucleus to cytoplasm and membrane. Our discovery challenges the common notion that PTK6 is not expressed in normal mammary gland. Our data suggest that PTK6 is de-regulated rather than induced during tumorigenesis. Although the function of PTK6 in normal breast is unclear, it is likely to be involved in maintaining the differentiated characteristics as knockdown of PTK6 in MCF-10A cells resulted in increased mammosphere formation. Interestingly, human and mouse appear to have different PTK6 expression pattern in the mammary glands. Although PTK6 is induced in mouse breast tumors, normal mouse mammary gland does not express PTK6 at any developmental stage, and *Ptk6* null mice showed no defects in mammary gland functions. The difference between human and mouse could have been caused by species specific structures, or by the exposure to stress and age-related stimulations.

PTK6 expression in normal tissues is developmentally regulated and coincides with epithelial cell differentiation (Haegebarth et al. 2006, Vasioukhin et al. 1995, Vasioukhin et al. 1997). Disruption of the *Ptk6* gene in the mouse revealed unique roles for this tyrosine kinase in promoting intestinal epithelial cell differentiation (Haegebarth et al. 2006) and stress-induced apoptosis (Gierut, J. et al. 2011, Haegebarth et al. 2009). PTK6 may also have distinct functions in normal and transformed mammary epithelial cells. For example, while PTK6 promotes EGF-induced proliferation in several breast cancer cell lines, it inhibited EGF-induced proliferation in h-TERT immortalized human mammary gland epithelial cells (Ostrander et al. 2010), and PTK6 localization and activation could be play a role.

The subcellular localization of PTK6 plays an important role in its function. PTK6 is structurally related to SRC family kinases, however, PTK6 lacks the SH4 domain and is not myristoylated/palmitoylated (Serfas et al. 2003). PTK6 also lacks a nuclear localization signal. Thus, it displays flexibility in its intracellular localization and has different functions in the nucleus and at the plasma membrane (Zheng et al. 2013). In normal prostate cells, total and active PTK6 is concentrated in epithelial cell nuclei, but nuclear localization is lost in prostate tumors (Derry et al. 2003). Knockdown of cytoplasmic/membrane-associated PTK6 proved to be growth inhibiting, while reintroduction of PTK6 into the nucleus also inhibited growth prostate cancer PC3 cells (Brauer et al. 2010). Targeting PTK6 to the cell membrane by addition of a myristoylation/palmitoylation signal resulted in oncogenic signaling (Ie Kim et al. 2009, Palka-Hamblin et al. 2010). Ectopic expression of membrane targeted PTK6 was sufficient to transform *Src/Yes/Fyn*$^{-/-}$ mouse embryonic fibroblasts (Zheng et al. 2012). Interestingly, active endogenous PTK6 was associated with the membrane in *Pten* null mouse prostates (Zheng et al. 2012, Zheng et al. 2013). Enhanced co-expression of membrane associated growth factor receptors such as ERBB2 with PTK6 might bring PTK6 to the membrane in the absence of amino terminal myristoylation/palmitoylation, leading to its activation and induction of oncogenic signaling.

Although total PTK6 was detected in most of the breast tumors, active PTK6 was only detected in half of them. The inconsistency between PTK6 expression and activation may have contributed to the puzzling results reported by Aubele et al. 2008, in which the disease-free survival of breast cancer patients was positively correlated with the protein expression level of PTK6. The highest levels of membrane associated active PTK6 were detected in high grade invasive ductal carcinomas, suggesting PTK6 may contribute to tumor growth and invasion. Interestingly, high levels of active PTK6 were also detected in the nucleus of lobular carcinoma in situ (LCIS). LCIS

is a non-invasive lesion arising from the lobules and terminal ducts of the breast. Unlike DCIS, LCIS is not known to be a premalignant lesion, but rather a marker that identifies women at an increased risk for subsequent development of invasive breast cancer. (Reviewed in Afonso et al. 2008). During long-term follow-up of women with LCIS, it was observed that most women with LCIS did not develop invasive breast tumor (Frykberg 1999, Gump et al. 1998). In summary, nuclear PTK6 is associated with normal gland and non-invasive lesions, while cytoplasmic and membrane associated PTK6 is associated with invasive carcinomas, these findings suggest PTK6 may be regulated and function similarly in mammary gland and prostate gland.

We also examined PTK6 overexpression and activation status in different intrinsic breast tumor subtypes. Breast tumors can be classified as Luminal A(ER/PR+, HER2-), Luminal B (ER/PR+, HER2+), HER2(ER/PR-, HER2+), and basal like (ER/PR/HER2 negative) according to the hormone receptors they express. Previously a correlation between PTK6 and HER2 overexpressing breast tumors (Luminal B and HER2) was reported (Irie et al. 2010). However, in our study, we detected highest PTK6 expression (scored 2-3+) in Luminal A (84.2%) and Luminal B (83.3%), followed by HER2 (75.0%) and triple negative (57.1%) (Table 5). Interestingly, active PTK6 does not always correlate with the expression pattern of total PTK6: Highest membrane localized active PTK6 (scored 2-3+) was detected in triple negative group (28.1%), followed by HER2 (25%), Luminal B (16.7%) and Luminal A (15.8%). Multiple studies showed that Luminal A breast tumors usually response well to the anti-cancer treatments while triple negative breast tumors indicated poor prognosis (Gyorffy et al. 2005, Diaz et al. 2007, Onitilo et al. 2009, Wang et al. 2011). It would be interesting to investigate the contribution of active PTK6 in the triple negative breast cancers.

TABLE V

EXPRESSION AND ACTIVATION STATUS OF PTK6 IN HUMAN BREAST TUMORS

	#	**PTK6 0-1+**	**PTK6 2-3+**	**P-PTK6 0-1+**	**P-PTK6 2-3+**
Triple Negative	14	6 (42.9%)	8 (57.1%)	10 (71.4%)	4 (28.6%)
HER2 Overexpression	4	1 (25.0%)	3 (75.0%)	3 (75.0%)	1 (25.0%)
Luminal A	19	3 (15.8%)	16 (84.2%)	16 (84.2%)	3 (15.8%)
Luminal B	6	1 (16.7%)	5 (83.3%)	5 (83.3%)	1 (16.7%)

[a] # = number of patients

4.2 PTK6 promotes breast tumor proliferation in mouse mammary glands

Our data, obtained by characterizing multiple independent lines of MMTV-PTK6 transgenic mice, indicate that PTK6 promotes breast tumorigenesis in vivo, but it is not a strong oncogenic driver. We detected an average 2.4-fold increase in tumor formation in virgin and multiparous animals compared with wild type control FVB/NJ mice. However, PTK6-promoted tumor formation was modest in contrast to the ERBB2 induced breast tumor formation. About 80% of the MMTV-ERBB2 mice developed tumors within 8 months compared with tumor formation in 10-13.6% of MMTV-PTK6 mice after 20 months. A previous study utilizing the whey acidic protein (WAP) promoter to drive PTK6 expression in the mouse mammary gland reported a three-fold higher incidence of tumor development in multiparous mice (Lofgren et al. 2011). Our study utilizing the MMTV promoter to drive PTK6 expression supports these findings. However, overexpression of PTK6 under the control of the WAP-promoter exhibited delayed mammary gland involution which was associated with increased prosurvival signaling. We did not detect any obvious changes in mammary gland development or involution in our MMTV-PTK6 transgenic lines. The differences between the two models could be due to distinctions in the timing and pattern of PTK6 transgene expression as a consequence of using different promoters.

Direct roles for PTK6 in the activation of STAT3 (Ikeda et al. 2009, Ikeda et al. 2010, Liu, L. et al. 2006) and STAT5b (Weaver and Silva 2007) have been reported. We show that STAT3 activation is increased in MMTV-PTK6 transgenic mammary glands and tumors and could contribute to the increase in tumor formation observed in these mice. STAT3 contributes to development of a variety of cancers and was shown to regulate growth of stem-like cells in human breast tumors (Marotta et al. 2011). Inhibitors of STAT3 activity inhibited breast cancer cell growth (Lin, L. et al. 2010). A tumor promoting role for PTK6 has been suggested in colon cancer, as *Ptk6* null

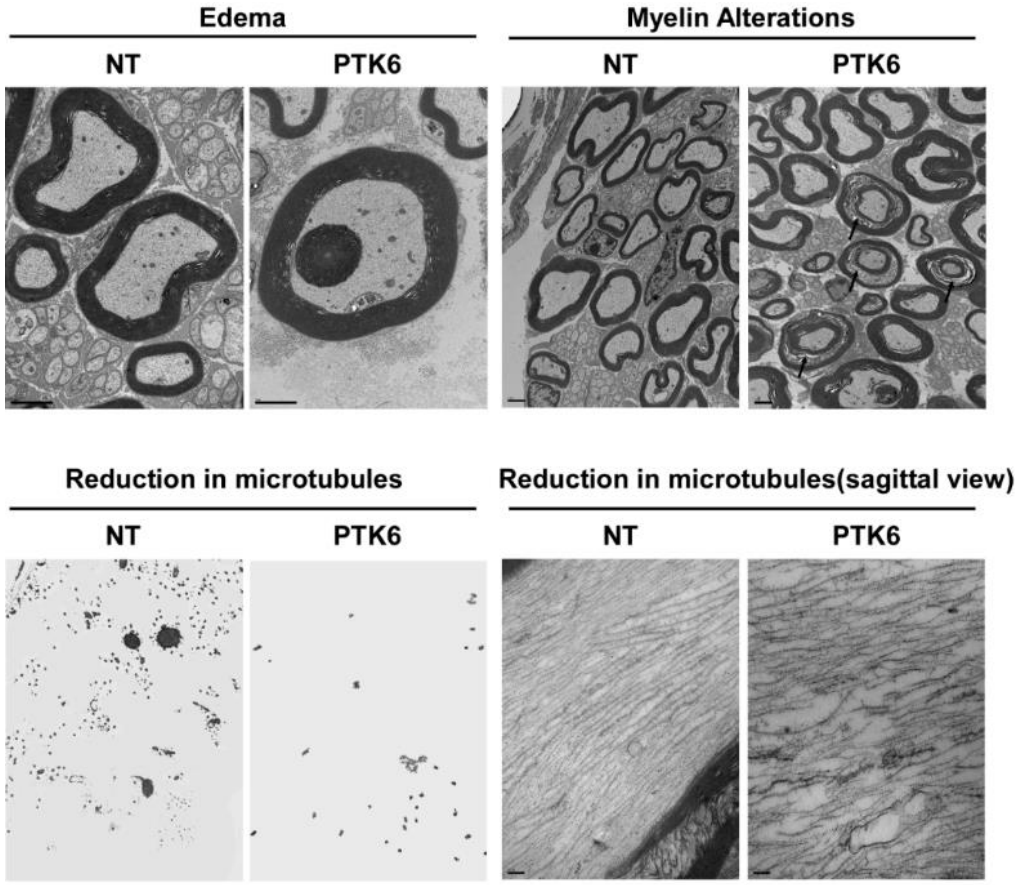
mice were resistant to an azoxymethane/dextran sodium sulfate tumorigenesis protocol and displayed reduced levels of activated phospho-STAT3 (Gierut, J. et al. 2011). PTK6 was also shown to promote EGF induced STAT3 activation in human colon cancer cells (Gierut et al. 2011).

MMTV-LTR is known to promoter expression in prostate gland, testis, seminal vesicle, skin, salivary gland and various cells of the immune system (Sinn et al. 1987). In our study, we detected MMTV-PTK6 expression in the testes of male mice and salivary glands of both genders in addition to the mammary glands of female mice. Other than breast tumor formation, we also noticed that some animals expressing human PTK6 became incontinent as they age, and this phenotype was likely to be caused by neuron dysfunction. Neurons of NT and PTK6 Tg animals were studied under electronic microscope by Dr. Ernesto R. Bongarzone (UIC) with the help of Dr. Jim Artwohl (BRL, UIC), and following defects/alterations were detected in the transgenic animal: edema, myelin alterations, reduction in microtubules, lower association of organelles with microtubular structures and axonal detachment from myelin sheath (Figure 34). It is not clear if these alterations were linked with PTK6 expression, but the phenotype is only detected in the animals expressing human PTK6 of different transgenic lines (B28 and B33).

PTK6 is expressed in a high percentage of human breast tumors, and its activities in cancer have been most extensively examined in breast cancer cell lines. A variety of studies indicate that PTK6 is involved in the signaling pathway of multiple ERBB receptor family members (Chen et al. 2004, Kamalati, T. et al. 2000, Kamalati, T. et al. 1996, Ostrander et al. 2007, Xiang et al. 2008). Orthotopic transplantation of an immortalized pluripotent mouse mammary epithelial cell line engineered to overexpress activated ERBB2 alone or activated ERBB2 plus PTK6 revealed reduced latency for tumor development when both ERBB2 and PTK6 were overexpressed (Xiang et al. 2008). In our studies, we detected increased proliferation in bi-transgenic PTK6/ERRB2

Figure 34. PTK6 transgenic animals exhibit neuronal defects

Some aged animals expressing human PTK6 became incontinent, most likely caused by neuron dysfunction. Neurons of NT and PTK6 Tg animals were studied under electronic microscope, and following alterations were detected in the transgenic animal: edema, myelin alterations, reduction in microtubules, lower association of organelles with microtubular structures and axonal detachment from myelin sheath. Figured showed a 22 months old B28 MMTV-PTK6 animals in comparison with age matched non-transgenic animals. Similar phenotypes were also observed in animals from B33 line.



mammary gland tumors, but also detected increased apoptosis that could counterbalance this increased proliferation. Increase apoptosis may also be an effect of overexpressing PTK6, as we previously showed that PTK6 regulates apoptosis in different tissues (Gierut et al. 2011, Haegebarth et al. 2005, Haegebarth et al. 2009).

While our *in vivo* data do not demonstrate synergy of tumor-promoting function between transgenic PTK6 and ERBB2, we cannot disregard contributions of endogenous PTK6. PTK6 is not expressed in the normal mouse mammary gland, but here we show that it is induced in mouse mammary gland tumors of different origins. We detected induction of mouse PTK6 in spontaneous mouse mammary gland tumors, as well as tumors caused by ectopic expression of ERBB2, activated RAS, and PyMT. Recently, expression of endogenous PTK6 was also reported in mouse mammary gland tumors induced by expression of an activated MET receptor transgene (Anderson et al. 2013). It is possible that induction of endogenous mouse PTK6 was sufficient to stimulate tumorigenesis and masked tumor promoting functions of ectopic transgenic human PTK6 *in vivo*.

To answer this question, we disrupted the endogenous *Ptk6* gene in ERBB2 transgenic mice and observed a significant delay in breast tumor formation (170 vs 210 days). ERBB2 induced proliferation in mammary gland epithelium was lower in *Ptk6* null animals, suggesting the abnormal growth in the mammary gland may require PTK6 induction. However, being a potent oncogenic protein, ERBB2 is able to induce breast tumor formation in a single step (Muller et al 1989), and disrupting *Ptk6* along is not sufficient to prevent ERBB2 induced breast tumor formation, as a majority of ERBB2; $Ptk6^{-/-}$ mice developed breast tumors after a year. Nevertheless, in these ERBB2; $Ptk6^{-/-}$ breast tumors, FAK and p130Cas activation were decreased while the total levels were not affected, suggesting PTK6 down-regulation may decrease the metastatic potential of breast tumors.

4.3 Targeting PTK6 enhances the response to chemo and irradiation therapy in breast cancer treatment

Several studies suggest that targeting PTK6 may have therapeutic benefits in breast (Xiang et al. 2008, Harvey et al. 2003, Irie et al. 2010, Ludyga et al. 2013, Ai et al. 2013), colon (Gierut et al. 2012), and prostate (Zheng et al. 2013) cancer cells, as knocking down PTK6 in cancer cells decreases proliferation, increases apoptosis and reverses EMT. However, earlier work also suggested a correlation between high PTK6 expression and differentiation (positive estrogen receptor status) (Zhao, C. et al. 2003), as well as increased patient survival (Aubele et al. 2007). Recent studies also suggest PTK6 has tumor suppressor functions in some cancers, including esophageal (Ma et al. 2012) and laryngeal (Liu et al. 2013) tumors. Clearly, complexities of PTK6 signaling are not yet fully understood, although PTK6 has been considered as a breast cancer therapeutic target for many years due to the high portion of breast tumors that overexpress PTK6, it will be necessary to determine if targeting PTK6 expression has specific benefits for treatment in different subtypes of human breast cancer. My study showed that knockdown of PTK6 in breast cancer cells sensitizes them to chemotherapeutic drug and irradiation treatment, regardless of the cells to be luminal A(MCF-7), luminal B (BT-474) or HER2 (SK-BR-3) subtypes. Down-regulating PTK6 increases apoptosis and decreases pro-survival signals after chemo or irradiation treatment. In ERBB2 overexpressing subtype SK-BR-3 cells, down-regulating PTK6 promoted trastuzumab induced growth inhibition. Still, more experiment need to be performed on cell lines such as MDA-MB-231 and MDA-MB-468 to evaluate the effect of targeting PTK6 in triple negative breast cancers.

However, down-regulating PTK6 should be proceed with caution. Until recently, knockdown of PTK6 has been regarded as a relatively practical and safe approach to inhibit breast cancer cell

proliferation without causing severe consequence to normal mammary glands, based on the common notion that PTK6 is not expressed in normal mammary glands. Indeed, PTK6 is not expressed in mouse mammary glands and *in vivo Ptk6* gene knockout data show *Ptk6* null mice do not suffer from any mammary gland defect or other severe health problems except some none-life-threatening changes in the small intestine (Haegebarth et al. 2006), suggesting that targeting PTK may produce few side effects in breast cancer treatment. However, my research found out that unlike mouse mammary gland, PTK6 is expressed in normal human mammary gland, and the function is still unclear. Since active PTK6 was not detected in the normal glands, PTK6 could be tightly regulated and only activate transiently, or function as an adaptor protein independent of kinase activity. Mammosphere formation assay in MCF-10A cells suggested down-regulating endogenous PTK6 caused the cells to be more stem-like, which is not the optimized outcome in cancer treatment. A possible approach for regulating PTK6 in breast tumor is by developing novel kinase inhibitors. Our data suggested PTK6 activation status may be more important than expression levels, and kinase inhibitors will inhibit PTK6-mediated pro-survival and pro-proliferation signaling pathways while keep the kinase independent functions intact.

4. Conclusion

Functions of PTK6 in normal and tumor tissues have been studied extensively over the years, and new discoveries about PTK6 regulators, substrates, pathways and expression patterns are accumulating day by day. When I started my work to address the function of PTK6 in breast tumorigenesis, no *in vivo* study has been conducted to test the oncogenic potential of PTK6 in mammary gland, and the relationship between PTK6 and ERBB2 was still poorly understood except the fact that they co-overexpressed in a portion of breast tumors. Now the role of PTK6 in breast tumorigenesis have been evaluated by us and another research group with different

transgene constructs, and the understanding of PTK6/ERBB interaction has advanced greatly in the past two years.

In this study, I determined that PTK6 is expressed but not activated in the normal human mammary gland, whereas in breast tumors PTK6 is overexpressed, activated and translocated from nucleus to cytoplasm and membrane. I discovered that knockdown of PTK6 renders non-transformed MCF-10A cells more "stem-like". I generated transgenic animals with mammary gland specific human PTK6 expression, and these PTK6 transgenic mice developed more than twice as many breast tumors compared with the non-transgenic controls. Ectopic PTK6 was activated in vivo and phosphorylated STAT3, a PTK6 substrate involves in pro-survival pathways, in the mammary gland epithelium of transgenic mice. The constitutive activation of PTK6 and STAT3 are likely to have contributed to the breast tumor formation. In the breast tumors that developed in transgenic mice, elevated levels of active PTK6 and STAT3 were detected. My experiment did not demonstrate synergy between transgenic PTK6 and ERBB2, possibly due to the masking effect conveyed by endogenous mouse PTK6 induction. Endogenous PTK6 induction was detected in mammary gland tumors of different origins including PTK6, H-Ras, ERBB2, and PyMT, and this induction was proven to be important for ERBB2-induce breast tumor formation. Disrupting *Ptk6* gene expression delays the initiation of breast tumor formation in MMTV-ERBB2 mice, and impairs ERBB2 induced FAK and p130Cas activation. These finding support the idea that targeting PTK6 in breast tumor may be beneficial, and my in vitro experiments proved that knockdown of PTK6 in BT-474, SK-BR-3 and MCF-7 can sensitize them to anti-cancer treatments.

My findings also bring up new questions such as: What are the functions of PTK6 in normal human mammary gland? How is it regulated? Is it possible to manipulate PTK6 in normal breast without causing severe side effects? How is PTK6 activated in breast tumors? How does PTK6 regulate

ERBB2 induced proliferation in mammary glands? Will PTK6 specific kinase inhibitor be more beneficial for invasive carcinomas? By answering these questions in future study, we will gain a better understanding of the role of PTK6 in mammary gland epithelium and breast cancer.

APPENDIX

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